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Mapping the Gene for Autosomal Dominant Restless Legs Syndrome in an Irish family

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Abstract

Restless Legs Syndrome (RLS) is a common neurological disorder affecting nearly 15% of the general population. Ironically, RLS can be described as the most common condition one has never heard of. It is usually characterised by uncomfortable, unpleasant sensations in the lower limbs inducing an uncontrollable desire to move the legs. RLS exhibits a circadian pattern with symptoms present predominantly in the evening or at night, thus leading to sleep disruption and daytime somnolence.

RLS is generally classified into primary (idiopathic) and secondary (symptomatic) forms. Primary RLS includes sporadic and familial cases of which the age of onset is usually less than 45 years and progresses slowly with a female to male ratio of 2:1. Secondary forms often occur as a complication of another health condition, such as iron deficiency or thyroid dysfunction. The age of onset is usually over 45 years, with an equal male to female ratio and more rapid progression.

Ekbom described the familial component of the disorder in 1945 and since then many studies have been published on the familial forms of the disorder. Molecular genetic studies have so far identified ten loci (5q, 12q, 14p, 9p, 20p, 16p, 19p, 4q, 17p). No specific gene within these loci has been identified thus far. Association mapping has highlighted a further five areas of interest. RLS6 has been found to be associated with SNPs in the *BTBD9* gene. Four other variants were found within intronic and intergenic regions of *MEIS1*, *MAP2K5*/*LBXCOR1*, *PTPRD* and *NOS1*.

The pathophysiology of RLS is complex and remains to be fully elucidated. Conditions associated with secondary RLS, such as pregnancy or end-stage renal disease, are characterised by iron deficiency, which suggests that disturbed iron homeostasis plays a role. Dopaminergic dysfunction in subcortical systems also appears to play a central role.

An ongoing study within the Department of Pathology (University College Cork) is investigating the genetic characteristics of RLS in Irish families. A three generation RLS pedigree RLS3002 consisting of 11 affected and 7 unaffected living family members was recruited. The family had been examined for four of the known loci (5q, 12q, 14p and 9p) (Abdulrahim 2008). The aim of this study was to continue examining this Irish RLS pedigree for possible linkage to the previously described loci and associated regions. Using informative microsatellite markers linkage was excluded to the loci on 5q, 12q, 14p, 9p, 20p, 16p, 19p, 4q, 17p and also within the regions reported to be associated with RLS. This suggested the presence of a new unidentified locus. A genome-wide scan was performed using two microsatellite marker screening sets (Research Genetics Inc. Mapping set and the Applied Biosystems Linkage mapping set version 2.5). Linkage analysis was conducted under an autosomal dominant model with a penetrance of 95% and an allele frequency of 0.01. A maximum LOD score of 3.59 at θ=0.00 for marker D19S878 indicated significant linkage on chromosome 19p. Haplotype analysis defined a genetic region of 6.57 cM on chromosome 19p13.3, corresponding to 2.5 Mb. There are approximately 100 genes annotated within the critical region. Sequencing of two candidate genes, KLF16 and *GAMT*, selected on the assumed pathophysiology of RLS, did not identify any sequence variant.

This study provides evidence of a novel RLS locus in an Irish pedigree, thus supporting the picture of RLS as a genetically heterogeneous trait.

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Abbreviations

°C	Degree Celsius
Θ	Recombination fraction
AD	Antidepressant
AGE	Agarose gel electrophoresis
bp	base pair
CEPH	Centre d'Etude du Polymorphime Humain
Chr	Chromosome
cM	Centi Morgan
CNS	Central Nervous System
COMT	Catechol-O-methyltransferase
CSF	Cerebrospinal Fluid
DA	Dopamine
DAT	Dopamine Transporter
ddNTP	Dideoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
DOPA	2,3-dihydroxyphenylalanine
DOPAC	Dihydroxyphenylacetic Acid
EDTA	Ethylene diamine tetra acetic acid
ECG	Electroencephalogram
EMG	Electromyography
GWAS	Genome-Wide Association Study
ICD	Impulse Control Disorder
IRLS	International RLS Study Group Severity Scale
IRLSSG	International Restless Legs Syndrome Study Group
JHSS	John Hopkins Severity Scale
Kb	Kilobase
LOD	Logarithm of the Odds
MAO	Monoamine Oxidase
Mb	Mega bases
$MgCl_2$	Magnesium Chloride

MRI	Magnetic resonance imaging
NCBI	National Center for Biotechnology Information
NOS1	Nitric Oxide Synthase
PC	Personalised Computer
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
PET	Position Electron Tomography
PLMI	Periodic Limb Movement Index
PLMS	Periodic Limb Movements during Sleep
PNS	Peripheral Nervous System
POP	Performance Optimized Polymer
PSG	Polysomnography
PTPRD	protein tyrosine phosphatase receptor type delta
QoL	Quality of Life
REST	RLS epidemiology, symptoms and treatment
RLS	Restless Legs Syndrome
RLS-DI	RLS diagnostic index
RPM	Revolutions per minute
SIT	Suggested immobilization test
SN	Substantia Nigra
SPECT	Singe Photon Emission Tomographic
TENS	Transcutaneous Electric Nerve Stimulation
TH	Tyrosine Hydroxylase
T_m	Melting Temperature
UCC	University College Cork
UV	Ultraviolet
VMAT	Vesicular Monamine Transporter
VTA	Ventral Tegmental Area
YKS	Yokukansan

Chapter 1

Restless Legs Syndrome

1.1 Introduction

Restless legs syndrome (RLS) is a neurological condition characterised by a distressing need or urge to move the legs (an akathisia focused on the legs), usually accompanied by an uncomfortable sensation in the legs (a paresthesia) described as a crawling, muscle ache or tension (Earley 2003). It is usually brought on by rest, worse in the evening or night and partially or totally relieved by movement. This scenario leads to nocturnal sleep disruption and consequently to daytime somnolence. Although the entity was mentioned in English literature by Thomas Willis in 1965, it was not until the Swedish neurologist Karl Axel Ekbom's lucid description in 1945, under the heading "Restless legs: a clinical study", that the scientific world became aware of this condition. Therefore, this disorder is often referred to as Ekbom's syndrome (Hening 2004).

RLS is a heterogenous disorder, the common feature of which is a circadian occurrence of disabling sensory symptoms at rest. However, in addition to sensorial symptoms such as dyesthesias, patients experience motor symptoms. These rhythmic or semirhythmic movements of the legs are otherwise known as periodic limb movements during sleep (PLMS) that occur every 5-90 seconds. PLMS, which occur in various sleep disorders as well as other neurological diseases, are observed in at least 80% of RLS patients (Montplaisir, Boucher et al. 1997).When present, PLMS support the diagnosis, and provide a measure of the clinical severity of this disorder.

RLS can be classified into two broad groups, according to the age of onset and the presence or absence of a familial history of RLS. Primary RLS is also known as idiopathic RLS. The name is applied to those cases of RLS where there is a positive family history of RLS and the age of onset is less than 45. The positive family

history and early age of onset indicate that genetic factors play a role in this RLS phenotype (Allen and Earley 2000). RLS familial aggregation accounts for up to 65% of reported cases (primary), with inheritance following an autosomal dominant pattern in at least one-third of familial cases (Montplaisir, Boucher et al. 1997; Ondo, Vuong et al. 2000). Secondary RLS is also known as "symptomatic RLS" or "sporadic RLS". The name "secondary RLS", represents cases of RLS where age of onset is usually greater than 45 years and there is no familial link with the syndrome. This indicates that environmental factors play a role in this phenotype. The sudden and late onset of the disease usually occurs secondary to other conditions, particularly iron-deficiency anemia and end-stage renal disease. Familial (primary) and sporadic (secondary) RLS cases present similar signs, symptoms and clinical course. However the symptoms of secondary RLS are usually resolved when the underlying conditions are corrected.

Epidemiological studies indicate that the symptoms of RLS are present in about 5-10% of the general population. Despite this high frequency of affected individuals, experience suggests the disease is often misdiagnosed, under-diagnosed or undiagnosed. It follows that there is a need to understand more about RLS and increase awareness of how it is most likely to present in a medical practice.

1.2 Diagnosing RLS

1.2.1 Clinical features of RLS

In 1995, the International Restless Legs Syndrome Study Group (IRLSSG) developed standardized criteria for the diagnosis of restless legs syndrome. Since that time, additional scientific scrutiny and clinical experience led to a better understanding of the condition. Subsequently a consensus conference held at the NIH in conjunction with the IRLSSG, resulted in revised diagnostic criteria for RLS to replace the previously proposed criteria. The primary revision from the previously proposed criteria include four criteria that are essential for the diagnosis of RLS in cognitively intact adult, three supportive criteria which may support the diagnosis in uncertain clinical cases, and three additional features of the disorder which deserve consideration when evaluating the patient with a potential diagnosis of RLS (Allen, Picchietti et al. 2003). These criteria are outlined in Table 1.1 below.

Table 1.1**:** The clinical features and diagnostic criteria for RLS

Essential Diagnostic Criteria for RLS
An **urge to move the lower limbs**, usually accompanied or caused by uncomfortable and **unpleasant sensations** in the lower limbs (sometimes the urge to move is present without the uncomfortable sensations and sometimes the arms or other body parts are involved in addition to the legs).
The urge to move or unpleasant sensations begin to **worsen during** periods of rest or **inactivity** such as lying or sitting.
The urge to move or unpleasant sensations are partially or totally **relieved by movement**, such as walking or stretching, at least as long as the activity continues.
The urge to move or unpleasant sensations are **worse in the evening** or at night than during the day or only occur in the evening or night (When symptoms are very severe, the worsening at night may not be noticeable but must have been previously present).

Table 1.1**:** The clinical features and diagnostic criteria for RLS (continued)

Supportive Clinical Features of RLS
• Periodic Limb Movements during Sleep (PLMS) Although the presence of PLMS is not specific to RLS an elevated PLM index (i.e. >5 movements/hr of sleep) is supportive of the diagnosis of RLS. Approximately 80% of patients with RLS have associated PLMS, which are usually present in the legs and less often in the arms. • Response to Dopaminergic Therapy Nearly all people with RLS show at least an initial positive therapeutic response to either L-dopa dopamine-receptor agonist at doses considered to be very low in relation to the traditional doses of these medications used for the treatment of Parkinson's disease. This initial response is not, however, universally maintained. • Family History The presence of RLS among first degree relatives is 3 to 5 times greater than in people without family history of RLS.
Associated Features of RLS
• Natural Clinical Course The clinical course of the disorder varies considerably, but certain patterns have been identified that may be helpful to the experienced clinician. When the age of onset of RLS symptoms is less than 50 years, the onset is often more insidious; when the age of onset is more than 50 years, the symptoms often occur more abruptly and more severely. In some patients, RLS can be intermittent and may spontaneously remit for many years.
• Sleep Disturbance Disturbed sleep is a common major morbidity for RLS and deserves special consideration in planning treatment. This symptom is often the primary reason the patient seeks medical attention.
• Medical Evaluation/ Physical evaluation The physical examination is generally normal and does not contribute to the diagnosis except for the identification of comorbid or secondary causes of RLS. Iron status, in particular, should be evaluated because decreased iron stores are significant potential risk factors that can be treated. The presence of peripheral neuropathy and radiculopathy should also be determined because the conditions have a possible, although uncertain, association with RLS and may require different treatment.

The uncomfortable sensations occur only when the limbs are at rest for a length of time, and they are typically relieved by movement. Individuals declare that they experience an almost irresistible urge to move the limbs. Symptoms of RLS can be very severe e.g. during flights, train or car journeys, at the cinema or the theatre. Symptoms can be especially troublesome in the late evening when patients are preparing for or attempting to sleep. Sensations may last for hours and persist, in some unfortunate suffers, into the early hours of the morning. Individuals often have to get up and walk around a few times per night to get relief. This form of coping behaviour has been named 'Night-walker's syndrome'. Loss of sleep is a grim consequence both to patients and their partners (Ekbom and Ulfberg 2009).

In addition to the standard criteria, the IRLSSG also outlined diagnostic criteria for RLS in special populations including children, adolescents and the cognitively impaired elderly (Allen, Picchietti et al. 2003). If a clear verbal description cannot be given, the diagnosis in children can be supported by a positive family history or a PLM index >5 per hour in overnight polysomnography. On the contrary, in elderly patients with cognitive impairment, diagnosis may be difficult when memory or language problems prevent a proper history taking. In this patient group, the consensus paper advises referral for observation, e.g., leg kicking, holding the legs and rubbing them etc.

1.2.2 Assessment Scales

RLS is said to be a subjective disorder, therefore it may be difficult for examiners to evaluate the exact severity of symptoms in individuals. However, in 2003, the IRLSSG rating scale was established. This scale is an easily administered instrument, which can be applied to all patients with RLS. It contains 10 items that are completed by patients themselves assessing the frequency and severity of symptoms over the preceding week. Responses are graded from 0 to 4 (e.g. 0 = absence of symptoms, 4 = very severe symptoms), with a maximum total score of 40. It can be used to accurately measure disease severity for clinical assessment, research, or therapeutic trials (Walters, LeBrocq et al. 2003). It is said to be the gold standard for measuring symptom severity in patients with RLS. A different scale, The John Hopkins Severity Scale (JHSS), predominantly assesses the time of daily onset of RLS symptoms. Other scales are used to examine quality of life in RLS. A list of assessment scales used in RLS are listed in Table 1.2.

Table 1.2: Scales for assessing RLS (Hogl, Garcia-Borreguero et al. 2009).

Severity scales
IRLS (International RLS Study Group Severity Scale)
John Hopkins Severity Scale (JHSS)
Quality of life scales
RLS Quality of life (QoL) questionnaire by Kohnen
RLS QoL questionnaire by Allen
RLS-QLI instrument
Other scales for RLS
Clinical Global Impression, Patient Global Impression
RLS diagnostic index (RLS-DI)
Augmentation Severity Rating Scale of the European RLS study group

1.2.3 Diagnosis

According to the mentioned diagnostic criteria, the diagnosis of RLS is based primarily on the patient's history. Approximately 60-80% of all RLS cases are idiopathic (primary). However, it is necessary that secondary causes or RLS-associated conditions are considered, and addressed where possible. Neurological examinations and neurophysiological examinations along with laboratory tests may be carried out to identify signs or symptoms of secondary RLS (Odin, Mrowka et al. 2002). Such laboratory tests include: complete blood panel; renal parameters; iron, transferrin and ferritin levels.

The diagnosis of RLS is based primarily on clinical criteria. Nonetheless, some of the following instrumental tools may be useful in doubtful cases, to confirm the diagnosis, or to aid in differential diagnosis. Instrumental diagnosis however is not commonly used to diagnose RLS (Zucconi and Ferini-Strambi 2004).

Polysomnography (PSG) enables recording of PLMS and periodic/non-periodic leg movements during wakefulness, either before or after sleep onset. These movements may be detected by Electromyography (EMG) recordings, most commonly of the tibialis anterior muscle. It is advised that PLM scoring is carried out according to the American Sleep Disorder Association rules; per hour of sleep, a pathological value is defined as more than five PLM/hour of sleep (Chaudhuri 2003). In using the PSG for assessment it should be noted that not all patients with RLS have demonstrated a Periodic Limb Movement Index (PLMI) greater than five, PLMS are present in other sleep pathologies, and at least 30% of older people have a PLMS index greater than 5 (Zucconi and Ferini-Strambi 2004)

Suggested immobilization test (SIT) is a new tool to evaluate the effect of immobility on sensory and motor symptoms. The patient attempts to maintain a seated posture without moving their legs for a 60 minute period, while the anterior tibialis EMG is again used to detect PLM. A PLM measure, along with quantification of the sensory symptoms, allows discrimination between RLS patients and controls (Michaud, Chabli et al. 2000).

Actigraphy is a method used to monitor muscle activity during the night, using an ankle placed portable activity meter. It has certain advantages such as avoiding the need for laboratory studies, allowing monitoring in the patients home, and increased accuracy and sensitivity since recordings can be made over multiple nights. However, the majority of actigraphical devices cannot differentiate between PLM and other involuntary movements associated with apnoea (Hening, Walters et al. 1999).

Other methods used to evaluate RLS include: Singe Photon Emission Tomographic (SPECT), Position Emission Tomographic (PET), Magnetic Resonance Imaging (MRI) and functional MRI.

1.2.4 Co-Morbid conditions & Differential diagnosis

The majority of RLS cases appear idiopathic, however a vast number of co-morbid conditions have been described in association with RLS. The majorities of these co-morbid conditions, with a few exceptions, have an unknown relationship to RLS, and most likely represent coincidental occurrence.

In recent years, several studies identified a higher prevalence of RLS in patients with migraine (d'Onofrio, Bussone et al. 2008). Migraine has been reported to have an association with poor sleep quality. It is uncertain if RLS is also associated with other primary headache disorders, however, it was found that the frequencies of RLS in patients with migraine were higher in those with tension type headaches or cluster headaches (Chen, Fuh et al. 2010). This study also demonstrated co-morbid RLS in migraine patients worsened sleep quality. It is thought that a shared underlying mechanism, related to the dopaminergic system, may account for the association between migraine features and co-morbid RLS.

Contrary to co-morbidity issues, differential diagnosis should distinguish RLS from conditions related to movement as well as sleep disorders. Conditions that can often be confused with RLS include those with prominent sensory symptoms in the legs such as neuropathies (patients complaining of numbness, tingling or pain), nerve conditions due to compression (meralgia paraesthetica), and radiculopathies. Several other conditions relating to motor restlessness and /or sensory symptoms include: painful legs and moving toes with segmental and semi-continuous distal movements during wakefulness, continuing during sleep and not alleviated by movements; neuropathic pain syndromes not consistently associated with rest and sleep; akathisia (excessive movement) which does not correlate with rest or time of day, which may

result from medications such as neuroleptics or other dopamine blocking agents; perplexing restlessness associated with insomnia, anxiety and panic attacks; nocturnal leg cramps unrelated to sensory symptoms or the urge to move the legs during the evening or the day, and different from nocturnal myoclonus; and propiospinal myclonus which is associated with relaxed wakefulness and recognized by axial and diffuse myoclonic jerks (Chaudhuri 2003; Garcia-Borreguero, Odin et al. 2004; Zucconi and Ferini-Strambi 2004). Apart from PLMS, most forms of "myoclonus" are either shorter in duration, suppressed during sleep or have a very different periodicity (Montplaisir 2004). Vesper's Curse, which is the combination of mild underlying congestive heart failure and lumbar stenosis, often causes nocturnal pain in the lower limbs and should be regarded in the differential diagnosis (Stiasny, Oertel et al. 2002). The majority of these conditions may not present with an urge to move the legs, may be present throughout the day, and symptoms may not be consistently worse in the evening or at rest. Nonetheless satisfying the four RLS diagnostic criteria outlined above will typically distinguish RLS dysthesias from other types of leg discomfort.

1.3.5 Diagnostic rates

With regard to the rates of diagnosing, the Dortmund Health Study, which was a population-based survey in Germany (n=1312), conducted face-to-face interviews in randomly selected individuals aged 25-75 years (Happe, Vennemann et al. 2008). They reported that the overall prevalence of people with a known doctor diagnosis of RLS was 2.3%; however, a further 6.5% fulfilled the four minimal criteria yet were not aware of their RLS. The overall prevalence was 8.8% in this sample, with a ratio of diagnosed:undiagnosed RLS of 1:3.

In the European context, the lowest diagnostic rate for RLS was reported from the UK. In a general practice research database involving 1,516,692 individuals, the prevalence of RLS with a registered diagnosis of RLS was only 0.25%. Only 0.6% of these patients had received a prescription for levodopa or a dopamine agonists, while many were prescribed medications not effective in RLS (e.g. oxerutins and quinine)(Van De Vijver, Walley et al. 2004). These findings are further supported by the French epidemiological survey where 60% of the RLS affected individuals had previously received a vascular diagnosis (Tison, Crochard et al. 2005) and an Irish study reported that only 16.7% of RLS sufferers were diagnosed with RLS (O'Keeffe, Egan et al. 2007). Furthermore, these findings are supported by the RLS epidemiology, symptoms and treatment (REST) studies where patients with RLS had been given a diagnosis of lower back pain, depression, spinal problems, depression, anxiety, peripheral neuropathy and were transferred to psychiatrists, vascular surgeons and orthopaedics (Hening, Walters et al. 2004).

1.3 Epidemiology

At least in Western countries, prevalence rates of RLS reveal this disorder as one of the most common neurological movement disorders. From the initial work of Ekbom to the end of the 1980s, a prevalence rate between 1 and 5% was estimated in the general population (Garcia-Borreguero, Odin et al. 2004). However, these studies were very limited and comprised of imprecise diagnostic criteria. Diagnostic criteria for RLS has provided data that is more detailed and population-based studies have shown that RLS affects 5 to 15% of the general population (Table 1.3). It has been noted however that prevalence estimates in the general population do not overlap across certain studies. Such discrepancies may be due to the different targeted patient population, the fluctuating course of initial symptoms and the numerous methodological tactics used such as telephone interviews, questionnaire surveys, direct observations and face-to-face interviews. It was found that in certain studies in western countries that used only the International Restless Legs Syndrome Study Group (IRLSSG) criteria the RLS prevalence rates ranged from 7.2 to 11.5% (Allen, Walters et al. 2005; Bjorvatn, Leissner et al. 2005).

In Western countries there is a female preponderance ranging from 13-17% (Lavigne and Montplaisir 1994) to 13.8% (Rothdach, Trenkwalder et al. 2000). A number of factors have been considered in explaining this sex difference. Firstly, patterns of inheritance may be a reason for female dominance. There will be a disproportionate number of women with RLS if the transmission of RLS is sex-linked and compromises females. A second contribution to the increased numbers of women in treatment studies may be a result of a gender bias in co-morbid medical disorders. Depression, anxiety and other common psychological disorders have a higher

prevalence in women. Such disorders may intensify the symptoms of RLS (Rothdach, Trenkwalder et al. 2000), or provide a secondary cause for insomnia, thus increasing the likelihood of RLS being reported. A third reason for the gender bias may be related to the impact of RLS on sleep. The gender bias in sleep complaints with RLS correlates with the gender bias found in other forms of insomnia. It was therefore proposed that female subjects with RLS are more likely to have increased sleep disturbances than their male counterparts (Bentley, Rosman et al. 2006). Furthermore it has been shown that female preponderance may be related to parity. Interestingly, nulliparous women had the same risk for RLS as age-matched men, whereas the risk for RLS increased gradually for women with one child (OR, 1.98; 95% CI, 1.25-3.13), two children (OR, 3.04; 95% CI;2.11-4.40), and three or more children (OR, 3.57;95% CI, 2.30-5.55)(Berger, Luedemann et al. 2004).

While RLS can occur in all ethnic backgrounds, epidemiologic studies suggest ethnic variation. Asian studies reported a prevalence rate of 0.6% in a selected healthy general population 55 years of age and older (Tan, Seah et al. 2001). A similar low prevalence rate of 1.5% and <1% was also reported in Japan (Mizuno, Miyaoka et al. 2005) and Singapore, respectively. Prevalence estimates also vary in Western countries; for example, prevalence is considerably higher in Norway (14.3%) than in Denmark (8.8%) (Bjorvatn, Leissner et al. 2005). Regional variations may be caused by environmental factors, awareness or the complex influence of variable genetic susceptibility.

Prevalence of RLS also increases with age (Lavigne and Montplaisir 1994; Allen, Walters et al. 2005). RLS is often associated with diseases of middle to old age. However, assessments indicate that the onset of the syndrome may occur before the

age of twenty years in up to 43% of adult cases (Chaudhuri, Appiah-Kubi et al. 2001). Thus far, no definite age of onset can be determined. Onset before 18 years of age has been reported (Walters, Hickey et al. 1996). However, children with RLS often describe symptoms that do not meet IRLSSG criteria but rather correspond to growing pains. One study, which used the newly established diagnostic criteria for RLS in children (Allen, Picchietti et al. 2003), concluded a prevalence rate of 5.9% in children (Kotagal and Silber 2004). Table 1.3 summarises the population prevalence studies which were carried out in recent years.

Table 1.3: Population prevalence of RLS (Mata, Bodkin et al. 2006).

Reference	Population	N/age	Frequency	Comments
Ekbom (1960)	Sweden	n=500	~5%	Healthy controls
Strang (1967)	Australia	n=320	2.5%	Relatives of P.D.
Lavigne (1994)	Canada	n=2019	0-15%	Face to face interview
Kageyama (2000)	Japan	n=4612	5-15%	Questionnaire survey
Rothdach (2000)	Germany	n=369	9.8%	Face to face interview
Berger (2004)	NE Germany	n=4310	10.6%	Face to face interview
Henning (2004)	Europe & N. America	n=23,052	11.1%	Questionnaire survey
Allen (2005)	Europe & N. America	n=15,391	2.7%	Face to face interview
Hogl (2005)	N. Italy	n=701	10.6%	Face to face interview
Bjorvtan (2005)	Denmark	n=2005	11.5%	Telephone interview
Hadjigeorgiou (2007)	Central Greece	n=3033	3.9%	Face to face interview & physical examination
Ulfberg (2007)	Sweden	n=1000	5.0%	Telephone interview
Happe (2008)	Germany	n=1312	8.8%	Face to face interview

(Lavigne and Montplaisir 1994; Kageyama, Kabuto et al. 2000; Rothdach, Trenkwalder et al. 2000; Berger, Luedemann et al. 2004; Hening, Walters et al. 2004; Allen, Walters et al. 2005; Bjorvatn, Leissner et al. 2005; Hogl, Kiechl et al. 2005; Hadjigeorgiou, Stefanidis et al. 2007; Ulfberg, Bjorvatn et al. 2007; Happe, Vennemann et al. 2008).

1.4 Pathophysiology

The pathophysiologic basis of RLS has not been fully elucidated. However, multiple etiologies including abnormalities of the central and peripheral Nervous System (CNS and PNS), vascular, metabolic, and genetic components have been proposed (Chaudhuri, Appiah-Kubi et al. 2001). In determining the pathophysiology of RLS, the primary areas of interest may be divided into three main themes, CNS dysfunction, the dopaminergic system and iron insufficiency. The genetics of RLS is discussed separately (section 1.9).

1.4.1 Localisation of CNS dysfunction in RLS

One important cue to pathophysiology may be the immediate and almost complete therapeutic response of RLS to dopamine treatment (Hening, Allen et al. 1999; Hening, Walters et al. 2004). This indicates rather clearly that RLS results from abnormal functioning of the CNS rather than the PNS. Treatment with centrally active dopamine antagonists causes intensification of RLS symptoms and thus the reversal of the dramatic benefits from treatment. However, such a striking effect is not experienced when treated with a peripherally active dopamine antagonist (Allen and Earley 2001). Although it is clear that RLS dysfunction involves the CNS, the exact areas of the CNS involved are somewhat unclear. So far no structural abnormalities have been noted in MRI imaging. However, Bucher and associates localized for the first time cerebral generators associated with sensory leg discomfort and periodic limb movements in 19 patients with restless legs syndrome, using high-resolution functional magnetic resonance imaging. It was therefore proposed that and thalamic activation may occur because of sensory leg discomfort and that the red nucleus and brainstem are involved in the generation of periodic limb movements in

patients with restless legs syndrome (Bucher, Seelos et al. 1997). These results correspond with the absence of a cortical prepotential in patients with RLS before they experience PLMS providing further confirmation of a subcortical site (Stiasny, Oertel et al. 2002). The subcortical dopamine systems, in particular, have been suggested as candidates given their involvement in sensory and motor regulation, including modulation of spinal activity.

Transcranial magnetic stimulation studies have also found that, compared with controls, RLS patients show reduced intracortical inhibition for both foot and hand, as well as an increased cortical silent period without other changes. These findings also suggest abnormal (i.e., subcortical) functioning of the CNS (Bucher, Trenkwalder et al. 1996).

1.5 Dopamine Dysfunction in RLS

Dopamine (DA) is a neurotransmitter that is involved in behaviours such as movement, motivaton, cognition, reward and feeding. Dopamine has various functions within the CNS, including regulation of sensory and motor functions. Dopaminergic systems have been connected with a number of movement disorders, such as Tourette's syndrome, Parkinson's disease and Huntington's corea. In addition, animal studies have supported suggestions that the dopaminergic system is involved in the regulation of sleep (Bagetta, De Sarro et al. 1988).

1.5.1 Dopamine pathways

Three dopamine pathways are seen in the central nervous system (figure 1.1). The first is the nigrostriatal DA pathway. It originates in the substantia nigra pars compacta neurons and targets dorsal striatum. It is thought to be involved in modulating movement. The second pathway is the mesocorticolimbic DA system, which originates in the midbrain ventral tegmental area (VTA) and targets the ventral striatum (nucleus accumbens), the limbic system, the hippocampus, the prefrontal cortex, and modulates cognition, emotion and reward. The third pathway is the tuberoinfundibular pathway which originates in the hypothalamic paraventricular and acuate nuclei and targets the anterior pituitary where is inhibits prolactin release.

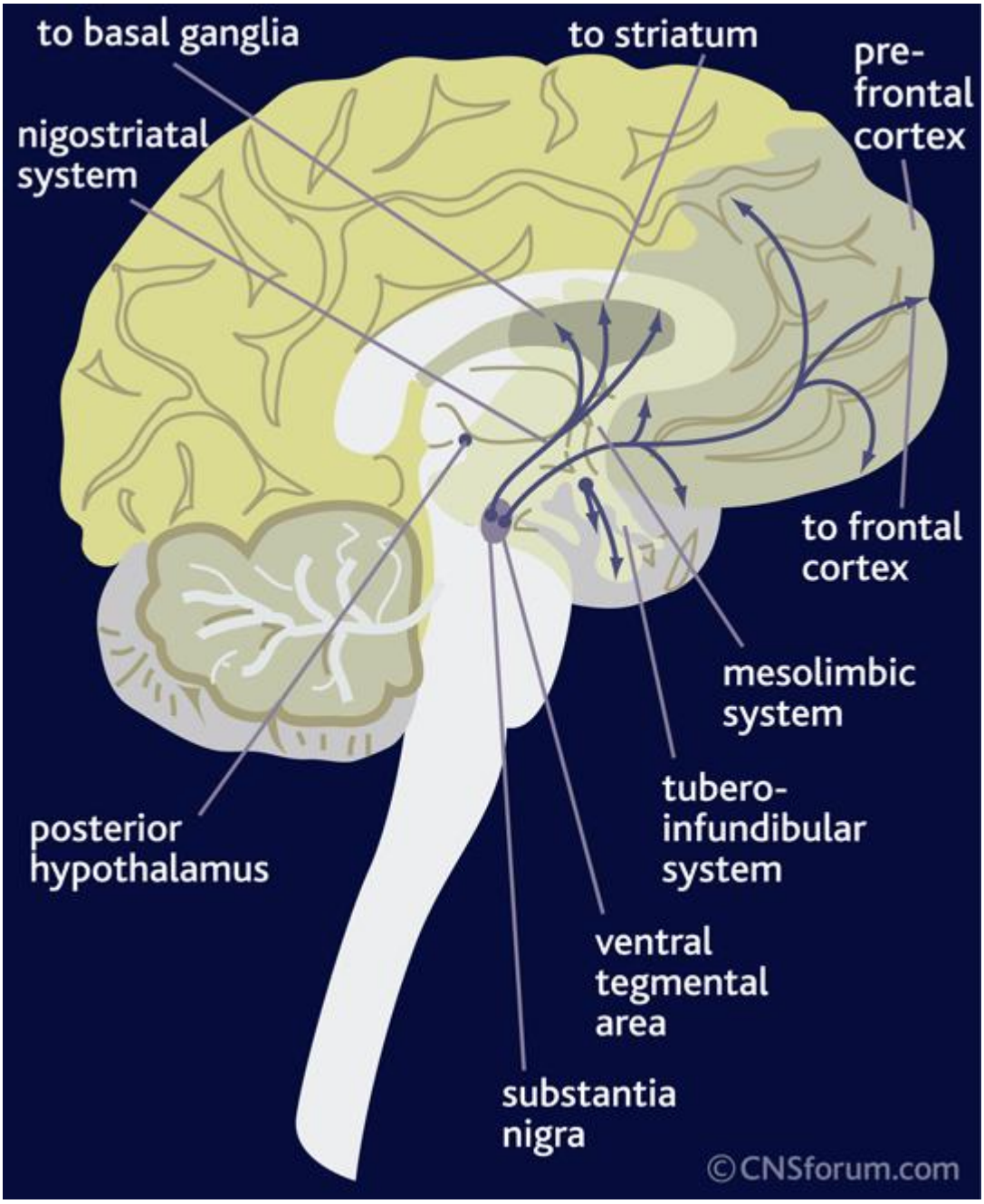


Figure 1.1: Dopamine Pathways

The three dopaminergic pathways; the nigrostriatal pathway which originates from the substantia nigra, the mesocorticolimbic pathway which originates from ventral tegmental area, and the tuberoindundibular pathway which originates from the hypothalamus (http://www.cnsforum.com/imagebank/item/Neuro_path_DA/default.aspx).

Tyrosine hydroxylase (TH) is the rate limiting enzyme in dopamine biosynthesis, which converts the amino acid tyrosine to 2,3-dihydroxyphenylalanine (DOPA). The conversion of DOPA to dopamine is catalysed by the enzyme DOPA decarboxylase (DDC) (figure 1.2).

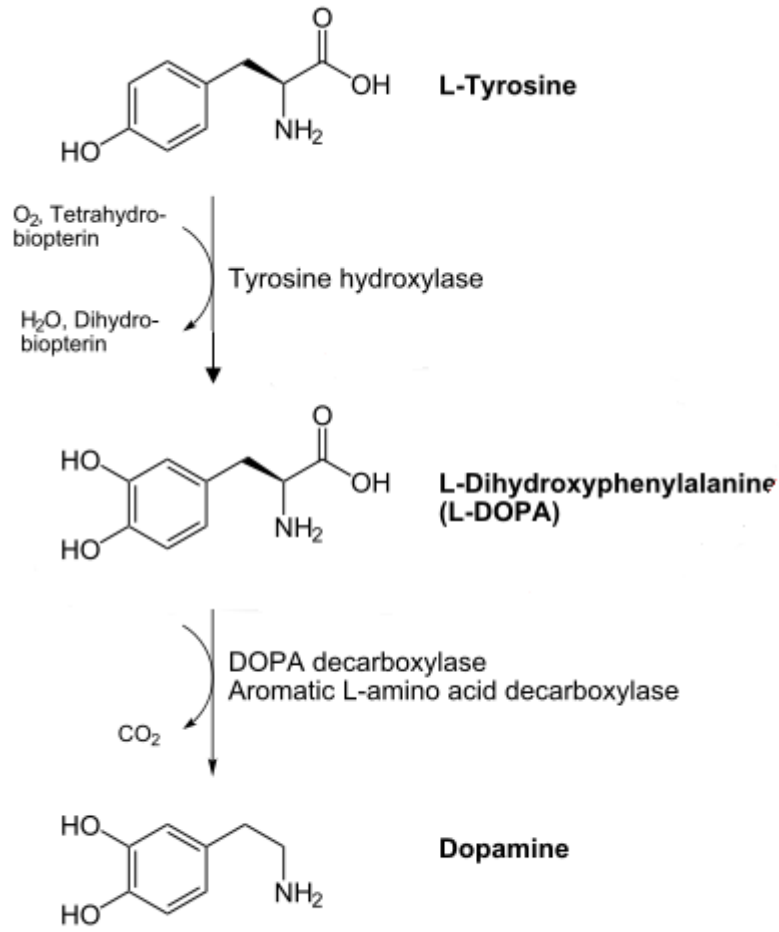


Figure 1.2. Dopamine Biosynthesis
A diagram of dopamine biosynthesis from tyrosine (http://scienceblogs.com/neurotopia).

Dopamine is moved into vesicles by the vesicular monamine transporter (VMAT), after being synthesised. The vesicles fuse with synaptic membrane with the electrical firing of the neuron, thus releasing DA into the synaptic cleft. DA then acts on pre- and post-synaptic DA receptors. There are at least five subtypes of DA receptors; D1, D2, D3. D4 and D5. They are divided into D1-like (D1, D5) and D2-like (D2, D3, D4) classes. The D2-like receptors have a high affinity for DA and dominate while asleep when DA levels are low. On the contrary D1-like receptors have a low affinity for DA and function while awake (during the day). The dopamine transporter (DAT) is a membrane spanning protein which terminates dopamine action. This rapidly reuptakes DA into the pre-synaptic terminal. Catechol-O-methyltransferase (COMT) also terminates DA activity by degrading it into homovanillic acid. Similarly, monamine oxidase (MAO) has the ability to degrade dopamine to dihydroxyphenylacetic acid (DOPAC).

1.5.2 Evidence for the involvement of Dopamine in the pathophysiology of RLS

Akpinar first suggested the dopaminergic deficiency theory of RLS in 1982. Pharmacologic and neuroimaging studies have subsequently provided evidence of the involvement of the central dopaminergic system, the striatonigral system particularly implicated.

Parkinson's Disease (PD)

RLS and Parkinson's disease (PD) both respond positively to dopaminergic treatments, both show dopaminergic abnormalities on functional imaging and are both associated with PLMS. RLS significantly improves with dopaminergic medications, usually at doses lower than those required to treat Parkinson's disease. Owing to the manner in which RLS responds to these medications, and observed coexistence within the same patients, an etiologic link between RLS and PD has been proposed.

The phenomenon of rebound and augmentation in RLS patients is essentially similar to fluctuations in motor responses and dyskinesias that can be observed in PD following long-term levodopa treatment (Appiah-Kubi, Pal et al. 2002). Ondo and colleagues (2002) suggested that RLS is not a risk factor for developing PD, as they observed that PD symptoms preceded RLS symptoms in 68% of cases (Ondo, Vuong et al. 2002). The study found a high prevalence of RLS symptoms; however, it failed to address the matter of whether or not they occur more often in PD patients than in the general population. Another population-based study with a larger sample did not find parkinsonian symptoms among RLS patients (Rothdach, Trenkwalder et al.

2000). The prevalence of RLS in PD patients is similar to that of the general population (Appiah-Kubi, Pal et al. 2002). In PD, the olfactory function is abnormal whereas it is normal in patients with RLS, thus reflecting the integrity of the A16 dopaminergic group (Adler, Gwinn et al. 1998). In addition, in patients with PD, an increase in total iron concentration in the Substantia Nigra (SN) was demonstrated by both pathologic and MRI approaches, but the opposite was demonstrated in RLS (Barriere, Cazalets et al. 2005). For these reason it has been suggested that the two diseases do not share the same pathophysiological mechanisms. Nonetheless, the positive response of RLS patients to dopmainergic treatments presents a strong argument in favour of the dopaminergic system involvement in the pathogenesis of RLS.

1.5.3 Central Nervous System (CNS)

Cerebrospinal fluid (CSF)

The dopmaine system has also been explored by measuring the metabolites of dopmaine synthesis and the degradation of CSF. It has been suggested that the dopamine system is largely involved in the circadian pattern of RLS (Section 1.5.1). Lower dopamine and homovanillic acid levels in nocturnal urinary excretion have been found in subjects with PLMS. Larger circadian changes in CSF dopamine metabolites have been reported in RLS patients compared to controls: in patients, all metabolites were higher in the morning (at 10am) compared to the evening values (at 10p.m.) values (Earley, Hyland et al. 2006).

Neuroimaging studies:

Some positron emission tomography (PET) and single-photon emission computed tomography (SPECT) studies have suggested mild but significant pre- and post-synaptic dopamine receptor abnormalities in the basal ganglia (Stiasny, Oertel et al. 2002), while other studies contradict these findings (Allen, Picchietti et al. 2003). Furthermore several studies have shown decreased striatal dopmaine uptake and dopaminergic (D2) striatal binding in RLS patients, while results of other studies have not demonstrated differences on functional imaging in RLS patients compared with controls (Trenkwalder, Walters et al. 1999; Turjanski, Lees et al. 1999). PET studies on 13 patients with idiopathic RLS showed significantly decreased D2 binding potentials in the caudate and putamen compared to controls (Michaud, Soucy et al. 2002). Similarly in a SPECT study the dopamine status was assessed in 10 RLS/PLMS patients, and significantly lower striatal D2 binding was observed in patients compared to controls (Ruottinen, Partinen et al. 2000). Furthermore, fluorodopa PET studies of 9 patients with RLS showed a statistically significant, 88% reduction in fluorodopa uptake in the caudate and an 89% reduction in the putamen compared to controls. These findings indicated striatal presynaptic and postsynaptic dopaminergic dysfunction in RLS (Trenkwalder, Walters et al. 1996). Differences in striatal dopamine transporter and dopamine D2-receptor binding on SPECT among 25 RLS drug-naive or L-dopa-treated patients and 10 controls could not be demonstrated in another study (Trenkwalder, Walters et al. 1999). Therefore brain-imaging studies of the dopmainergic system failed to reveal consistent abnormalities. Dysfunction of dopaminergic pathways other than the nigrostriatal axis, e.g the diencephalospinal system, may play an important role in the pathogenesis of RLS (Chahine and Chemali 2006).

1.5.4 Pharmocological evidence

The strongest argument for a dopaminergic role in RLS is the excellent response to low-dose dopaminergic agents (DA) (Hening, Allen et al. 1999). To date, all evaluated agents that enhance dopamine activity in the body have been found to reduce RLS symptoms in open-labelled uncontrolled trials. Double-blind, placebo controlled studies have demonstrated the treatment benefit for Levodopa (Brodeur, Montplaisir et al. 1988; Trenkwalder, Stiasny et al. 1995) and for the currently used major dopamine agonists, i.e. pergolide (Wetter, Stiasny et al. 1999; Trenkwalder, Hundemer et al. 2004), pramipexole (Montplaisir, Nicolas et al. 1999) and ropinirole (Bliwise, Freeman et al. 2005). These medications are effective at remarkably low doses relative to that given for Parkinson's disease, often at the minimum tablet size available to the patient e.g. carbidopa/Levodopa at 25/100 mg or pergolide at 12.5 mg (Allen 2004). Furthermore, they are effective immediately after the first therapeutic dose for almost all RLS patients. The immediate, universal and almost complete relief of RLS symptoms by low doses of Das provides strong support for a possible dopaminergic abnormality in this disorder.

Reversal of beneficial effects of dopamine agonists by antagonists further support the role of dopamine. This was first reported in a study published in 1991, when Pimozide, a dopamine agonist was shown to reverse treatment benefits of the opioid Codeine in an RLS patient (Montplaisir, Lorrain et al. 1991). In a later study, Metoclopramide given intravenously produced RLS symptoms on a suggested immobilization test in five out of six subjects evaluated and produced an increase in prolactin release that was at least three times greater than that expected from age-matched normals (Winkelmann, Schadrack et al. 2001).

1.6 Iron pathology in RLS

The connection between iron deficiency and RLS was first proposed in the 1950's when intravenous infusions of iron were used for the treatment of RLS. Norlander reported that the creeping sensations in the legs of his patients ceased with iron therapy (Norlander 1953). Two years later in 1955, K.A. Ekbom discussed the relationship between RLS and iron deficiency when he described two male patients with malignancies that were severely iron deficient and also suffered from RLS (Ekbom 1955). Ekbom also noted that one in four of his RLS patients were iron deficient and that the RLS was increased among blood donors. This hypothesis of the relationship between RLS and low iron levels has prompted numerous studies into the role of iron in RLS. Investigations have shown a correlation between low body stores of iron and symptoms of RLS.

1.6.1 Evidence for the involvement of iron in the pathophysiology of RLS

Pregnancy

Pregnant women have a two to three times higher risk of experiencing RLS than the general population (Manconi, Govoni et al. 2004). In 11-27% of pregnant women in Western countries, RLS symptoms are reported, despite supplementation with iron and folate. In a Japanese survey, a similar prevalence rate of 19% was reported (Suzuki, Ohida et al. 2003). Those women affected by RLS were also found to have a lower haemoglobin level and lower average red blood cell size, or Mean Corpuscular Volume (MCV), than those who did not experience RLS symptoms. In 2007, Tunc et al agreed that low iron levels were a risk factor for RLS in pregnant women. First signs and symptoms of RLS may only appear during pregnancy. However, pre-existing RLS is considerably exacerbated during pregnancy, mostly during the third trimester (Goodman, Brodie et al. 1988; Suzuki, Ohida et al. 2003; Manconi, Govoni et al. 2004). In the study carried out by Manconi and associates (2004), 16.7% of the women never experienced RLS symptoms before the pregnancy, whereas 9.9% had already experienced symptoms (Manconi, Govoni et al. 2004). In 2009 Dzaja et al found RLS to occur in some pregnant women during the third trimester, however this study concluded that transiently elevated Estradiol levels were responsible for triggering the RLS among this patient group (Dzaja, Wehrle et al. 2009). Notably, this study was conducted on a particularly small number of subjects, therefore analysis could not have fully assessed all of the factors influencing RLS symptoms. In 2008, Ghorayeb et al also discussed female hormonal changes as a factor in RLS symptoms severity, but on a larger number of patients,

and concluded that in those female patients with RLS, hormonal changes during pregnancy could not account for the worsening of symptoms (Ghorayeb, Bioulac et al. 2008). This study agreed with the findings by Manconi and associates that approximately 23% of women with RLS admitted worsening of symptoms during pregnancy and concluded that these changes in iron status could best explain these changes in symptom severity. Pregnancy related RLS is usually mild and transitory, with a tendency to disappear after delivery. One of the largest studies on RLS during pregnancy reported complete resolution of RLS in 96% of women 4 weeks after delivery (Goodman, Brodie et al. 1988).

Uremia and End-stage renal disease (ESRD)

One of the best-studied secondary causes of RLS is end-stage renal disease (ESRD) with dialysis. Reports found 62% of individuals with ESRD presented with mild RLS. Furthermore, prevalence rates of 20-25% were observed in patients with more severe RLS (Winkelman, Chertow et al. 1996; Hui, Wong et al. 2000). RLS symptoms with ESRD have been associated with increased mortality, with kidney transplantation resolving the renal disease and, also leading to complete resolution of RLS within 1-21 days after transplant (Allen 2004). Uremic patients who also were diagnosed with RLS had lower hemoglobin than those who did not have RLS (Takaki, Nishi et al. 2003). Moreover, RLS symptoms improved in patients on dialysis by regulation of anemia with erythroprotein and intravenous iron (Benz, Pressman et al. 1999). Another study linked low parathormone levels in uremic patients with RLS (Rijsman and de Weerd 1999). These studies show that it is the iron insufficiency associated with ESRD that produces the RLS symptoms.

Iron Deficiency

Anaemic patients have been studied and oral iron suuplements has been found to improve RLS symptoms in some of these patients (O'Keeffe, Gavin et al. 1994). In 2004, Earley et al showed successful treatment of 7 out of 10 RLS patients with IV iron treatment (Earley, Heckler et al. 2004).

Other conditions which have been investigated include low-density lipoprotein apheresis (Tings, Schettler et al. 2004), and these studies agree with the hypothese put forward by Allen et al in 2007 that "all conditions that compromise iron availability will increase the risk of RLS leading to a higher than expected prevalence of RLS in these conditions" (Allen and Earley 2007).

The hypothesis that iron status correlated with RLS symptom occurrence led to further studies of iron and how it affected RLS patients.

1.6.3 Peripheral Iron Status

Ferritin generally provides a measure of iron storage, with low values indicating low iron storage. Serum ferritin reflects the levels of iron in the peripheral stores and CSF ferritin reflects the levels of iron in the brain stores. Similarly, transferrin provides a measure of the tissues need for iron ,where the serum transferring reflects the peripheral tissue's iron needs, and the CSF transferrin reflects the brain's iron needs. As the iron requirements of a tissue increase, the level of transferring found in the fluid associated with that tissue also increase.

The serum ferritin values of patients believed to be iron deficient was compared to the iron status of their bone marrow in a study carried out by Guyatt and colleagues (Guyatt, Patterson et al. 1990). It was discovered that a serum ferritin of below 45 μg/L indicated that the patients peripeheral iron stores were depleted. Nonetheless it is important to keep in mind that ferritin has phase-reactive properties and can give falsely elevated results. Therefore measurements of percentage transferrin saturation and total iron binding capacity (TIBC) are more useful for clinically determining iron store status. TIBC is a measurement of the maximum amount of iron that the blood can carry. In 2006 a double-blind controlled study found that for those RLS patients with low ferritin values oral iron treatment was successful in treating their symptoms , thereby agreeing with the 1994 study by O' Keeffe et al (O'Keeffe, Gavin et al. 1994), but disagreeing with the findings of the 2000 study by Davis et al (Davis, Rajput et al. 2000). This study by Davis et al, found no improvement of RLS symptoms following administration of oral iron, however the study did not include a sufficient number of patients with low serum ferritin. The American National Heart, Lung and Blood institute suggest that in RLS patients with a serum ferritin of less

than 50 μg/L, iron deficiency can be implicated in the syndrome (National Heart 2000). Thereby in a 2004 study it was recommended that RLS patients with a serum ferritin of less than 50 μg/L should be considered for oral iron treatment (Silber, Ehrenberg et al. 2004).

1.6.4 Central Nervous System (CNS)

Autopsy Studies

Generally when cells are iron deficient they respond to this deficient state by increasing the quantity of Transferrin Receptors (TfR) on their surface, thereby increasing the chance that transferrin will bind to the cell and supply iron (Aisen, Enns et al. 2001). The ability to either increase or decrease the cells TfRs concentration is achieved by post-transcriptional regulation by two iron regulation proteins (IRPs); IRP1 and IRP2.

Autopsy studies which have been performed so far have focused primarily on the substantia nigra. This is due to its dopmaine producing activity. These autopsy studies have demonstrated the following:

a) Neuromelanin cells are the darkly pigmented neuronal cells present in the brain. These cells produce monoamine neurotransmitters e.g. dopamine. The neuromelanin cells of the substantia nigra are similar in density between the brains of RLS patients and those of controls (Connor, Boyer et al. 2003).
b) Compared to the brains of control subjects, the neuromelanin cells of the substantia nigra of RLS patients have an increased concentration of Tyrosine Hydroxylase (Connor, Wang et al. 2004).
c) In comparison to the brains of control subjects, iron concentration in the neuromelanin cells of the substantia nigra of RLS patients is decreased (Connor, Boyer et al. 2003).
d) In RLS patient brains, IRP1 activity is decreased (Connor, Wang et al. 2004).

e) In RLS patient brains, IRP2 activity is normal/increased. (This may be a compensatory mechanism in response to the decreased IRP1) (Connor, Wang et al. 2004).

f) In comparison to the brains of control subjects , the expression of the TfR on the neuromelanin cells of the substantia nigra of RLS patients is decreased, thereby consistent with the reduced activity of IRP1 (Connor, Wang et al. 2004).

g) Thy-1 is a T-cell protein which is expressed on some neuronal cells i.e. at the dopmainergic synapses (Morris 1985). The function of Thy-1 is believed to be in the association of pre-synaptic and post-synaptic membranes, and the fusion of vesicles to the pre-synaptic membrane (Jeng, McCarroll et al. 1998). Thy-1 is responsive to iron status (Ye and Connor 2000). It is thought that a Thy-1 deficiency would lead to the decreased structural integrity of the cells on which it is expressed.

   It was found that Thy-1 expression is decreased not only in RLS brains but in models which were iron deficient. If this deficiency results in loss of structural integrity, this might explain why no morphological differences have been observed between the brains of RLS patients and controls, and the reason that RLS symptoms appear to improve with the treatment of dopmainergic agonists (Wang, Wiesinger et al. 2004).

Cerebrospinal Fluid (CSF)

CSF was utilised for the indirect measurement of brain iron status. As previously mentioned, in iron deficiency, serum ferritin levels are usually low and serum transferrin levels high. In 2000, Earley and associates carried out a study which analysed the CSF concentrations of ferritin and transferrin in RLS patients (Earley, Connor et al. 2000). They discovered that CSF ferritin concentrations were low and transferrin levels were high, thus indicating brain iron insufficiency in RLS patients. In 2005 these results were further verified (Mizuno, Mihara et al. 2005).

In the report published by Earley and associates in 2000, it was found that the CSF ferritin levels of the controls improved as their peripheral ferritin status moved closer to the normal 100 μg/L. However, the normal CSF ferritin concentrations observed in the control subjects with normal serum ferritin concentrations would only occur for the RLS patients with a serum ferritin of over 400 μg/L. This would be considered highly elevated in a normal population. Therefore one hypothesised that RLS patients had an impaired ability to get brain iron from peripheral iron, which could in turn be due to impaired iron transport in the brain and/or an impaired ability of the brain to retain iron.

Neuroimaging Studies

To date Neuroimaging studies include Magnetic Resonance Imaging (MRI) which measures the brain iron status; Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT), both which examine the dopaminergic system;.MRI studies have been performed to measure regional brain iron concentrations. These studies found that decreases in iron concentration in the

nigrostriatal areas correlate with RLS severity (Allen, Barker et al. 2001). The decrease in iron was most evident in patients with a severe form of RLS. This finding was confirmed in a large sample of subjects with early-onset RLS (Earley, B. Barker et al. 2006), as well as in other studies using a transcranial ultrasound method (Schmidauer, Sojer et al. 2005). A more recent MRI study showed that the decrease in brain iron insufficiency extended beyond the nigrostriatal areas, indicating that the iron deficiency was brain-wide, thus emphasizing the importance of correct interpretation of MRI results when assessing brain iron status (Lee 2007).

1.6.5 Role of Iron in the Dopaminergic Pathway

Iron distribution in the brain is highly heterogenous and is higher in the dopaminergic regions such as the substantia nigra and striatum. The strong evidence of the role of iron deficiency and the role of the dopaminergic system raises the question of the relationship between iron and dopamine. There are two leading arguments indicating that decreased iron would lead to abnormal dopaminergic transmission (Krieger and Schroeder 2001). Firstly, iron is a known co-factor for tyrosine hydroxylase, a rate-limiting enzyme involved in the synthesis of dopamine. This enzyme catalyses the hydroxylation of troine into dihydroxyphenylalanine (DOPA) which is ultimately transformed into dopamine. The second is that iron is involved in the physiological regulation of the dopamine D2 receptor binding sites and the effect of iron deficiency is specific for these receptors, however, the exact mechanism remains to be resolved (Earley, Allen et al. 2000). Iron deprivation in rats results in a 40-60% reduction of D2 postsynaptic receptors, while dopamine type-1 receptors are not affected (Erikson, Jones et al. 2001).

1.7 Circadian Pattern of RLS

RLS symptoms display a true circadian pattern, with an increase in both sensorial and motor symptoms occurring most frequently in the evening and at night. Typically, the untreated patient reaches a maximum symptomology between 11pm and 4am, with the symptoms being reduced between 6am and 12pm (Garcia-Borreguero, Larrosa et al. 2002). Since the dopamine system is proposed to play a central role in the pathophysiology of RLS, it is likely that alteration in the circadian variation of the dopaminergic system or related compounds might account for RLS symptom fluctuations. For example, changes in melatonin secretion, as a maker of the circadian rhythm, preceed the increase in sensory and motor symptoms in patients with the syndrome (Trenkwalder, Paulus et al. 2005). Nevertheless, the circadian rhythymicity is overridden and symptoms are present 24 hours a day in patients with increased disease severity.

In 2009, Sharifan et al published a study which investigated the relationship between shift work patterns and the occurrence of RLS. The study was conducted in an automobile manufacturing factory in Iran. During the study they noted that there was a greater percentage of antihitamanic and antidepressant drug consumption, a higher percentage of chronic disorders and a higher percentage of smokers among the RLS individuals than in controls. The study found that the prevalence of RLS was significantly higher in rotational shift workers (15%) than in worker with permanent morning work schedules (8.5%). Work during the night shift is much more likely to have adverse effects on health than work during morning or afternoon shifts because the circadian organisation of the body is disrupted. This indicates that the association

between rotational shift work and RLS is most likely mediated by disruption of circadian rhythmicity (Sharifian, Firoozeh et al. 2009).

1.8 Periodic Limb Movements during Sleep (PLMS)

Periodic limb movements during sleep (PLMS) have been classically described as rhythmic extension of the big toe and dorsiflexion of the ankle, with occasional flexion at the knee and hip (Allen, Picchietti et al. 2003). PLMS may be accompanied by brief awakenings that result in sleep fragmentation and subsequent excessive daytime sleepiness. Diagnosis of periodic limb movements during sleep is based on the definition of the American Sleep Disorders Association (Trenkwalder, Paulus et al. 2005). These movements are measured by surface electromyography from the tibialis muscle and are scored only if they occur in a series of four consecutive movements lasting 0.5-5 s and recurring at intervals of 5-90 s. The muscle contractions must have amplitude of at least 25% of the toe dorsiflexion during calibration. An index (number of PLMS per hour of sleep) greater than five for the entire night is considered pathologic.

The presence of PLMS in patients with RLS was first documented in 1965, and was originally called "nocturnal myoclonus". Since then, several studies have been carried out, and it was estimated that about 80% of patients with RLS have periodic limb movements during sleep (Montplaisir, Boucher et al. 1997). A recent study carried out by Boehm et al., (2008) characterised PLMS and their association with sleep disturbances in RLS patients and healthy controls without sleep complaints (Boehm, Wetter et al. 2008). Measures of PLMS showed that the RLS patients have a significantly longer mean duration of single PLM during wakefulness and non rapid eye movement (NREM) sleep. For future studies this mean duration of single PLM might be an appropriate parameter to discriminate between RLS patients and

healthy subjects with PLM. Decreased sleep efficiency was associated with a higher number and shorter duration of PLM sequences (Boehm, Wetter et al. 2008).

It should be emphasized that RLS remains a distinct clinical diagnosis. However, PLMS occur frequently in several other sleep disorders, including narcolepsy, rapid eye-movement sleep behaviour disorder, and obstructive sleep apnoea; as well as an isolated occurrence (Trenkwalder, Paulus et al. 2005). PLMS also appear more abundant among the elderly, possibly indicating a general aging process. Therefore, although the presence of PLMS is not specific to RLS, an elevated PLMS is supportive of the diagnosis of RLS. Effective treatments for RLS generally decrease episodes of PLMS.

1.9 Genetics of RLS

1.9.1 Familial Aggregation

Primary (genetic or familial) RLS may be difficult to distinguish from RLS associated with uraemia, iron deficiency, neuropathy, or other disorders (secondary RLS). Ekbom first described the familial component of RLS in 1945, estimating the frequency of hereditary RLS compared to sporadic cases as 'one-third' (Ekbom 1945). In 1960, Ekbom also reported that 49 out of 112 unselected probands with severe, typical RLS knew of one or several close relatives with creeping sensations in their legs (Ekbom 1960). Since then many studies have been published on the substantial genetic contribution in the aetiology of this sleep disorder. Clinical surveys have shown that up to 60% of idiopathic RLS patients report a positive family history (Walters, Hickey et al. 1996). Furthermore, RLS is 3-5 times greater amongst first degree relatives of subjects suffering from RLS than relatives of subjects without RLS. Clinical manifestations and the course of the disease appear similar in familial and sporadic cases (Winkelmann, Wetter et al. 2000). However, familial RLS undoubtedly correlates with an earlier age at onset, a much slower progression of symptoms, and a limited relation to serum iron status in contrast to late-onset RLS patients (Walters, Hickey et al. 1996; Allen and Earley 2000).

One of the best methods for evaluating the genetic and environmental contributions to a disease is twin study. Two twin studies have been published to date (Ondo, Vuong et al. 2000; Desai, Cherkas et al. 2004). One study examined twelve pairs of monozygotic (MZ) twins in which both members of ten pairs had definite RLS based on the IRLSSG criteria. The high concordance rate suggested, similar to other investigations, an autosomal dominant mode of inheritance with a high penetrance.

Despite this, however the symptom descriptions and age of symptom onset varied markedly (Allen and Earley 2001). Desai and associates evaluated RLS symptoms in 933 MZ pairs and 1004 dizygotic (DZ) pairs. Concordance rates were found to be 61% and 45% respectively (Desai, Cherkas et al. 2004).

1.9.2 Pattern of Inheritance

Pattern of inheritance or mode of inheritance, refers to the way in which genes are passed down from generation to generation. By analyzing the pattern of inheritance, the relative risk for and penetrance of an illness can be estimated. An autosomal dominant mode of inheritance with variable expressivity was suggested following several investigations of single families with RLS (Walters, Picchietti et al. 1990; Trenkwalder, Seidel et al. 1996). The possibility of anticipation – i.e. the disease starts earlier with each new generation – has been described in three large pedigrees of familial RLS. In 1996, Trenkwalder et al., (1996), demonstrated evidence of anticipation in one large German pedigree. In this German kindred which included 20 affected members, the mean age of onset fell from 51.5 years in the second generation to 19.8 years in the fourth generation (ANOVA, $p = 0.025$) (Trenkwalder, Seidel et al. 1996).

1.9.3 Linkage studies

To identify the gene(s) involved in a disorder the most powerful method is pedigree analysis. This involves using linkage statistics to compare the distribution of disease inheritance in high-risk families with the inheritance of a specific chromosomal region (locus) and disease-causing gene mutations. Linkage studies typically use polymorphic microsatellite repeat markers, spaced evenly at about 10 cM (10 Mb) intervals. A likelihood of ~3.3 (described in section 1.12), at or adjacent to a marker in a genome wide study, is considered significant evidence that a disease and a chromosomal region co-segregate within a family (P-value <0.05). Linkage studies in families with RLS have identified ten loci that are associated with RLS, however no causal sequence variant has yet been recognised.

The first major susceptibility locus for RLS was identified in a French-Canadian family in 2001 (Desautels, Turecki et al. 2001). About 380 genetic makers spaced throughout the genome, were analysed in 25 individuals (14 affected, 4 at risk and 7 healthy). The locus was found on the long arm of chromosome 12 and connected with a series of adjacent microsatellite markers with an autosomal recessive mode of inheritance. The linkage was followed up by a study of 276 individuals from 19 families. Five of these lineages, which had a French-Canadian origin, were consistent with the chromosome 12q linkage. The results also indicated the presence of other loci involved in this disorder due to six of the larger pedigrees not demonstrating any linkage to chromosome 12 (Desautels, Turecki et al. 2005). Furthermore a linkage signal was found in the Icelandic population (D.B. Reye 2005), and in a number of German families using the transmission disequilibrium test (Winkelmann, Lichtner et al. 2006).

Almost two years later, a second locus was identified on chromosome 14. In this study, 24 individuals (13 affected) in a large family from Northern Italy were genotyped for 382 polymorphic microsatellite markers. Five potential candidate loci were recognized. However, only a locus on chromosome 14 remained significant following the incorporation of more markers and increasing the number of individuals analysed. An autosomal dominant model was obtained, with variable penetrance depending on whether patients have periodic limb movements with or without RLS symptoms. The locus, which spans 9.1 cM on chromosome 14, contains at least 60 genes (Bonati, Ferini-Strambi et al. 2003).

In 2004, Chen and associates identified significant linkage to a third locus on the short arm of chromosome 9 (Chen, Ondo et al. 2004). In this investigation, 15 multiplex American families were genotyped for more than 400 markers and significant linkage to a third locus was found in two of the larger families. Chen and colleagues (2004) used an autosomal-dominant model-based analysis for this study. This study represented the first model-free linkage analysis used to genetically dissect RLS. The locus spans 34.43 cM and is located on chromosome 9p24-22.

In 2005, a large Irish family with autosomal dominant RLS was studied with the subsequent identification of a novel locus on chromosome 5q. Microsatellite markers spaced throughout the genome were analysed in 29 family members (12 affected). A genome scan for linkage was performed. Recombination events in two of the affected family members defined the flanking markers, thus placing the locus in an 11.5cM region. This interval is 16.8Mb long and contains approximately 120 known or predicted genes. The authors also highlighted four candidate genes for mutation analysis. These genes included the neurolysin (*NLN*) gene, which is involved in

central dopaminergic regulation; the *CART* gene involved in the regulation of dopamine levels; the *HTR1A* gene that encodes the 5-hydroxytryptamine (serotonin) receptor; and the *PMCHL2* gene, which exhibits diurnal variation and is active during certain phases of sleep (Parfrey et al., 2006).

In 2006, Levchenko and associates reported the first autosomal dominant locus in the French-Canadian population, mapping to chromosome 20p13. Desautels et al., had previously described an RLS locus in a French-Canadian population, but under an autosomal recessive model. Genomic DNA from fifty-six members of this French-Canadian family was used, and investigators performed a 10-cM genome-wide scan using a panel of 377 microsatellite markers. The chromosome 20 candidate region is flanked by the telomeric end of chromosome 20p and by a 0.56cM interval between markers D20S849 and D20S835. This region spans approximately 16cM or 5.2 Mb containing 84 annotated genes (Levchenko, Provost et al. 2006).

Later that same year Pichler et al., (2006) published a paper in The American Journal of Human Genetics providing strong evidence for the identification of a new susceptibility locus for RLS at 2q33. An isolated population in the South Tyrolean Alps was identified and 530 adults took part in the study, 37 of which were identified with having idiopathic RLS. A 4-cM genome-wide linkage scan was performed on the DNA from all the 530 participants and the data was analysed using both nonparametric and parametric analysis. A shared disease haplotype from two of these families defined a candidate region of 8.2 cM on chromosome 2q. This study illustrates the use of isolated populations i.e. the western Alps of South Tyrol (Italy), where there are few founders, expansion is slow, traditional lifestyle has been maintained, and historical documents are highly available and accessible. The use of

such population can reduce genetic complexity and potentially the environmental heterogeneity (Pichler, Marroni et al. 2006).

Two loci were identified in a single RLS family on chromosome 4q and 17p under the assumption of an autosomal-dominant model (Winkelmann J 2006). A genome-wide linkage analysis was conducted on a large RLS family of German origin. Two-point linkage analysis of this pedigree revealed a LOD score of 2.3 and 2.1 and a multipoint LOD score of 3.08 and 2.78 on chromosome 4 and 17 respectively. The disease associated region on chromosome 4 was refined between D4S406 and D4S402, defining a 8.4 Mb (7.39 cM) region. The disease associated region on chromosome 17 was located between markers D17S1857 and D17S1294, thus defining a 9.05 Mb (7.73 cM) region. The latter finding was replicated in an independent family-based linkage study of 159 RLS trios from 8 European countries.

A novel locus for RLS on chromosome 16p12.1 was reported by Levchenko and colleagues in October 2008 (Levchenko, Montplaisir et al. 2008). The locus was identified in a French-Canadian family and supporting evidence of linkage was presented in a smaller pedigree of similar origin. An overall candidate region of 1.18 Mb was revealed. This region was divided in two linked regions of ~850 kb and 350 kb each by an uncommon and rare double recombination in one individual. It was hypothesized that this recombination may have taken place in the situation of genomic rearrangement, e.g. duplication, therefore it was decided to follow with a karyotype analysis and copy number variation (CNV) analysis. Both analyses showed that the region has a normal chromosomal region. Furthermore, no disease-causing variants were uncovered following sequencing of eight positional candidate genes (Levchenko, Montplaisir et al. 2008).

Kemlink and associates (2008) provided suggestive evidence for a new susceptibility locus for RLS on chromosome 19p. A genome-wide linkage study was conducted in a large RLS family of Italian origin with 12 affected members in 3 generations. 5,861 SNPs were used initially, and 13 additional microsatellite markers on chromosome 19p and 7 markers on chromosome 10p were subsequently genotyped to confirm the SNP genotyping and to reduce the candidate regions. The genome-wide scan resulted in suggestive linkage on chromosome 19p with a maximum multipoint logarithm of the odds score of 2.61. Although this value is below the threshold typically used for a Mendelian trait (i.e., a LOD > 3), Kemlink and associates did not observe a simulated result above this threshold. This may be due to the small pedigree used for the study. To confirm linkage to chromosome 19p, the locus was replicated in a family-based association study in a set of 159 trios of European origin. This study presents a further susceptibility locus for RLS mapped within a 1.90 cM (0.3 Mbp) interval between D19S429 and D19S915. Two candidate genes, *RAB3A* and *KCNN1* were subsequently chosen based on location and the believed pathophysiogy of RLS. However, no mutation was identified (Kemlink, Plazzi et al. 2008).

1.9.4 Molecular genetic studies

Few molecular genetic studies have been undertaken to determine genes that predispose to RLS, despite the many reports that have been published suggesting a genetic contribution.

In the studies that were performed, emphasis was put on the genes encoding for the GABA A receptor subunits and the gene for the alpha1 subunit of the glycine receptor (Winkelmann and Ferini-Strambi 2006). However, no significant findings were reported.

Desautels and colleagues (2002) investigated the role of Monoamine Oxidase (*MAO*) A and B genes in association with the RLS phenotype. Females were found to have high activity alleles, consequently resulting in an elevated *MAO-A* activity. It was concluded that an elevated MAO-A activity would lead to an increased dopaminergic catabolism and cause a reduction in synaptic levels of this neurotransmitter. It is postulated that the high activity of the *MAO-A* gene may represent a modifying factor in the severity of the RLS manifestation. Estrogens may in turn interact with specific *MAO* alleles (Desautels, Turecki et al. 2002). The involvement of the dopaminergic system in the etiology of idiopathic RLS has also been suggested. Eight relevant candidate genes involved in metabolism and dopaminergic transmission were examined in a study. The study was carried out on 92 patients and 182 controls; however, no significant difference was established in the genotypic or allelic distribution between the two groups. Nonetheless, the significance of the dopaminergic system in the pathogenesis of RLS has not been ruled out. Other loci implicated in the dopaminergic system remain to be studied (Desautels, Turecki et al. 2001).

1.9.5 Genome-wide Association Studies

Association studies compare the frequencies of alleles in case and control populations. A higher frequency of the allele tested in the cases is accepted as evidence that the allele or genotype is associated with an increased risk for the disease.

In 2007, Stefansson et al., reported the results of a genome-wide association study in an Icelandic discovery sample of patients with RLS and PLMS. The study revealed that one single nucleotide polymorphism (SNP) in the BTB (POZ) domain-containing 9 (*BTBD9*) gene was associated with RLS. This association was replicated in a second smaller sample from Iceland and a United States sample. It showed that the discovery originated form a subgroup of patients with RLS who presented with PLMS. The absence of such an association for RLS without PLMS suggests that a genetic determinant of PLMS has been identified. Furthermore the serum ferritin levels were decreased by 13% per allele of the at-risk variant (95% confidence interval, 5 to 20; p=0.002). This inverse correlation of the variant with iron stores is consistent with the suspected involvement of iron reduction in the pathogenesis of the disease (Stefansson, Rye et al. 2007).

Also in 2007, Winkelmann et al. performed a similar analysis for patients with RLS who were of European decent. A total of 236,758 SNPs were used for the first stage of the study. For stage 2, 13 SNPs (based on P value) and 15 neighbouring SNPs, were replicated in a German and French-Canadian case/control sample. The study highlighted SNP associations in four genes in three chromosomal loci; *MEIS1*, *BTBD9* and *MAP2K5/LBXCOR1*. The second region mentioned here is interesting as it confirms the association with *BTBD9* found also by Stefansson et al.,(2007).

BTB stands for broad complex, tramtrack and bric a brac, genes that in *Drosophila melanogaster* are required for embryonic development, cell fate determination in the eye and pattern formation in the limbs. In humans, *BTBD9* is widely expressed in parts of the brain, such as the amygdala, cerebellum, hippocampus, and caudate and subthalmic nuclei, and in other organs, such as the heart, kidneys, pancreas and liver. The BTBD9 protein is not well characterised, and its function has not yet been determined (Winkelmann, Schormair et al. 2007).

Further analysis was carried out in the Mayo clinic in 2008, and genotyping was carried out on 11 SNPs spanning all three reported loci for disease susceptibility. The subjects used in this study consisted of 244 patients with RLS and 497 controls. Statistical analysis verified the association with *MEIS1* and *BTBD9* genes but showed only a trend for variants in *MAP2K5/LBXCOR1* and RLS. The odds ratio for the associated risk alleles ranged from 1.8 to 2.9 for *BTBD9* and from 2.6 to 3.7 for *MEIS1*. It is interesting that *MEIS1* was not detected in the original study of the Icelandic population, given the magnitude of the results. There could be many reasons for this such as, the use of different genotyping platforms or the diversity between populations (Vilarino-Guell, Farrer et al. 2008).

Furthermore, Kemlink and associates investigated if these three variants, which had been previously identified in German and Canadian RLS cases, were relevant among other European (Czech, Austrian, and Finnish) RLS cases (Kemlink, Polo et al. 2009). The study proved that these three loci present consistent disease risks in patients of European descent. Therefore these genetic determinants, observed in *BTBD9*, *MEIS1*, and *MAP2K5*/*LBXCOR1*, are risk factors for RLS in multiple populations. The study also found that *BTBD9* was the most consistent in its effect

on RLS across populations and was also more independent of familial clustering than the other two variants.

The genome-wide association studies that were carried by Winkelmann and associates and by Steffansson and colleagues (2007) identified four genes in total. However none of these genes are located in any of the previously described linkage regions for RLS. Schormair et al., (2008) analysed the five known loci (RLS1-RLS5) in a genome-wide association study, which resulted in significant signals in RLS3 on 9p23-24. From this 31 Mb region (9p, 0.5-31.5 Mb), an association study with 3,270 SNPs was carried out. For the exploaratory genome-wide scan, 628 RLS cases and 1,644 population-based controls were genotyped from the KORA-S3/F3 study, as previously used by Winkelmann et al., 2007. Of the 3,270 SNPs analysed in RLS3, 8 SNPs passed the criterion for replication. In the replication phase, these SNPs were genotyped in German, Czech, and Canadian samples. Two independent SNPs (rs4626664 and rs1975197) in the 5' UTR of splice variants showed highly significant *P* values. The association signals were located 0.41 Mb apart and mapped to introns 8 and 10 *of protein tyrosine phosphatase receptor type delta* (*PTPRD*) gene within two separate linkage disequilibrium (LD) blocks. Several *PTPRD* mRNA isoforms are expressed in developmental and tissue-specific manner. Both RLS-associated SNPs are located within the 5'UTR, which consists of ten noncoding exons contained in two known long splice variants expressed predominantly in fetal and adult brain tissue. The involvement of *PTPRD* in RLS remains unknown. These two RLS-associated SNPs are common. Rare alleles with strong effects within this gene that could explain the linkage signal were not detected (Schormair, Kemlink et al. 2008).

To further identify genes related to RLS Winkelmann and associates (2008) performed another association study. This time they carried out a three-stage association study in two Caucasian RLS case-control samples, which contained 918 independent cases and controls. In the first stage (explorative study), 1536 SNPs in 366 genes in a 21 Mb region encompassing the RLS1 critical region on chromosome 12 were screened. Three genomic regions were identified which were significant ($P < 0.05$). The second stage (the replication stage) consisted of genotyping the most significant SNPs of stage 1. Following correction for multiple testing, an association was found with SNP rs7977109. This SNP is found in the neuronal nitric oxide synthase (*NOS1*) gene. In addition to the five SNPs typed in the first two stages, an extra 29 SNPs were used for fine mapping the *NOS1* gene in the third and final stage, thus further confirming the significant association results to *NOS1*. Nitric oxide synthase action in the CNS has been associated with pain perception as well as the control of sleep regulation. Nitric oxide (NO) performs as an intracellular messenger in the CNS, and moreover the NO-arginine pathway is intimately connected to the modulation of the dopaminergic transmission. A positive response to dopaminergic agents supports the diagnosis of RLS. The pathophysiology of the disorder is thought to be related to an alteration of the dopaminergic neuro-transmission. A relationship between the opioidergic system and NO could also be relevant in the pathophysiology of RLS. In conclusion this study provides indication for an association of variants in the *NOS1* gene and RLS, and points to the involvement of the NO-arginine pathway in the pathogenesis of RLS (Winkelmann, Lichtner et al. 2008).

1.10 Management

The high prevalence of RLS does not necessarily mean that all patients with RLS should be treated with pharmacological therapy. Patients with sporadic or only mild symptoms who do not suffer from considerable impairment are unlikely to require pharmacological treatment. The proportion of such patients within the whole RLS population is currently the subject of ongoing studies. According to previous studies it is estimated that approximately 2-3% of the population have RLS that is severe enough to merit treatment (Trenkwalder, Hogl et al. 2009). The latest epidemiological study from Germany reported that among 1312 individuals the prevalence of RLS was 8.8%; and only 1.6% of the study population wanted treatment. Unfortunately, there is no data available for mild to intermittent RLS and its response to treatment. Before beginning pharmacological treatment, it is important that sleep hygiene measures be suggested, and that all causes of secondary RLS, such as iron deficiency, be ruled out.

1.10.1 Nonpharmocological Treatments

Behaviour therapy- Many physicians recommend certain lifestyle changes and activities to reduce or eliminate symptoms. Researchers have found that alcohol, tobacco, and caffeine may aggravate or trigger symptoms in RLS patients. Decreased use or elimination of such substances may relieve symptoms. Physicians suggest a program of regular moderate exercise. A small randomized controlled trial of 23 patients illustrated improvement in symptoms of RLS with a program consisting of lower body resistance training and aerobic exercise (Aukerman, Aukerman et al. 2006). Some patients also benefit from hot or cold baths, whirlpool baths, acupuncture, chiropractic therapy, stress/relaxation therapy, and mental alerting

activities such as math/computer work. More recently, advances in additional psychotherapeutic interventions have been described and are presently under investigation (Hornyak, Grossmann et al. 2008).

Iron supplementation – Treatment of RLS should initially be directed toward any underlying illness, if possible. For example, clinicians should consider determining the serrum ferritin levels in RLS patients, especially those with a history of gastrointestinal blood loss, frequent blood donation or menorrhagia. A serum ferritin concentration lower than 45 to 50 μg/ml has been observed in patients with increased severity of RLS (O'Keeffe, Gavin et al. 1994; Sun, Chen et al. 1998). If the serrum ferritin level is low, or percent iron saturation is low, replacement treatment is recommended.

Transcutaneous Electric Nerve Stimulation (TENS) - TENS is a non-invasive pain control treatment, which involves applying electrical stimulation to an area of the feet or legs. A session typically lasts from five to fifteen minutes and treatment may be applied as often as necessary depending on the severity of the pain.

Clinicians should also consider if certain medications (e.g. antidepressants, neuroleptic agents or dopamine-blocking antiemetics) are associated with initiation or worsening of RLS. Discontinuation of such medication should be considered, ensuring no harm is caused to patients.

1.10.2 Pharmocological Treatment

If pharmacological treatment is necessary, the medication that is initially selected is based upon individual's specific RLS symptoms. It is recommended that medications should be initiated at a low dose and be taken an hour or two before bedtime, thus to allow sufficient absorption and commencement of a reaction. If symptoms cause awakening during the night, additional doses can be taken. To prevent development of tolerance to one drug, monthly rotation of two to three drugs may be effective. In severe cases, a combination of drugs may be favorable. It is important to recognize that a variety of medications may be prescribed and eventually discontinued before the most effective drug(s) – and the suitable dosage level(s) - are determined. It is accepted that dopamine is the neurotransmitter most closely associated with the pathophysiology of RLS based on the class effect of levodopa (L-dopa) and Dopamine Agonists against RLS symptoms Generally, physicians chose from dopaminergics, dopaminergic agonists, opioids and benzodiazepines.

Dopaminergics – The first-line drug for most RLS patients are dopaminergic agents, which are largely used to treat Parkinson's disease. Treatment with levodopa plus carbidopa, have received positive results when used for RLS that occurs intermittently in the evening, at bedtime, or during specific activities such as airplane or lengthy car journeys. Studies have consistently shown that Levodopa reduces PLMS and nocturnal RLS symptoms (Stiasny, Wetter et al. 2001). For maximal absorption, it is recommended that levodopa be not taken with high-protein foods.

The manifestation of augmentation and rebound are the two most frequently encountered problems associated with Levodopa treatments.

Rebound refers to the appearance of symptoms at a time coinciding with the end of the half-life period of the drug. The recurrence of RLS in the early morning occurs in 20% to 35% of patients taking levodopa (Hening, Walters et al. 1999; Hening, Walters et al. 2004).

Augmentation is the most important complication of dopaminergic therapy. It involves earlier onset of RLS symptoms during the day, commencement of symptoms during the day, an involvement of other body parts such as the arms, an increased severity, or a shorter latency to the beginning of RLS symptoms while at rest (Allen and Earley 1996). Up to 70% of patients, taking levodopa daily will develop augmentation, and the risk increases with daily doses of 200 mg or more. Augmentation, however, can start soon after therapy is begun or not until months or years later. The mechanisms causing the development of augmentation are still unknown. The main hypothesis is that a dopaminergic overstimulation of the striatal dopamine D1-receptors may play a role (Paulus and Trenkwalder 2006). Low serum ferritin levels may also enhance the symptoms of RLS (Trenkwalder, Hogl et al. 2008). Furthermore, regardless of the difficulty of comparing augmentation rates from different studies it has become evident that higher dopaminergic doses must be considered as a risk factor for augmentation (Allen and Earley 1996).

Tolerance to treatment has been described in some cases of dopaminergic therapy. It is the loss of therapeutic efficacy of an RLS treatment that had previously been efficacious.

Punding refers to an intense fascination with complex, stereotypical, purposeless, and repetitive behaviours e.g. sorting and hording of objects, cleaning and grooming. It has been previously described in high-dose psychostimulant users and patients with PD on dopamine agonists or levodopa. In October 2008, Evans and associates described two individuals with RLS who were treated with dopamine agonists and developed punding along with a variety of impulse control disorders (ICDs). Nevertheless, patients treated with agonists for RLS generally show a predisposition to punding (Evans and Stegeman 2009). Further studies are needed to fully evaluate punding and other ICDs with regards to RLS.

Dopaminergic Agonists stimulate dopaminergic receptors. Generally, agonists which do not cross the blood-brain barrier have peripheral effects and agonists which penetrate well into the brain have mainly central effects. Agents such as mesylate, pramipexole, and roprinirole hydrochloride have proved to be effective in some patients. Although augmentation may be observed in some patients, it does not appear to be a major problem (Odin, Mrowka et al. 2002).

Pergolide mesylate (Permax) is a potent, long acting dopamine agonist, which has been proven effective in RLS (Walters 1995). Pergolide was found to deliver better subjective relief and greater decreases in PLMS when compared with Levodopa (Staedt, Wassmuth et al. 1997). It was also found to be effective in patients who are unresponsive to levodopa (Earley and Allen 1996). However, some patients taking pergolide to treat the symptoms of Parkinson's disease have been shown to develop valvular heart disease, even though this is usually associated with higher doses than those used in RLS (Van Camp, Flamez et al. 2004).

Pramipexole (Mirapex) is a D3 and to a lesser extent a D2 dopaminergic receptor agonist. According to experimental studies, Pramipexole could have a neuroprotective effect. It can induce various adverse effects which include hallucinations and sudden onset of sleep. A recent study by Inoue and associates, was performed to evaluate the safety and efficacy of the long term use of Pramipexole in patients with primary RLS. The study was carried out in a cohort of 141 Japanese patients. The IRLS score improved from 22.3 +/- 4.7 to 11.1+/-7.7 at week 8 and 4.9+/-5.9 at week 52. Adverse events were typical of nonergot dopmamine agonists, mild in intensity, and decreased in frequency as the study progressed. RLS augmentation was not observed. Overall the study found that Pramipexole showed good efficacy, particularly in patients with an IRLS total score <20 (Inoue, Kuroda et al. 2010).

Bromocriptine mesylate (ParLODel), which was first reported in 1988, was reported to decrease PLMS and provide subjective relief in RLS patients.

Cabergoline (Dostinex) is an ergot derivative, and a rather selective D2 receptor agonist. It is the strongest acting dopamine agonist available, with a half-life of at least 65 hours. It has the advantage of giving a 24-hour effect, which is useful for reducing/preventing the effects of augmentation.

The action of dopamine agonists does not commence for 90 to 120 minutes after ingestion; thus, these agents cannot be used effectively once symptoms have started. In addition, agonists can cause nausea; therefore, slow dosage increase is important, especially for pergolide.

Opioids – These agents are usually recommended for the treatment of more severely affected patients, patients who are unresponsive to other medications or the uncommon patient with pain as a prominent symptom (Clark 2001). Although never formally investigated in large scale trials, opioids are often used following unsuccessful treatment with dopaminergic agents. Low-potency opioids include codeine and propoxyphen, and have proven to benefit patients with mild or intermittent symptoms. Higher-potency agents, such as methadone hydrochloride and oxycodone hydrochloride are thought to have a role in refractory cases. Oxycodone at an average dose of 15.9 mg reportedly decreases PLMS, and provides decreased arousals and improved sleep efficiency. Nevertheless, because of the recognized risk of addiction, most physicians are reluctant to use opioids.

Benzodiazepines – Studies of benzodiazepines have focused on clonazepam therapy. Clonazepam has shown to ease the sensory symptoms and decrease PLMS. Other benzodiazepines include temazepam and alprazolam. Although this drug has been proven to be well tolerated in older patients, its long duration of action may cause effects that are more adverse. Such side effects include daytime drowsiness and confusion, unsteadiness and falls, and cognitive impairment in the morning.

Gabapentin and other anticonvulsants - Anti-epileptic medications such as carbamazepine and gabapentin can also provide a therapeutic effect, especially in patients with painful RLS. Gabapentin has been shown to be an effective treatment, however, similar to opioids, large scale trials and long-term observations are minimal. Anticonvulsants such as carbamazepine and valproic acid, which have been previously investigated for the treatment of RLS, are no longer regarded as first-line

options due to their side effects. Dizziness, fatigue, and sleepiness are among the possible side effects (Happe, Klosch et al. 2001).

Yokukansan **(Herbal)** – Yokukansan (YKS) was developed as a remedy for restlessness and agitiation. It has been approved for patients with insomnia in Japan, and in 2008, Shinno and associates reported that YKS improved sleep quality in patients with dementia (Shinno, Inami et al. 2008). In 2010, Shinno reported that three patients with RLS were successfully treated with YKS. It was speculated in the paper that actions on GABAergic, serotonergic and dopaminergic systems might account for some of the therapeutic effects of YKS in RLS patients (Shinno, Yamanaka et al. 2010).

1.10.3 Medications that may exacerbate RLS

RLS as a side effect is ascribed to a number of drugs. Prior reports exist on RLS associated with drugs such as olanzapine (Kraus, Schuld et al. 1999), risperidone (Wetter, Brunner et al. 2002), and clozapine (Duggal and Mendhekar 2007). A number of case reports have been published on the provocation of RLS by "modern antidepressants (AD)" with specific receptor binding profile. One case was reported for each one of the selective serotonin reuptake inhibitors (SSRIs) sertraline, fluoxetine, paroxetine and citaloprame. For the nonadrenergic-specific serotenergic AD (NASSA) mirtazapine, a total of seven cases were reported (Rottach, Schaner et al. 2008). Some papers focused on the relationship between AD and RLS. Brown et al., investigated the possible association between RLS and AD, in 200 patients with sleep onset disturbance. Of these 200 patients, 45% met the diagnostic criteria of RLS, 56% suffered from depression, and 38% were on AD. A statistically significant association between RLS and AD was not demonstrable (Brown, Dedrick et al. 2005).

In 2007, Kang et al., reported that the prevalence of RLS was more than twofold higher (21.4%) in schizophrenics taking antipsychotics than in healthy individuals (Kang, Lee et al. 2007). However these reports are often disregarded or believed to be psychotic agitation or a somatic copmplaint because its symptoms are nonspecific, unclear and diverse, and are aggrevated or even happen exclusively at night time.

In 2008, Kang et al., published another report in the Journal of Psychopharmacology in which five case studies were reported of RLS and PLMS that were probably associated with olanzapine. For example, the first case, a 36-year-old Korean female

with schizophrenia, showed a good response to olanzapine. However the RLS symptoms associated with olazapine resulted in poor long-term compliance, which ultimately led to the recurrence of psychotic symptoms. These report high-lights the fact that antipsychotic-induced RLS and PLMS are under-recognized side effects of antipsychotics, with the symptoms often misdiagnosed as psychotic agitation. It also aims to make clinicians aware that although the antipsychotic olanzapine has few extrapyramidal symptoms, it can be the cause of RLS and PLMS and therefore affect a patients compliance with the medication and lead worsening of psychotic symptoms. Even though the cause of RLS and PLMS caused by antipsychotics has not been established, it is thought to be due to the dopamine receptor blocking of antipsychotics.

1.11 Prognosis and Impact of RLS

1.11.1 Prognosis

RLS is generally a lifelong condition. Symptoms tend to gradually progress with advancing age. At times individuals may experience spontaneous improvement, in which symptoms decrease or disappear for days, weeks, or months, although symptoms tend to recur. Improvement or resolution of symptoms is often observed in patients with RLS secondary to an underlying condition, if the underlying condition is treated.

1.11.2 Impact on quality of life

Depression is common in RLS patients and may be related to the chronic unrelenting nature of symptoms, to the persistent sleep loss or to the underlying brain abnormalities that cause RLS. In a study carried out on a headache and control groups, higher scores for depression and anxiety were more frequent in subjects with RLS compared to those without (d'Onofrio, Bussone et al. 2008). Treating depression patients with RLS may be difficult since, as mentioned above, some of the antidepressants may individually exacerbate RLS symptoms.

The most disturbing feature of RLS may be the social disruption which RLS sufferers experience. This is based on the need to decrease evening social or work engagements that involve inactivity. Social dinners, board meetings or theatre can be very challenging. Furthermore, individuals often have difficulty concentrating on daily tasks such as those demanded by their work. Problems with short term memory loss are often encountered, which can lead to cognitive deficits i.e. learning difficulties (Pearson, Allen et al. 2006).

RLS patients are more prone to headaches than healthy subjects. This is most likely secondary to the sleep disturbances and sleep loss. Headaches are usually comparable to migraine in character, though with an element of tension-type headache (d'Onofrio, Bussone et al. 2008). In a study conducted between headache and control groups, headache patients with RLS reported sleep disturbances more frequently compared to those without RLS (50.0% vs. 32.7%; $p<0.0001$) (d'Onofrio, Bussone et al. 2008).

1.12 Linkage analysis

1.12.1 Basic concepts in Genetics

For centuries the hereditary basis of human disease has fascinated both scientists and the general public. An Austrian monk, Gregor Mendel provided the foundation for the study of human genetics. By carefully creating crossing experiments with peas and recording observations of the frequency of variable characteristics in the pea plant (e.g. seed texture, colour, plant height), he hypothesised what are now called Mendel's laws (Mendel 1866). Mendel's fundamental unit of inheritance is named the gene, which contains the information necessary for manufacturing proteins. DNA or deoxyribonucleic acid is the molecule that contains the gene and encodes information for producing both proteins and RNA. The locus is the physical location of a gene. Each individual carries two copies of each gene of which one was received from the mother and the other received from the father. At any particular locus there can exist a number of different forms of the gene, known as alleles.

Normal chromosomes have a single centromere that is seen under the microscope as the central constriction. It separates the p (short) arm from the q (long) arm. It is essential for segregation during cell division. The normal human chromosome consists of 23 pairs of chromosomes, with one member of each pair inherited from each parent. Non-gamete cells are diploid as there are two copies of each chromosome in the cell. The first 22 pairs of chromosomes are called autosomes. The two remaining chromosomes, X and Y, are called sex chromosomes of which females carry two X and males carry one X and one Y.

Genes located on the same chromosome are said to be syntenic. Although two genes may be syntenic they may be far enough away from each other on the chromosome to segregate independently from one another i.e. unlinked. If two syntenic genes fail to be passed on to gametes independently from one another they are said to be linked.

In sexual organisms such as humans the reproductive method involves the union of haploid gametes (sperm and egg cells). The process by which these haploid gametes are formed is termed as meiosis. Meiosis consists of meiosis I and meiosis II. In meiosis I each chromosome in the cell is replicated to generate two sets of duplicated homologous chromosomes. Each homologous chromosome therefore consists of two strands known as chromatids. Physical contact may occur between the chromatids, resulting in the formation of chiasmata. Chiasmata are a sign of the occurrence of crossing over (*recombination*) between two chromatids, thus leading to the exchange of DNA between two of the four chiasmata. A chiasma occurs at least once per chromosome pair, resulting in recombinant haplotypes. Meiosis II is identical to a mitotic division in which genetic material is transmitted equally and without recombination to daughter cells.

As a consequence of the process of meiosis, gametes contain only a haploid set (one copy) of chromosomes. This explains why parents pass only a single gene onto their offspring. It also explains why the gender of an individual is determined by the father of the child.

1.12.2 The principles underlying linkage analysis

In the accompanying figure (Figure 1.3), each bar represents a chromosome, paternal (P) and maternal (M). "D" represents the disease gene in a disorder with an autosomal dominant mode of inheritance and "d" is its normal correspondent. The numbers denote particular alleles at a series of genetic markers that span the chromosome (genetic markers will be explained later in section 1.11.3).

When a gamete is produced, crossover events (recombinations) can occur at the points indicated with arrows during meiosis. (Four chromatids are formed during meiosis, but it is not shown here for the purpose of simplicity). The resulting offspring will inherit a "new" sequence of alleles.

Subsequently a different pattern of marker alleles is seen in the next generation. Only the markers adjacent to the disease gene "D" (marked with a red bar) remain unchanged. During gamete formation in this individual, a

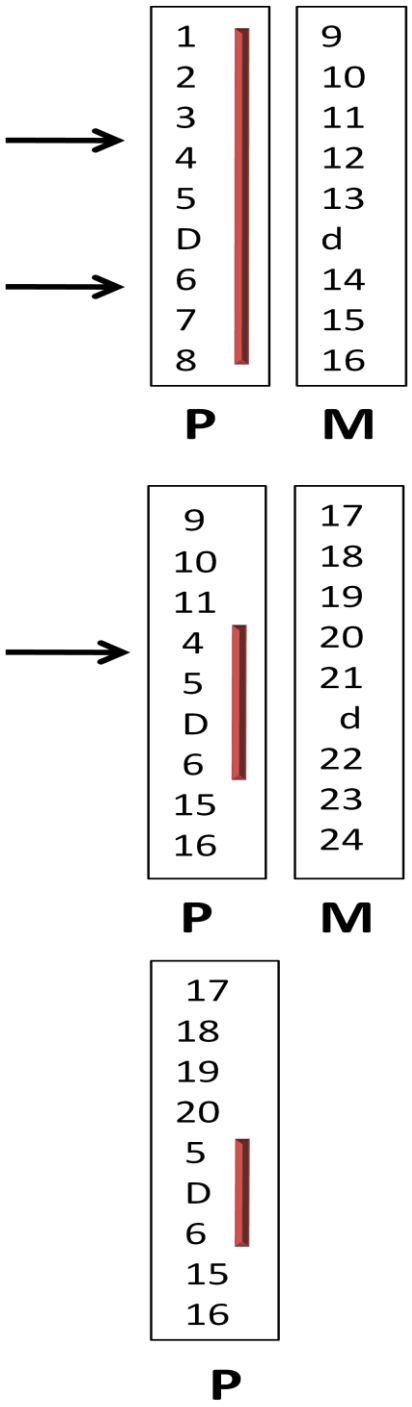


Figure 1.3: The principles underlying linkage analysis
Each bar represents a chromosome, paternal (p) and maternal (m). The numbers denote particular alleles at a series of genetic markers that span the chromosome. Crossover events (recombinations) have occured at the points indicated with arrows during meiosis. Thus, it is only the marker which are adjacent to the disease gene 'D' which remain unchanged i.e. are inherited with the disease gene.

further recombination event may occur, thus separating some alleles from the disease gene.

It is only the marker alleles closest to the disease gene “D” that remain unchanged. It is clear that allele 5 of the marker above the disease gene and allele 6 of the marker below the disease gene have remained “associated” with the disease gene “D”.

These two markers are said to be linked to the disease gene because recombination events are not likely to isolate them from it. This is because of their close physical proximity.

From figure 1.3 it can be perceived that if all the numbers in the bars were “2” it would be impossible to demonstrate the recombination events. This is an essential detail and indicates why genetic markers and in particular microsatellite markers are so valuable in linkage studies.

1.12.3 Genetic Markers

If, in the above example the alleles were all numbered “2” i.e. if all individuals were homozygous for the allele 2, then no information on recombination events could be acquired. The rationale behind why the example shown above succeeds is that two different alleles are present at each locus, in other words the individuals are heterozygous. When individuals are heterozygous at these locations, recombination events can be identified and information can be obtained from the family.

Genetic markers can be defined as sequences of DNA inherited in a mendelian manner. They demonstrate polymorphism, which means a difference in DNA sequence is observed among individuals, groups or populations that gives rise to different forms of the marker. Traditionally autosomal marker traits included the blood groups and the ability to taste phenylthiocarbamide (PTC). The X-linked marker groups include the Xg blood group and colour blindness. The demonstration of genetic linkage by pedigree studies was first described by Bell and Haldane in the 1930’s, when they showed that the locus for haemophilia was close to that for colour vision (Bell and Haldane 1986).

Microsatellite markers

The genetic markers used in this study were polymorphic repeats which belong to a group of genetic markers called microsatellite repeats. Microsatellites were first described in 1989 as small blocks of tandemly repeated DNA in which the repeated element is usually a di-, tri-, or tetra-nucleotide sequence (e.g. [CA]n, [CAG]n, [CAGT]n, where n represents the number of repeats). The number of repeat elements in these blocks is often highly polymorphic, and show simple Mendelian inheritance. Information on several of thousands of microsatellite repeats is now electronically accessible to investigators through databases such as the Marshfield Institute, Entrez (at NCBI), the Whitehead Institute, the Genome Database and Généthon. Although these highly polymorphic microsatellites supply the investigator with important maps for linkage analysis, it is vital to remember that all genetic and physical maps are not constructed alike or with the same reliance.

Dinucleotide Repeats

The most common type of repeat is the dinucleotide CA (GT) repeat, occurring on average every 0.4 cM (Weber, 1990). The function of these repeats is unknown. A suggestion that they may be "hot-spots" for recombination is unlikely because of the linkage disequilibrium seen between them. Dinucleotide repeats have been used extensively as genetic markers. Two factors determine whether these repeats are polymorphic. Firstly the size of the repeat, and secondly whether it is an imperfect repeat (with interruptions) or a perfect repeat. A large percentage of the markers with 15 or more perfect repeats are polymorphic. Dinucleotides while powerful have several technical disadvantages. Often dinucleotides have a "stutter" or "shadow band" that produces a background ladder of bands two base pairs apart. Also since

the alleles are only two base pairs apart, it can be difficult to make a distinction between one allele and the next.

Tri- and Tetranucleotide repeats

Trinucleotide repeats are approximately ten times less common than dinucleotides. They occur once every 300-500 kb. The most common form is [AAB]n (where B is C, G or T). Although certain trinucleotide repeats which are prone to expansion have warranted a lot of attention because of their disease causing characteristics (e.g. fragile X syndrome, Huntington's disease, myotonic dystrophy), the majority of trinucleotide repeats are not associated with disease and are very stable. Tetranucleotide are even more rare, and the most common form is [AAAB]n (where B= C,G,T]n. Tri- and Tetranucleotides have fewer stutter bands than dinucleotide repeats, typically generating unique PCR alleles without the laddering artefact. They are also easier to read when using fluorescently labelled primers and automated detectors such as the ABI 3130. Accurate allele identification is imperative, making these markers more favourable than their di-nucleotide match. However their lower occurrence in the genome restricts their extensive use.

1.12.4 Centre d'Etude du polymorphisme Humain (CEPH)

Centre d'Etude du polymorphisme Humain (CEPH) is a centre for genetic studies in Paris, France. The primary URL for CEPH is *http;//www.cephb.fr*. This website provides information about CEPH and provides access to the CEPH genotype database which contains data on polymorphic markers. Researchers from over 100 laboratories around the world have submitted genotyping data and from this CEPH have assembled a large panel of families to construct maps of genetic markers. These large families generally consist of pedigrees with three generations, living grandparents and an average sibship size of 8.5. The standard family set comprises of 40 families, while an extended family set of 64 families is available. These families are generally of European descent (Dausset, Cann et al. 1990).

The CEPH database provides information on the marker including allele sizes and frequencies, and heterozygosity. It can also provide the actual genotypes for individuals in the CEPH pedigrees. Blood from each member is stored at CEPH and can be made available to researchers around the world, therefore being able to access the actual genotypes of these individuals can greatly improve the consistency of allele calling between gels, projects and laboratories. CEPH however does not provide primer sequence data or the position of the marker on the chromosome in question.

1.12.5 Using Microsatellite Markers to find Disease-causing Genes

When the disease causing gene in a family is unknown, a common technique used to find the gene is to genotype family members for a collection of microsatellite markers dispersed throughout the genome. If a distinct allele of one of these makers is found to be "linked" with the disease phenotype, then the disease gene is likely to be physically close to that marker. Complex statistical analyses are required to determine the level of linkage between the disease phenotype and the marker. A number of factors such as family structure, mode of inheritance and marker allele frequencies are all taken into account.

Following sufficient evidence that the disease is linked to a given marker, other markers in that area need to be genotyped. When a set of alleles from adjacent microsatellite markers are found on a short segment of the same chromosome and tend to be transmitted together through a family, it is referred to as a haplotype. Within this haplotype there will be a number of candidate genes present, one of which will be the disease gene.

1.12.6 LOD scores

By analysing the pattern of segregation of a gene and potential marker genes in families, it is possible to generate a mathematical measure of the likelihood of linkage, known as the LOD score or Z score. The LOD score was devised by Morton in 1955 and is defined as the logarithm of the odds (LOD) ratio (R) that the loci are linked rather than unlinked. The LOD score is therefore a statistical estimate that measures the probability of two loci being physically close together and thus being inherited together.

The recombination fraction and genetic distance

Recombination will rarely separate loci that lie very close together on a chromosome, as only a crossover located precisely in the small space between the two loci will produce recombinants. Thus sets of alleles on the same small chromosomal segment tend to be transmitted as a block through a pedigree. This block of alleles is known as a haplotype. The further apart two loci are on a chromosome the more likely it is that a crossover will separate them. The recombination is a measure of the distance between two loci. The further apart two loci are on a chromosome, the greater will be the frequency of recombination between them up to a maximum of 50%. Recombination fractions (θ) never exceed 0.5, however far apart the loci. If there are no recombinants, the LOD score will be maximum at $\theta = 0.00$. Recombination events can therefore be used as a measure of how close two loci are (genetic distance). Two loci that show 1% recombination are defined as being 1 centimorgan (cM) apart on a genetic map (Strachan and Read 2003).

Genetic distance and physical distance are not equivalent. Genetic maps show the order of features along the chromosome and the chances that they will be separated by recombination; however, physical maps show the distance of features along the chromosome in kilobases (kb) or megabases (Mb). Areas such as the centromere show very few crossovers; therefore, genetic distances in these areas would be small in comparison to physical distance. In contrast, areas of equivalent physical size in crossover "hotspots" such as the telomeres would show large genetic distance. For large distances i.e. two loci that have a recombination value greater than $\Theta = 0.50$, it is not possible to use the recombination fraction as an additive distance measure. It must therefore be transformed by a map function into a certain additive map distance, under the assumption of a certain level of genetic interference.

Interference is thought of as the process whereby the presence of one cross-over in an interval inhibits the formation of another cross-over in the same region. Subsequently negative interference suggests that the presence of one cross-over will in turn encourage the formation of another cross-over event within an interval. Numerous levels of interference in humans have been hypothesized; however, the level of interference used in this study was the Kosambi (Kos) level. This method adopts an intermediate level of interference whereby double cross-over are seldom observed at small genetic distance, although the probability increases at greater distances (Keats, Ott et al. 1989). Another common map function employed to covert the estimated recombination fractions to map distances is the Haldane level, however it appears to produce less realistic values than does Kosambi's formula. For example the Haldene map function converts $\Theta = 0.27$ into 39 cM and the Kosambi map function translates $\Theta = 27$ into 0.30 Morgans (30 cM). Higher recombination rates are seen in the female than in the male. (Kosambi 1944)

Some allowance must therefore be made for the likely distance (in addition to recombination fraction) that may be present between a particular marker and the disease locus. Hence, it is normal practice to calculate all LOD scored at a range of recombination fractions.

1.12.7 Calculating LOD scores

To find the odds of linkage it is necessary to calculate the overall likelihood that the two loci are linked ($\Theta = 0.00$) compared to the likelihood that they are not linked ($\Theta = 0.50$). The ratio of the latter probability to the former, expressed as a logarithm (base 10) is then the 'LOD score' between those two markers. Therefore, the LOD score is a statistical estimate that measures the probability of two loci being physically close together and thus being inherited together.

Each meiosis in the family is looked at in turn and the likelihood of the observed genotype and disease gene being transmitted together is calculated. This is moderately straightforward when all individuals are genotyped and recombinations are easily determined. Nonetheless problems do arise when recombinations cannot be easily identified and when gaps arise in a pedigree e.g. when individuals are unavailable for DNA sampling or perhaps deceased. Therefore when uncertainties like this arise the meiosis must be examined under the various alternatives that exist, and each alternative given an appropriate weighting. In order to calculate the full LOD score for a family various factors exist that need to be taken into account e.g. the genotypes of other individuals and population allele frequencies. Consequently this results in a large "branching tree" of genetic probabilities (Strachan and Read 2003). Therefore, apart from very simplistic cases, human linkage analysis is completely dependent on computer programs.

1.12.8 LOD score values for linkage and exclusion

The degree of linkage is a function of the distance between the disease gene locus and the maker locus. This can be measured by the number of crossovers between the two loci among the observed meioses (i.e., recombination fraction, θ). If there is no recombination, $\theta = 0.00$, there is "complete linkage" and observed meiosis ($\theta < 0.50$). However if the observed recombination fraction is 50% ($\theta = 0.50$), there is no linkage. LOD scores (θ) are usually recorded at $\theta = 0.00, 0.05, 0.10, 0.20, 0.30, 0.40$ and 0.50.

If all chromosomes were of equal size and if two loci were picked randomly, the chance that they were found on the same chromosome would be 1 in 23. However the probability that two randomly picked loci would be close together and likely to be linked is estimated to be a one in fifty chance, thus the prior probability of linkage. Therefore, a large amount of evidence would be required to confirm linkage if there were only a one in fifty chance the two loci were actually linked.

The LOD score is the logarithm of the odds that the loci are linked rather than unlinked, therefore, $\text{Log}_{10}1000 = 3$ represents the minimum LOD score required for statistical proof of linkage.

As stated the conventional threshold for claiming linkage is a LOD score of 3.0, corresponding to 1000:1 odds that the loci are linked. LOD scores of -2.0 or less are indicative of nonlinkage (Morton, 1955). Values between -2.0 and 3.0 are inconclusive and require additional data before an assumption can be made. These criteria are based on the probability of type I and type II errors. Type I errors refers to the probability of concluding that there is linkage between the loci in question

when in fact there is no linkage (false-positive); and type II refers to assuming that there is no linkage when in fact there is (false-negative). A cautious value of $z > 3.00$ for inferring linkage was chosen by Morton (1955) to prevent the risk of false positives. Therefore, if the total LOD score for one family or over all families is 3.0 or more, one can conclude that there is extensive evidence for linkage.

1.12.9 Drawing family trees

There are a number of computer programs available for pedigree drawing, and they have huge advantages over some of the older methods such as by hand. The pedigree drawing package used for this study was *Cyrillic* v2.01. Aside from being a drawing program *Cyrillic* v2.01 has a number of useful features. Such features include the ability to store vital information about individual patients and their families; to scale pedigree drawings to fit on one page; and to store up to 10000 individuals per family, up to 250 markers per chromosome and up to 150 alleles per marker. It can also colour haplotype bars to show inheritance and multiple crossovers.

1.13 Haplotype Analysis

A haplotype is a set of alleles on the same small chromosomal segment that tends to be transmitted as a block through a pedigree. A haplotype may refer to as few as two loci or to an entire chromosome depending on the number of times recombination takes place between a given set of loci. Although a LOD score can point us in the direction of whether linkage has taken place or not, it is simply a statistical estimate and does not give us a mental picture of what is occurring.

If a particular candidate locus is being examined with a series of markers, it is often useful to draw the haplotype at this locus for the family in the study. It can provide a visual explanation of the LOD score. (It may also be useful in identifying cases with possible reduced penetrance or other useful information.) Figure 1.4 explains this concept. Each individual in the pedigree has two columns of numbers, one column representing a paternal chromosome and the other representing a maternal chromosome. The numbers signify the set of alleles found using a series of adjacent markers. Therefore, each column is a haplotype and using colours for different haplotypes makes it easier to follow through the family. In the figure, one can see that the affected daughter has received the haplotype 2-1-5-7 from the father and haplotype 4-5-7-6 from the mother. Nonetheless there are times when it is not possible to determine which parent the haplotype has been passed on from. For example, for marker D in figure 1.4 the father is homozygous for allele 7, therefore, we are unable to determine if the allele 7 inherited by the affected son comes from the “red” chromosome or the “green” chromosome.

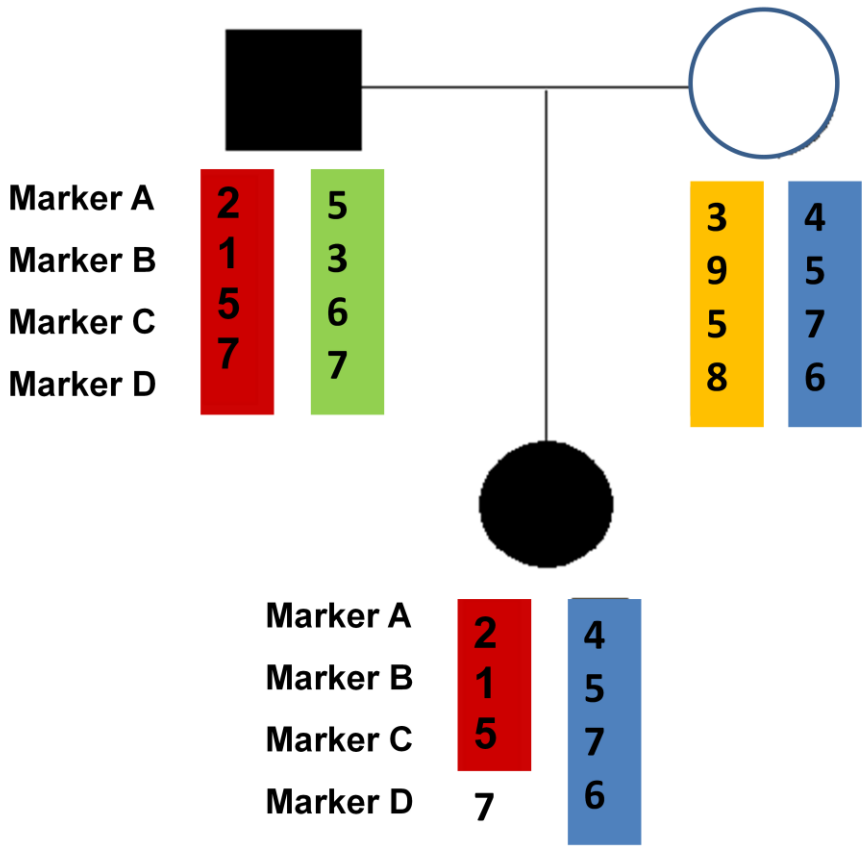


Figure 1.4: The concept of haplotypes and haplotye analysis
This figure shows an affected father (filled square), an unaffected mother (circle) and an affected daughter. Four adjacent markers are examined here, marker A, B, C and D. The resultant allele for each marker is given by the numbers under each individual. The series of alleles (haplotypes) is given a coloured vertical bar. In this example, the daughter has inherited the red haplotype from the father and the blue haplotype from the mother. However for marker D, it is not possible to determine if the allele 7 inherited by the affected daughter comes from the fathers 'red' chromosome or the 'green' chromosome as the father is homozygous for this marker.

1.14 Summary

Restless legs syndrome (RLS) is one of the most common neurological sensorimotor disorders in Western countries, affecting approximately 10% of the population. It is often associated with periodic limb movement during sleep (PLMS) leading to severe insomnia. However, it remains largely underdiagnosed and its underlying pathogenesis remains to be elucidated.

RLS is generally idiopathic, with familial association in 40-60% of the cases, but may also be symptomatic of such associated conditions (secondary forms) as peripheral neuropathies, uremia, iron deficiency, diabetes, Parkinson's disease and pregnancy. In comparison to non-familial cases, familial RLS usually has a younger age at onset and a more slowly progressive course. The symptoms within a single RLS family can vary significantly, with some members displaying mild symptoms on only a few occasions during their life, while those more severely affected suffer from restlessness and sleep disturbances.

Linkage studies in RLS families have revealed ten loci to date, however, no causally related sequence variant has yet been identified using this approach. Based on the hypothesis that RLS follows a recessive mode of inheritance, linkage to chromosome 12q (RLS1) was identified in 2001 in a French-Canadian family. All of the nine other loci identified followed a dominant mode of inheritance.

Genome wide association studies have identified variants within intronic or intergenic regions of *MEIS1, BTBD9, PTPRD, MAP2K5/LBXCOR1,* which has raised new pathological hypothesis for RLS.

Treatment options for RLS are advanced and have been investigated in large trials. In order to optimize treatment strategies, future studies should concentrate on the remaining problems of augmentation, rebound and its pathomechanism. Furthermore, genetic insights could reveal further specific treatment regiments other than the present therapeutic strategies.

Notable progress has been made in distinguishing the diagnostic criteria of RLS from mimic conditions. However, much more must be done to fully elucidate the pathophysiology and etiologies of both primary and secondary forms of RLS.

Chapter 2

Materials and Method

2.1 Family recruitment and clinical assessment

In the University College Cork (UCC) Department of Pathology, research into the genetic features of RLS is continuous. Families for this ongoing study are recruited by various methods e.g. word of mouth, contact with GPs, and articles in the press. Contact was made with the department by one member of this family interested in participating in the study. Subsequently other family members were primarily contacted by phone, and those with interest in participating were visited individually in their homes located in various parts of Ireland. The requirements and aims of the study were explained to the members of the kindred and those willing to take part were asked to sign a consent form. Participants were then questioned as to the presence, frequency and severity of symptoms suggestive of RLS with particular reference to:

- The presence of the revised essential diagnostic criteria proposed by the RLS epidemiology and diagnosis workshop with IRLSSG, 2002.
- Obtaining RLS severity rating scale (proposed by IRLSSG).
- Periodic limb movements during sleep and sleep disturbance.
- The duration, frequency and severity of these symptoms.
- The effect of pregnancy on symptoms (female patients).
- Their past medical history.
- Current medications and drug history.
- Family history including anecdotal information on the affection status of deceased family members.

Physical examination included a full neurological examination. Venous blood samples (approximately 20 mls) were obtained from affected and unaffected family members. Blood samples were collected in EDTA coated Vacutainers (to prevent clotting) and subsequently stored at -20 °C.

2.1.1 RLS3002 Pedigree

The pedigree, name RLS3002, is outlined in figure 2.1 below. In this family RLS appears to follow an autosomal dominant mode of inheritance. Blood samples were collected from 18 members of the family, and following genomic extraction each family member was assigned their own unique "PATH" number. Of the 18 family members 11 are affected, all of whom met the essential diagnostic criteria. The age of onset was seen to vary from childhood, to teenage years and some early twenties. Although the majority of affected individuals did not endure sleep disturbances, one individual did experience severe disturbance and another had moderate disturbance. 4 of the 14 family members remained unaffected. Table 2.1 lists the clinical data from all 11 affected members of RLS3002

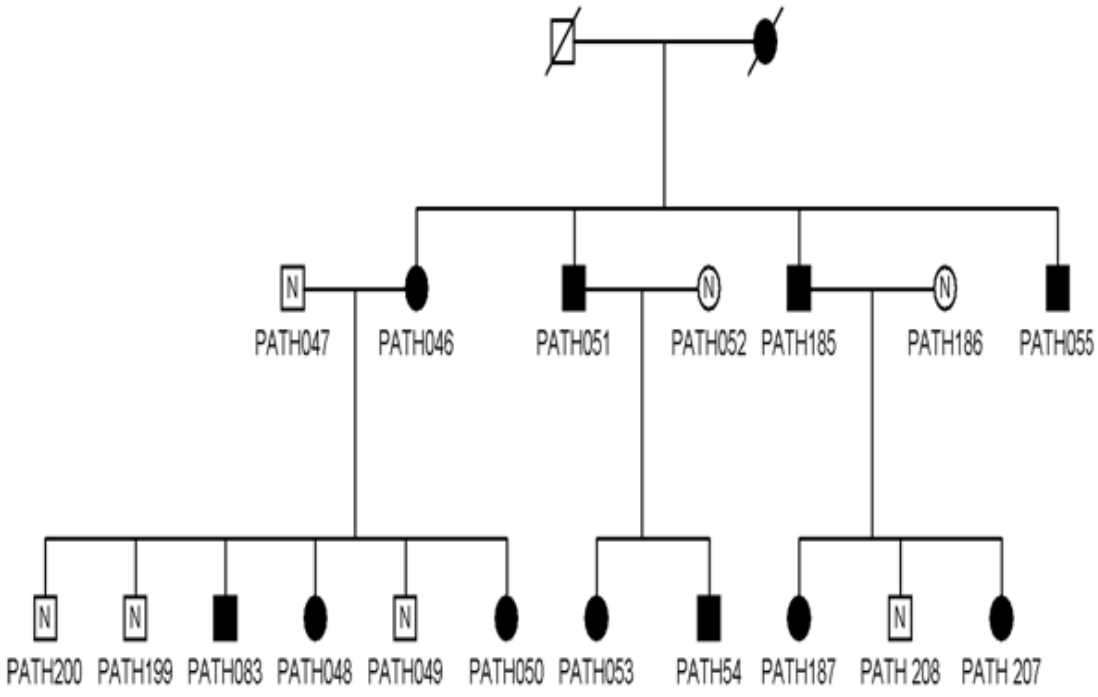


Figure 2.1: RLS3002 Pedigree
This is a three generation family. Circles represent females, squares represent males. Coloured symbols represent affected members and symbols with the letter N represent unaffected members. The lines through the grandparents symbols indicate that they are deceased. Members of the family were each assigned their own unique PATH number.

Table 2.1: Clinical Features of RLS in the affected members of Family RLS3002.

Individual	Sex	Age at study (yr)	Age at onset (yr)	Severity (IRLS)	Parasthesia in legs	Parasthesia in arms	Symptom Prevalence
PATH046	F	58	3	4	Yes	No	Night
PATH048	F	31	10	14	Yes	No	Evening/ Night
PATH050	F	21	11	13	Yes	No	Evening/ Night
PATH051	M	57	20	15	Yes	No	Sitting for long periods/ Evening/ Night
PATH053	F	25	13	17	Yes	No	Sitting for long periods/ Evening/ Night
PATH054	M	23	15	11	Yes	No	Evening/ Night
PATH055	M	53	10	10	Yes (Lateralization of Symptoms; left leg)	No	Evening/ Night
PATH083	M	27	13	10	Yes	No	Night
PATH185	M	57	13	16	Yes	No	Evening/ Night
PATH187	F	29	13	8	Yes	No	Evening/ Night
PATH207	F	19	15	10	Yes	No	Sitting for long periods

2.2 DNA Extraction

DNA was extracted from venous blood using a QIAamp Blood Kit from Qiagen. It involves cell lysis, nuclear lysis and protein salt presipitation stages. This method retains the DNA in a QIAamp spin column while contaminants are washed out and typically yields approximately 3-12 µg in 200 µl of final volume per 200 µl of whole blood. Before the extraction process the blood samples were allowed to thaw at room temperature, and a check was performed to ensure that the Buffer AL, Buffer AE and QIAGEN Protease were prepared correctly, according to the accompanying manual. The extraction was carried out according to the manual. The DNA was eluted with Buffer AE or distilled water and a NanoDrop was used to measure the DNA concentration. The DNA extraction was also confirmed by amplification using the polymerase chain reaction and efficient primers, then checked by agarose gel electrophoresis. The manufacturers state that the typical DNA yield from a 200 µl sample volume of Human Whole Blood is 3-12 µg of DNA in 200 µl water. The yield however depends on the quantity of white blood cells present and the performance of the procedure.

2.2.1 Determination of DNA Concentration

A NanoDrop ND-1000 spectrophotometer was used to measure the DNA concentration following extraction. A NanoDrop is a cuvette-free spectrophotometer. It uses just 1 µl of sample and is accurate from 5 ng/µl up to 3,000 ng/µl. By eliminating the need to carry out dilutions, and thus reducing human error and variability factors, analyses can de done quickly and easily. The NanoDrop Spectrophotometer is linked to the computer that also runs the digital imaging system.

The NanoDrop was blanked using buffer as the DNA was eluted with Buffer TE. When adding the sample it is essential that the liquid column is formed between the upper and lower measurement pedestals.

Operation: A 1 µl sample is pipetted onto the lower pedestal (the receiving fiber). The second fiber or the upper pedestal is brought into contact with the liquid. A pulsed xenon flash lamp within the machine provides the light source and a spectrometer is used to analyse the light passing through the sample. As previously mentioned, the instrument is controlled by special software run from a PC. The data is then saved in an archive file on the PC. The sample concentration is recorded in ng/µl and is based on absorbance at 260 nm and 280 nm.

2.3 Polymerase Chain Reaction (PCR)

The polymerase chain reaction is a rapid and versatile cell free procedure for in vitro enzymatic amplification of specific segment's of DNA. The template DNA is usually genomic DNA from a particular tissue or cultured cells, in which case the target DNA is typically a tiny fraction of the total DNA. In order to permit selective amplification of the target DNA, some prior DNA sequence information from the target sequence is required. This information is used to design two oligonucleotide primers, usually about 18-25 nucleotides long. These primers are specific for sequences flanking the target sequence. For the majority of PCR reactions, the goal is to amplify a single DNA sequence and so it is important to reduce the possibility of the primers binding to other locations in the DNA than the desired ones. Therefore, in order to avoid this a variety of considerations need to be factored into primer design e.g. the melting temperature (T_m) of the two primers should not differ by >5°c, self-complimentary sequences should be avoided, and the GC content should be between 40 and 60%.

In addition to template DNA and primers, a PCR needs other reagents in order to be successful. A heat stable enzyme known as DNA polymerase, e.g. Taq polymerase, is employed. Small segments of DNA are generated when the polymerase adds complimentary deoxynucleotides (dNTPs) to the template DNA. The primers in this case are used as the starting point for the polymerase by providing double stranded DNA. During PCR, the DNA is heated and the double strands separate. Upon cooling, the primers bind to the template DNA thus providing a region of double stranded DNA from which the polymerase can begin to synthesise the new strand.

The PCR process usually consists of a series of twenty to thirty-five cycles. Each sequential cycle is composed of three steps:

Denaturation: The double-stranded (template) DNA has to be heated to 94-96°C in order to separate the strands. This is referred to as denaturing; it breaks apart the hydrogen bonds that connect the two DNA strands. Also certain polymerases are activated at this time.

Annealing: After separating the DNA strands, the temperature is lowered. This allows the oligonucleotide primers to bind to the single stranded DNA template. The two oligonucleotide primer sequences are specific for the taget DNA and are constructed using prior knowledge of the DNA sequence of the target sequence. The annealing temperature depends on the primers and is usually 5°C below their melting temperature (45-60°C).

Extension: This is the final stage which involves the DNA polymerase copying the template DNA strand. The heat stable DNA polymerase initiates the synthesis of new DNA by adding the four bases, the deoxynucleoside triphosphates – dATP, dCTP, dGTP and dTTP, to the annealed primers in the order complimentary to the single stranded DNA template. The elongation (extension) temperature depends on the DNA polymerase. Taq polymerase elongates optimally at a temperature of 72°C. The thermostable DNA polymerase is possibly the most important component of the PCR technique as it remains active despite being repeatedly exposed to high denaturing temperatures. Taq polymerase isolated from the bacteria *Thermus aquaticus,* is thermostable up to 94°C. The Taq polymerase and buffers used in this study were obtained from Promega.

The newly synthesized DNA strands act as templates for further DNA synthesis in subsequent cycles. After about 30 successive cycles of DNA synthesis, the products of the PCR will lead to a 10^5 - 10^6 fold increase in the amount of target DNA. This amplification results in sufficient quantities of the target DNA for direct visualization by ultraviolet fluorescence after ethidium bromide staining.

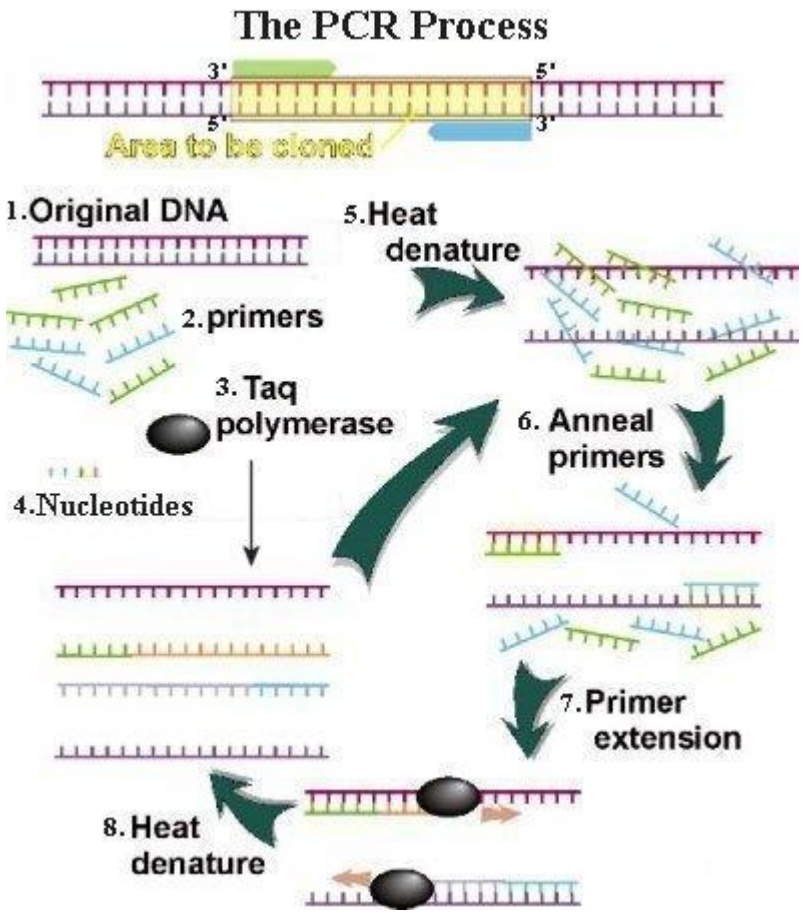


Figure 2.2: The PCR process
Double-stranded DNA in the sample is heated to generate single strands. Sequence specific primers are added, which anneal to desired sites on the DNA. Nucleotides and heat-tolerant DNA polymerase allow for primer extension at elevated temperature. The result is two new copies of double-stranded DNA. The process is repeated to generate multiple specific dsDNA molecules (www.universe-review.ca).

2.3.1 Determination of optimal PCR conditions

When carrying out a PCR reaction some variables may need to be optimized as conditions are specific to each primer pair. The most important variables are the $MgCl_2$ buffer concentration and the cycling temperatures. The number of cycles, amount of template DNA and the primer concentration may also need to be optimized.

The selection of the annealing temperature is one of the most critical factors for optimizing the specificity of a PCR reaction. The higher the temperature the more stringent the base-pairing; hence, the more specific the PCR amplification. However, if the temperature is too low non-specific priming may occur. The choice of optimum annealing temperature for each primer depends on the Tm (melting temperature) of the expected duplex between primer and the DNA. This is the temperature in which half of the primer binding sites are occupied. The melting temperature increases with the length of the primer. The T_m is based on the nucleotide composition of the primer sequence and can be calculated using the following formula:

$T_m(°C) = 2(A+T) + 4(G+C)$.

A preferred method of determining the optimum annealing temperature is when a temperature gradient is generated across a "gradient" block. This can be carried out on a thermocycler. A temperature gradient is built up across the thermoblock, where multiple reaction mixtures can be simultaneously run at temperatures that differ only slightly. With the aid of only one single PCR reaction an optimum temperature for the reaction can be quickly identified.

Lower salt concentration gives a higher degree of specificity by 1) affecting the primer annealing and 2) lowering the activity of the Taq polymerase, which in turn correlates with higher accuracy of the polymerase.

PCR is a highly sensitive technique; therefore, it is important that sufficient measures are used to avoid contamination with other DNA (bacteria, viruses, own DNA etc.). Such measures include changing gloves for each PCR step; and storing aliquots separately from other DNA samples. A control reaction, omitting template DNA, should also be carried out, to ensure the absence of contamination.

2.3.2 PCR Method

The Polymerase chain reactions were carried out in 15 µl reaction volumes. Genomic DNA at a concentration of 100 ng/µl was used as a template. The reagents required were gathered and thawed. The Taq polymerase was always kept on ice. Reagent volumes were calculated using the following table (Table 2.2), in order to prepare a premix solution covering the required number of samples (n). The table contains the name of the reagent, the stock concentration, the concentration used, and the amount of each reagent in a 15 µl reaction volume.

Table 2.2: Reagents used for PCR in 15µl reaction volumes

Reagent	**Stock**	**Final**	**n x (µl)**
DNA	20ngμl^{-1}	100ng	n x 1.5
10x Buffer	10x	1x	n x 1.5
$MgCl_2$	100mM	1.5mM	n x 0.225
dNTP	10mM	0.2mM	n x 0.3
F/R Primer	100ng μl^{-1}	100ng	n x 0.15
Taq Polymerase	5 U μl^{-1}	0.75 U	n x 0.05
H_2O	Variable	Variable	n x 11.35
Final Vol (µl)			1 x 15

The reagents (with the exception of the DNA) were added to a mastermix tube. The Taq polymerase was added last and kept on ice thereafter. Once a premix solution of reagents was prepared 13.5 µl of this solution was transferred into each 0.2 ml labelled PCR tube. The 1.5 µl DNA sample was then added to give the total 15 µl reaction volume.

PCR was carried out on a G-Storm Thermal cycler, under tube temperature control using the following program:

- 95°C X 5minutes
- 95°C X 30seconds
- 40-60°C X 30seconds
- 72°C X 45seconds

} 30 cycles

- Incubate at 72°C X 5minutes
- Hold at 4°C

2.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis is the easiest and most common way of separating and analysing DNA. An electric current is applied and due to the fact that DNA has a negative electric charge, the DNA molecules move across the agarose gel. The DNA fragments are therefore, separated according to their size. By adding ethidium bromide to the gel the DNA is visualised. The ethidium bromide is an intercalating dye which means it binds strongly to DNA. It is also fluorescent, therefore, capable of absorbing invisible UV light and transmitting the energy as visible orange light.

Agarose gel electrophoresis was used after PCR had been performed on any given marker; the products from a number of samples together with the CEPH product and negative control were run on an agarose gel. This was to assess the success of that particular PCR and to confirm that there was no contamination of the reaction.

2.4.1 Procedure

A number of different gel concentrations were used (1.0 to 1.5%), however a 1.5% gel will be described as it was used most often.

1. Molecular grade agarose (1.5g) was dissolved in100 ml of 1X TBE by heating in a microwave until the agarose was seen to have dissolved.
2. The molten solution was poured into a gel plate and when set then tape and combs were removed.
3. Each sample was assigned an electrophoresis lane/well. For each sample 5µl of PCR product was mixed with 2 µl of 2x gel loading buffer (Promega) and loaded into its well.
4. A 100 bp ladder (Promega) was added allow determination of the PCR product size. The ladder contains multiple bands of known sizes, yielding 12 bands suitable for use as molecular weight standards for agarose gel

electrophoresis. The digested DNA includes fragments ranging from 100-1,517 bp. The 500 and 1,000 bp bands have increased intensity to serve as reference points (Figure 2.3).

The DNA ladder also allowed for qualitative estimation of the level of staining and exposure of the gel that was independent of the PCR itself (i.e. if there were no bands seen in the sample lanes and the 100 bp ladder was seen to stain well, then this was due to the absence of the PCR product and not the under-exposure or under-staining of the gel).

5. A voltage of between 100 and 150 volts was applied to the gel for between 10 and 30 minutes.
6. The gel was then immersed in a solution of ethidium bromide (0.5 ug/ml) for between 30 and 50 minutes, depending on the quality and age of the ethidium bromide solution.
7. After staining the gel was examined with a UV transilluminator. (A UV protective visor was used when viewing to avoid eye injury caused by exposure to light from the UV light) Photographic record of the appearance of the gel was obtained and the gel was carefully disposed of.

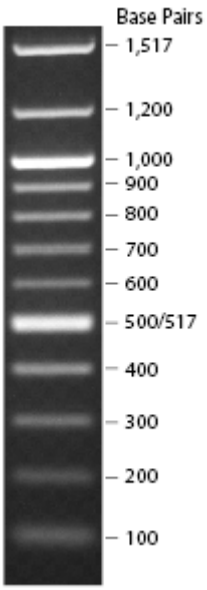


Figure 2.3: 100 bp DNA Ladder visualised by ethidium bromide staining on an agarose gel

The 100 bp DNA Ladder consists of 10 blunt-ended fragments between 100 and 1,000 bp in multiples of 100, plus additional fragments at 1,200 and 1,500 bp. The 500 and 1,000 bp bands are approximately 2 to 3 times brighter than the other ladder bands, and these serve as reference bands.

2.5 Genotyping

One of the essential components of any gene mapping study is obtaining the genotypes for the genetic markers used to test for linkage. Genotyping encompasses a range of applications used to analyse genetic differences between individuals. Amplification by PCR of microsatellite markers using oligonucleotide primers results in alleles of the microsatellite markers that differ by their number of integral repeat units. In this study, genotyping is used for analyzing the differences in microsatellite marker repeats between individuals.

2.5.1 ABI 3130 Genetic Analyser

Genotyping was carried out by direct electrophoreses on an ABI 3130 sequencer analyser (Applied Biosystems). Instead of an agarose gel there is a capillary containing a gel matrix through which the samples are electrophoreised. The fragments of DNA (and all other negatively charged molecules) are attracted to the platinum cathode electrode attached to the end of the capillary (4 capillaries in total), which are filled with Performance optimized Polymer (POP). POP Polymers dynamically coat the capillary wall to control electro-osmotic flow and are specifically formulated to separate DNA fragments of a known size range at a desired resolution and run time. A current is applied to the DNA fragments which travel through the polymer. The capillary has a glass window through which laser light is passed. The fluorescent labels on the PCR product are excited by laser resulting in the emission of light of a particular wavelength (wavelength depends on the fluorescent label). The light is collected and separated according to wavelength by a spectrograph. It is collected onto a charge-coupled device camera. The ABI 3130 has the ability to collect up to five types of fluorescent emissions simultaneously. Using software selectable filters, the data collection software gathers the emissions of light and stores them on the hard disk for eventual processing.

Within each sample a size standard is also run. The internal-lane size standard permits accurate estimation of the peak sizes. The internal-lane size standard used is GeneScan™ - 500 ROX™ developed by Applied Biosystems, which allows accurate sizing of DNA fragment in the 35-500 bp range, and provides 16 single-stranded fragments of 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490, and 500 bases. These fragments are uniformly spaced to provide extremely accurate

base calling. Using this size standard for comparison, the analysis software assembles a graph with fragment size on the X-axis and coloured peaks complementary to the various fluorescent signals. The position of the peaks on the X-axis will depend on the time point these signals were detected in comparison to the time the size standards were detected. The smaller the DNA fragments the faster they will move through the gel and therefore, the earlier they will be detected. The height of the coloured peaks depend on the amplitude of the fluorescent signals, and therefore, on the quantity of the PCR product in the sample. Table 2.3 outlines the advantages of the ABI 3130.

Table 2.3: A list of the advantages of the ABI3130

Advantages of the ABI3130
Continuous, unattended 24-hour operation, with fully automated delivery system, sample injection, separation and data analysis.
Accurate determination of allele size. This is vital when dealing with dinucleotide or trinucleotide repeats, where some alleles may differ by only two or three base pairs.
Capillary electrophoresis is used, which is a significant advantage over slab-gel based technologies. Samples can be re-run and the arrays are guaranteed for 100 runs (rather than one slab per gel).
It is also less time consuming when compared with alternative techniques e.g. polyacrylamide gel electrophoresis succeeded by silver staining.
Markers of similar size labelled with different fluorescent labels can be analysed together, thereby increasing the efficiency of sample analysis.
The results may be saved, which allows easy review of previous ABI runs.

As previously mentioned it is possible to distinguish between the different markers because they have colours on the graph (due to fluorescent markers) or have different size ranges. The operator can accurately view allele sizes for each marker. However interpretations of the graph and deciding which are the true alleles resides with the operator.

For each microsatellite marker, the DNA samples from the family members was analysed with two positive controls in order to accurately determine the correct allele sizes. These positive controls were individual 1 and individual 2 of the CEPH 1331 family. These DNA samples were obtained from CEPH and amplified by PCR in the same manner as the family DNA. The genotypes of these CEPH samples is known for a large number of microsatellite markers and that data is available from the CEPH database. By comparing the known allele sizes with those obtained experimentally, corrections can be made to the allele sizes which ensures that the correct fequency data is applied in the statistical calculation of LOD scores. These controls were used for comparison when sizing the unknown alleles, as the genotype of the CEPH individual is known. The Foundation Jean Dausset-Centre d'Etude du Polymorphisme Humain (CEPH) maintains a database of genotypes for genetic markers that have been typed, and the allele sizes of the positive controls can be viewed on this database (section 1.11.4).

2.5.2 Preparing an ABI3130 run

To analyse samples on the ABI3130, the samples need to be prepared and then a Genemapper software plate record is created in order to inform the of the location of each sample within the sample plate.

1. Sample Preparation
    - For each analysis, a premix was prepared for all samples in the run allowing 12 µl of de-ionised formamide and 0.5 µl of GeneScan^{TM} - 500 ROX^{TM} size standard for each sample. [To maximise use on the ABI3130, multiple markers were analysed simultaneously. For each family member, 1 µl of products from different PCR reactions were pooled together in one sample tube, making sure they were compatible i.e. if they could be distinguished from one another on the basis of different fluorescent primer labels, or different expected sizes.]
    - 12.5 µl of the premix (step 1) was then added and mixed with the pooled PCR products.
    - All sample tubes were checked for air bubbles. Air bubbles can interfere with the injection step in the ABI analysis.
    - The samples were then denatured by heating to 95°C, for five minutes. After denaturing the samples were snap cooled by placing on ice before the samples were placed onto the autosampler tray of the machine.

2. Plate Preparation
    - Before running the samples on the ABI3130 it was necessary to prepare a plate using GeneMapper softer, which told the machine the positions of the samples on the plate. To open a GeneMapper software plate information in the New Plate dialog must first be entered. This includes information on the name of the plate, description of the plate, the plate type (96-well or 384-well), the name of the owner, and the name of the operator. This enables the operator to find and review previous ABI runs easily. Once this is completed the Genemapper software plate editor opens.

- Preparing a Genemapper software plate is simply a way of labelling each sample in the analysis software by indicating its position on the autosampler tray. The following information and conditions were also detailed: Sample Type (Positive control/Negative control/Sample), Size Standard and Analysis Method.

3. Analysis of samples

- When the ABI3130 run was finished, it was necessary to analyse the results and assign alleles to each individual. GeneMapper® Software was used to size and genotype the data. Fragment sizes were determined automatically relative to an the size standard. The software then allocated fragment sizes to the previously calibrated genotypes. The genotypes for each individual were then adjusted based on the two CEPH controls. The alleles for the family members were then recorded on an page which had the family pedigree. This was to ensure that each individual had been assigned the correct alleles, i.e. that a child had one allele from his/her father and one from the mother. Once alleles were assigned it was possible to generate LOD scores (Linkage analysis).

2.6 Programs for Linkage analysis

LOD scores can easily be calculated on any desktop PC. The original LINKAGE program was compiled by J. Ott. (Ott 1985), and this is the program most commonly used for linkage analysis. This program accepts genotype data as a direct input or from the Cyrillic pedigree drawing software. LINKAGE calculates LOD scores between a chosen marker (a disease locus) and one or more other markers, and gives an output which indicates where the chosen marker lies relative to the others. The so called direct method of linkage analysis proceeds by directly observing and counting recombinants and non-recombinants.

For this study two-point linkage analysis was performed using the subprogram MLINK of the Linkage program (version 5.0) as implemented in FASTLINK (ftp://linkage.rockefeller.edu/software/fastlink) (Lathrop et al., 1984: Lathrop et al., 1985). MLINK calculates the likelihood of the pedigree under user-specified values of θ for two different loci (http://linkage.rockefeller.edu/soft/linkage/sec3.3.html). Subsequently, these likelihoods are used to calculate the LOD scores. In this case, analyses performed with MLINK involved a disease locus and a marker locus.

Prior to running the MLINK program two input files are required;

1. A pedigree (*marker.ped*) file. This file contains numeric representation of the family structure, disease affection status and details the alleles present in each individual.
2. A parameter (marker.dat) file. This file includes information specific to the genetic model to be analyse such as disease and marker allele frequencies, and disease penetrance.

In this study, RLS was analysed as an autosomal dominant disease with the assumption of a disease-gene frequency of 0.001, and a penetrance rate of 95% (Bonati, Ferini-Strambi et al. 2003). Microsatellite maker allele frequencies were obtained from the CEPH database (www.cephb.fr).

Using the above files, MLINK was used to generate LOD scores for specified θ values. The output from the two-point LOD score is put into the output (*marker.out*) file.

While genemappers hope for a positive LOD score i.e. linkage, negative LOD scores are not without value. Such values are informative and show where the disease gene is **not** located, otherwise known as exclusion mapping. Consequently if enough of the genome is excluded only a few possible locations may remain. To find the genetic distance actually excluded when a negative LOD score < -2 is observed, a program called the map function (*mapfun*) is used. This program is available at URL ftp://linkage.rockefeller.edu/ott/linkutil.htm. A map function allows conversion between recombination frequencies (θ) and genetic distance in centimorgans (cM), under the assumption of a certain level of genetic interference (section 1.11.6).

2.6.1 Calculating sample size and power

Before commencing linkage analysis, it is important to know whether the available pedigree information is sufficient to allow detection of gene(s) causing the disease of interest. Computer simulation methods are often employed to investigate this. The simulation method can also be helpful in determining if an observed maximum LOD score is significant for a pedigree. For example, if a LOD score of 2.0 was found in the pedigree in question with a given marker and disease, according to the established LOD-score-of-3 criterion this result is not significant. However, in a small pedigree with a sample size that is not sufficient to generate a LOD score of 3 such a LOD score could be of interest. Such information can help the researcher decide whether or not to devote further time into typing more markers in this region, to try finding the disease causing gene in question.

For this study we used the SLINK package to investigate if the RLS3002 pedigree was powerful enough to detect linkage. The program SLINK is a general computer program that uses a variation of the algorithm described by OTT (1989). It is based on the LINKAGE programs version 4.91 (Lathrop, Lalouel et al. 1984). The SLINK package consists not only of the simulation program (SLINK) but also of several analysis programs (MSIM, LSIM, ISIM, ELODHET). It is essential to remember that the data are simulated conditional on the phenotypes given in the pedigree file, at the recombination fraction(s) given in the data file.

2.7 Primer Design

Primer design is aided greatly by software and includes checks for balanced annealing temperatures and for possible dimers and hairpins. There are many software packages available such as Primer Express (Applied Biosystems) and some can even be downloaded from the internet. All of the primers which were designed for this project were done so with the assistance of *PrimerSelect, EditSeq* and *SeqBuilder*, which are components of the *DNAStar* software package (Lasergene). This software supports a large number of criteria for primer design including checks for internal primer stability, energy profile, primer dimer and hairpin formation and a mismatch primer deign.

The following points should be adhered to when designing primers regardless of the software used:

- Primers should be between 16 and 24 bases long
- Avoid long runs of any single base, particularly the tetranucleotide GGGG as it frequently occurs throughout the human genome.
- If possible, the G and C content should be approximately 50% to prevent excessive primer dimer formation
- Ensure that the ends of the primers are not complimentary, thus avoiding excessive primer dimer formation
- Preferably the T_m of the two primers should be within 2°C of each other.

Primers had to be designed for this project for two different purposes;

1. The first was when trying to minimize the RLS locus, by selecting repeats within specific areas of the locus in the hope that the number of repeats would differ between family individuals thus giving information.
2. The second was when selecting a gene for screening within the RLS locus.

When selecting an area for genotyping or a gene for screening:

- UCSC (www.genome.ucsc.org), ensemble (www.ensembl.org) and NCBI (www.ncbi.nlm.nih.gov/) websites were consulted.
- The location of interest was chosen and the DNA sequence was found using UCSC website.
- Di-, tri-, or tetranucleotide repeats in the area of interest were identified. This area containing the repeat region was selected, with approximately 200 bp flanking either side
- The sequence of interest was entered into *EditSeq* by a simple copy and paste procedure. The sequence became a (seq)-format when saved there.
- The sequence was imported to *PrimerSelect.*
- For each microsatellite marker primers were designed. Each of these primer files were saved as D(chromosome no.)S(physical position).
- The primers were located on the original SeqBuilder File and it was ensured that the amplified region contains the repeated region.
- The 5' end of one of the primers was tagged with a fluorescent dye such as 6-FAM or HEX as genotyping was carried out on an ABI automated DNA analyser.
- By using the PCR tool in UCSC or using BLAST from NCBI it was verified that the primer pairs were specific. It was vital to ensure that each primer would bind to the correct genomic location. If both primers also bound a location on another chromosome, it was essential to investigate if a PCR product would be generated.
- When all primers had been validated primers were ordered from www.biomers.net .

Or when selecting a gene for screening:

- Once a candidate gene was chosen for screening the UCSC (www.genome.ucsc.org), Ensembl (www.ensembl.org) and NCBI (www.ncbi.nlm.nih.gov/) websites were searched for gene information. If more than one transcript existed it had to be ensured that all exons were screened.
- The genomic DNA, mRNA and protein sequences for the gene was obtained, and saved using *EditSeq*. Looking at the genomic sequence the number of exons in the gene were confirmed i.e. the sequence in uppercase.
- It was important to consult another commonly used genetic website such as Ensembl, and check that no additional transcripts or alternate exons existed. Also it was confirmed that the protein sequence corresponded with the UCSC protein.
- Also it was examined if the exons found in the ensemble site were identical or different to the UCSC exons.
- Each exon sequence was saved as a file named GENEx1.
- All the exons were spliced together to make an in silico mRNA. This GENEspliced file was opened in MapDraw, the ORFs were found and the protein translated. It was confirmed that it was the expected protein.
- Once the exons were confirmed, the UCSC genomic seq file was revisited and each exon with approximately 300bp flanking sequence was selected. Each of these files was saved as GENEx1 exonic region.
- Using primerSelect and the exonic region files, primers were designed for each exon. The primers had to be positioned so that at least 50bp upstream of the start of the exon would be sequenced
- If a large exon existed, a group of overlapping primer pairs was designed to ensure that the entire region was covered. These primers were labeled x3a, x3b, x3c etc
- Each of these primer pairs were saved as GENEx1 primer files.
- The primers were detected on the exonic region file.It was also confirmed that the exon was adequately covered. This file was saved with the sequence of the PCR product as a GENEx1 amplicon file.

- A word file of primers to be ordered was generated and the primers were copied from PrimerSelect. It was confirmed that the orientation of the primers was correct by comparing them with the amplification summary in PrimerSelect.
- It was also confirmed that the primer pairs were specific for the selected gene by using the PCR tool in UCSC or using BLAST from NCBI selecting human only. It was important that each primer hit the correct location. If both primers also hit a location on another chromosome, it was necessary to investigate if a PCR product would be produced.
- When all primers had been validated primers were ordered from www.biomers.net.

2.8 PCR Purification

PCR Purification prior to sequencing was carried out using the JETQUICK PCR purification kit (Genomed). PCR purification kits provide rapid and efficient removal of short primers, dNTPs, enzymes, short-failed PCR products, and salts from PCR products. The kit contains two solutions: Solution H1 (binding) contains guanidine hydrochloride, isopropanol and Solution H2 (wash, reconstituted) contains ethanol, NaCl, EDTA and Tris/HCl. Before starting the procedure, the solution H2 is reconstituted in alcohol (96-100%) as indicated in the instructions. The kit also contains spin columns or 'micro-spin units' which have a silica membrane in microcentrifugation units. These silica membranes initially trap the PCR products and during the final steps the PCR products are released from the membranes. All centrifugation steps are carried out at >12,000 RPM in a table-top microfuge.

1. Sample Preparation

400 µl of solution H1 was added to 100 µl of PCR assay and mixed thoroughly.

2. Column Loading

A JETQUICK spin column was placed into a 2 ml receiver tube. The mixture from step 1 was loaded into the prepared spin column and centrifuged at >12,000 RPM for 1 min. The flowthrough was discarded.

3. Column Washing

The spin-column was inserted into an empty receiver tube. 500 µl of reconstituted solution H2 was added and centrifuged at >12,000 RPM for 1 min. The flowthrough was discarded. The JETQUICK column was placed back into the same receiver tube and centrifuged at maximum speed again for 1 min.

4. DNA Elution

The spin-column was inserted into a new 1.5 ml microfuge tube. 50 µl of sterile water (or TE buffer or 10 mM Tris/HCL, pH 8.0) was added directly onto the center of the silica matrix of the spin column. It was then centrifuged at >12,000 RPM for 2 min. The eluted sample was stored at 1–5° C.

2.9 Sequencing Reaction

Prior to sequencing the following steps were essential:

- The fragment was amplified by PCR (50 µl).
- Amplification was confirmed on an Agarose gel.
- PCR product was purified (JETQUICK kit).
- The purified product was then used as the template for sequencing. This step is based on Fred Sanger's dideoxy sequencing method which will be discussed in more detail in section 6.1/6.2. This will result in differently sized DNA fragments of the desired sequence with each base in the sequence tagged with a different fluorescent label.

Table 2.4: Reagents used for PCR in 15µl reaction volumes

Big Dye Ready Reaction mix	0.5 µl
Buffer	1.0 µl
Primer (100ng μl^{-1})	0.5 µl
H_2O	0.5 µl
Template DNA (purified)	2.5 µl
Total	5.0 µl

Sequencing was carried out on a thermocycler, using the following sequence cycle programme:

- 93°C X 30 seconds
- 50°C X 15 seconds
- 60°C X 4 minutes

} x 25 cycles

- Incubate at 10°C X 15 minutes

2.10 Post-Sequencing Purification

Purification of sequencing products was carried out using the AmpliPurify™ ExTerminator Kit (AME Biosciences). This optimised purification procedure will remove unincorporated dyes, dNTPs and excess salt. It will also remove the sequencing enzyme. All of these components, if not removed, will greatly reduce the quality of the capillary electrophoresis data.

The post-sequencing purification was carried out as follows:

1. 5 µl of Mix Blue was added to the sequencing reaction mixture (10-20 µl).
2. 100 µl of bind/wash solution was added.
3. The sample was loaded onto a minicolumn and spun at 3,000-4000 RPM for 30 seconds.
4. Another 400 µl of bind/wash solution was added to the minicolumn and spun at 12,000 – 14,000 RPM for 2 minutes.
5. The minicolumn was transferred to a new 1.5ml tube and 25 µl of was applied directly to the membrane.
6. The column and solution were incubated at room temperature for 2 minutes, and then spun again at 12,000 – 14,000 RPM for 1 minute.
7. The samples were loaded directly onto the ABI3130 Sample Plate.

2.11 Sequencing analysis

After loading the samples onto the ABI3130 plate, a Plate Record was created as for genotyping (section 2.5.2), however the application “Sequencing” was chosen instead.

Following completion of the run, the generated sequences were analysed from raw data. This was carried out by opening the Sequence analysis software on the ABI3130 PC, adding the samples, and analysing. The success/failure of the ABI3130 system run was recorded. It was noted if the run was completed correctly or if there had been an interruption to the run. The success of the sequencing for each individual sample was also noted, e.g. failed to sequence or sequence present but short/unreadable etc.

2.11.1 Seqman Software

After a sequence was run successfully on the ABI3130, it was necessary to compare generated sequences to each other, and also to generate a contig of sequences. DNAstar has a software tool available to do this, known as SeqMan. It allows users to perform alignments and discover variants, along with many other analyses on de novo or template assemblies.

Sequence assembly and Contig management

- The Seqman program was opened from the Lasergene menu.
- The sequences to be assembled were located and opened.

The following options for assembly were chosen:

1. Minimum match percentage = 80%
2. Trace evidence (compare) 95%
3. Majority (primary) 100%

- To detect any variants between samples the traces were compared.
- The sequences were examined carefully and any changes were recorded. It was important to see if any of the variants observed were in fact SNPs, and this was discovered by consulting with either the dbSNP or UCSC databases.
- All files were saved so that additional sequences could be added as they were generated.

Chapter 3 Investigation of known RLS Loci and Associated Regions

3.1 Introduction

Familial aggregation of RLS has been recognized since Ekbom formally described the condition in 1945 (Ekbom 1945). Since then genetic contribution to idiopathic RLS has been well-documented, is substantial, and has been consistently recognised from population and family studies. In 1960 Ekbom described familial aggregation in one-third of RLS patients (Ekbom 1960). A high concordance rate of 83% in monozygotic twins adds to the evidence(Ondo, Vuong et al. 2000).

To date several major susceptibility loci for RLS (termed RLS1-5 and RLS7) have been proposed by linkage analysis. In addition 3 other loci on chromosome 16p, 4q and 17p were recently identified (table 3.1). A study of 25 individuals from a French-Canadian family was the first to identify a major genetic locus for the RLS phenotype. While findings were consistent with an autosomal recessive mode of inheritance, the researchers suggested a pseudodominant mode of inheritance may be more appropriate (Desautels, Turecki et al. 2001). Linkage to chromosome 12q was the only locus found under this model of inheritance. This locus has since been reported in several families as well as in the Icelandic population. According to Winkelmann et al the genetic traits found in these families could be explained by the described pseudominant model (described by Desautels et al) as well as by an autosomal dominant model (Desautels, Turecki et al. 2001; Winkelmann, Lichtner et al. 2006). Nonetheless, the susceptibility locus for RLS on chromosome 12q could not be confirmed in a study of 51 South Tyrolean family members or in two Northern Italian families affected by RLS (Ferini-Strambi, Bonati et al. 2004). Linkage in all other instances assumed an autosomal dominant inheritance model and is limited to single or a small number of kindreds. Further studies in Italian,

American, Canadian and German families showed linkage to chromosome 14q (RLS2), 9p (RLS3), 20p (RLS4), and 2q (RLS5). A study carried out in 2006 by Winkelmann and associates identified two loci in a single RLS family of German origin on chromosome 4q and 17p (Winkelmann J 2006). In 2008, suggestive evidence for linkage was found on chromosome 19p13 (Kemlink, Plazzi et al. 2008).

Genome-wide association studies are also providing a better picture of how variants in the sequence of the human genome impact RLS. Association studies compare the frequencies of alleles in case and control populations. A higher frequency of the allele tested in cases is taken as confirmation that the allele or genotype is associated with an increased risk for the disease. Two such studies used a large-scale high density genome-wide approach. The first study was performed using the German and Canadian population in 1600 RLS cases and 2600 controls. Three genomic regions were identified. The first was RLS6 which was found to be associated with SNPs in the *BTBD9* gene on chromosome 6p21 (Stefansson, Rye et al. 2007; Winkelmann, Schormair et al. 2007). Two other variants were found within intronic regions of *MEIS1* and *MAP2K5/LBXCOR1* (Winkelmann, Schormair et al. 2007). Individuals who carry one risk-allele have a 50% increased risk of developing RLS (Stefansson, Rye et al. 2007). Coincidently, in the same year a similar study was carried out in the Icelandic and US population, and an association was found to the identical variant in *BTBD9* (RLS6) (Stefansson, Rye et al. 2007). In this study the presence of PLM was used to support the diagnosis of RLS and the association of *BTBD9* was based on individuals being PLM-positive. However, none of these genes are located in any of the previously described loci (RLS1-RLS5). In 2008, an association study which included 2458 RLS cases and 4749 controls focused on the RLS3 linkage region, on chromosome 9p. Two independent signals within splice variants expressed in the

central nervous system of *PTPRD* showed genome-wide significant association to RLS. *PTPRD* located on chromosome 9p23-24 is the fourth locus associated with RLS (Schormair, Kemlink et al. 2008). Also in 2008, a further association study in two Caucasian RLS samples of 918 independent cases and controls focused on the RLS1 linkage region. A total of 1536 SNPs in 366 genes within the 21 Mb region of RLS1 were analysed. Association was observed with SNP rs7977109 which is in the neuronal nitric oxide synthase (*NOS1*) gene, and therefore, suggests the involvement of the NO/arginine pathway in the pathogenesis of RLS (Winkelmann, Lichtner et al. 2008). These molecular findings suggest substantial genetic and clinical heterogeneity of RLS.

Table 3.1 lists the previously known RLS loci and associated regions, the ancestry, the number of generations or number of cases/controls, the method used to locate the region, the chromosomal location and locus name.

Table 3.1:Known RLS loci and associated regions.

Study	**Ancestry**	**No. Of generations (No. of members) or No. of cases/controls**	**Method**	**Chromosomal location**	**Locus**
	Irish	2 (29)	Linkage analysis	5q	
Desautels et al. (2001)	French-Canadian	1 (25)	Linkage analysis	12q12-q21	RLS1
Bonati et al. (2003)	Italian	1 (30)	Linkage analysis	14q13-q31	RLS2
Chen et al. (2004)	United States	2 (23)	Linkage analysis	9p24-22	RLS3
Pichler et al. (2006)	South Tyrolean	Population Isolate (530)	Linkage analysis	2q33	RLS4
Levchenko et al. (2006)	French-Canadian	1 (59)	Linkage analysis	20p13	RLS5
Winkelmann et al (2006)	German	Unknown	Linkage analysis	4q	
Winkelmann et al (2006)	German	Unknown	Linkage analysis	17p	
Winkelmann et al (2007)	German and Canadian case/controls	1600 cases and 2000 controls	Association study	6p21	RLS6
Stefansson et al (2007)	Icelandic	429 cases and 16,866 controls	Association studies	6p21	RLS6
Winkelmann et al (2007)	German and Canadian case/controls	1600 cases and 2000 controls	Association studies	2p14	RLS7
Winkelmann et al (2007)	German and Canadian case/controls	1600 cases and 2000 controls	Association studies	15q	
Schormair et al (2008)	German, Austrian, Czech, Canadian	2458 RLS cases and 4749 controls	Association studies	9p23-24	
Winkelmann et al (2008)	Caucasian	918 independent cases	A three-stage association study	12q	
Levchenko et al (2008)	French-Canadian	4 (46)	Linkage analysis	16p	
Kemlink et al. (2008)	Italian	3 (12)	Linkage analysis	19p	

The newly recruited Irish family RLS3002 had been previously examined for linkage to known loci. At the time of the initial research there were only four known loci: chromosome 12q, 14q, 9p and 5q and hence only these loci were examined. The research successfully excluded the entire chromosome 14 locus. However, although the other three loci were excluded for linkage to RLS in this pedigree by haplotype analysis, gaps still remained which required further investigation.

Therefore, the aim of part one of this study was to completely exclude linkage from the three loci already examined, and to examine the newly identified loci and associated regions.

3.2 Materials and methods

DNA extraction

The same QIAamp Blood Kit from Qiagen described in section 2.2 was used to extract DNA from peripheral venous blood samples.

Primer selection

Polymorphic markers from the regions of RLS loci and associated SNPs were selected. Primer information was accessible from the UCSC genome browser (www.genome.ucsc.edu) and from the NCBI website (www.ncbi.nlm.nih.gov). Primers were ordered from biomers.net and the forward primer of each pair was fluorescently tagged to allow detection by the ABI 3130 system. The microsatellite markers for each of the RLS loci and associated SNPs examined are outlined in the tables below.

Polymerase Chain Reaction (PCR)

For this section of the study and for the purpose of genotyping, Polymerase chain reaction was carried out in 15 µl reaction volumes. Table 3.2 provides details of the reagents used.

Table 3.2: Reagents used for PCR reaction

Reagent	**[Stock]**	**[Final]**	**1 sample (µl)**
10x buffer	10x	1x	1.5
$MgCl_2$	25mM	1.5mM	0.9
dNTP	10mM	0.2mM	0.3
Forward Primer	100ng/µl	100ng	0.5
Reverse Primer	100ng/µl	100ng	0.5
Taq polymerase	5U/µl	0.05U	0.1
H_2O			6.2
DNA		50-100ng	5.0
Total Volume			15.0

Once the samples were prepared, each 15 µl reaction was placed in a 0.2 ml capped PCR tube and placed on the GStorm Thermal Cycler PCR machine. A temperature gradient was initially used to discover the optimal annealing temperature for each individual marker (as outlined in section 2.3.1). The success of PCR was verified by agarose gel electrophoresis (as outlined in section 2.4).

Genotyping and Linkage analysis were successfully performed for all markers under investigation (as per section 2.5 to 2.7). Figure 3.1 below is an example of ABI analysis reading of genotyping for two parents and two children for marker D13S218.

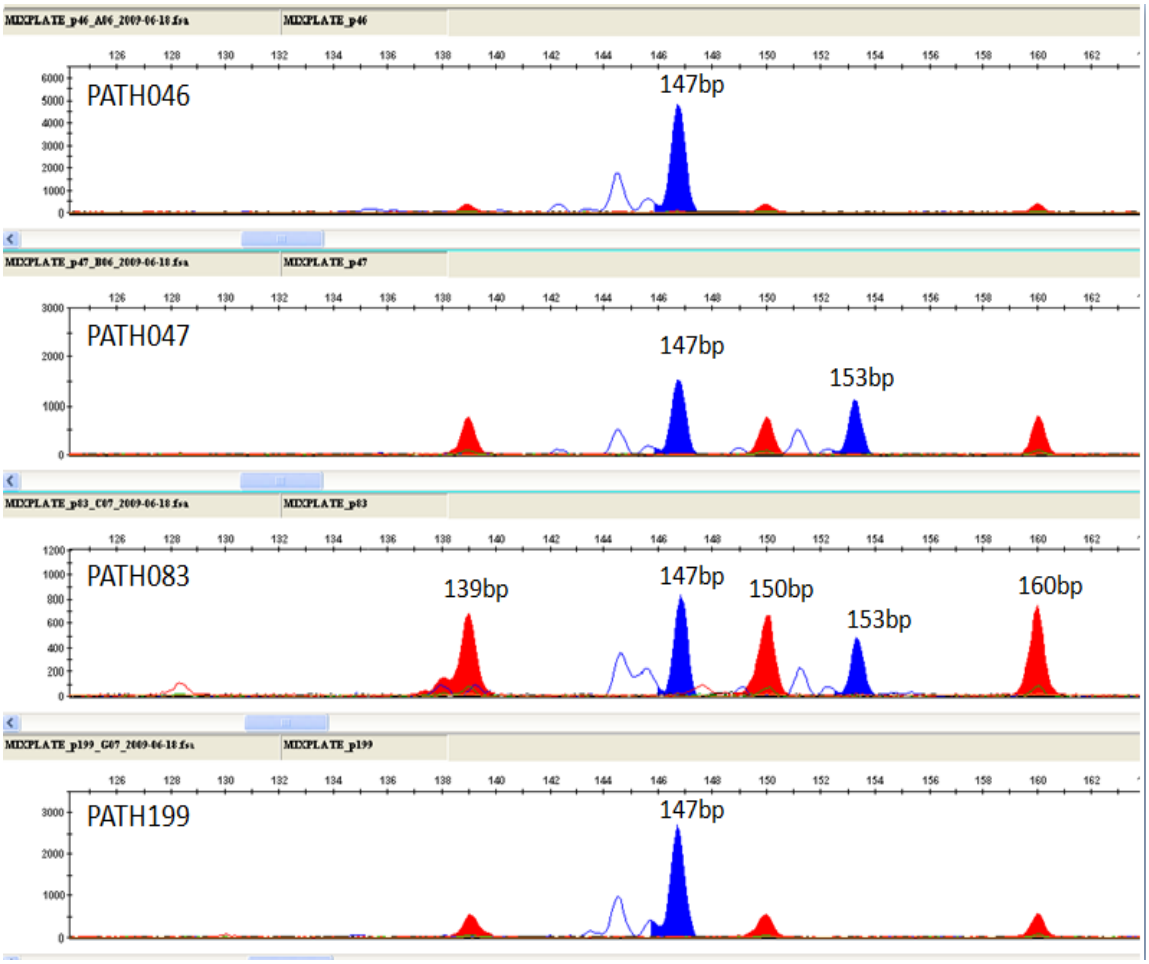


Figure 3.1: An example of ABI genotyping of related individuals
Marker D13S218 using samples PATH046 (mother), PATH047 (father), PATH83 (child1) and PATH199 (child 2). The red peaks refer to the internal size standard (ROX) used to determine allele sizes. These size standards are labelled for PATH083, at 139 bp, 150 bp and 160 bp respectively. The alleles are shown as blue peaks. The colour of the peaks correspond to the colour of the fluorescent label, which in this example is 6-FAM. The mother (PATH046) is homozygous for this marker (one peak at 147 bp) and the father (PATH047) is heterozygous (two peaks at 147 bp and 153 bp). The positions of the peaks corresponds to the size of the allele which in this case is 141-153 bp. Child 1 (PATH083) is heterozygous and the alleles can be traced through the generations. He receives the 147 bp allele from his mother and the 153 bp allele from his father. Subsequently child 2 (PATH199) is homozygous for this marker (one single peak) with the inheritance of one 147 bp allele from the mother and another 147 bp from the father.

The allele fragment sizes obtained from genotyping were correlated with the allele numbers according to the CEPH database (www.cephb.fr). LOD scores were generated and distances excluded were determined. In addition, when gaps remained to be excluded in a locus, haplotype analysis was performed.

Chromosomes 5q, 12q, and 9p had been analysed previously and needed some further work; therefore, they were examined first. The Linkage analysis result of each locus and associated region is described separately.

3.3 Results

3.3.1 Chromosome 5 locus

A novel locus for autosomal dominant RLS was previously mapped to chromosome 5q. Based on linkage analysis and haplotype analysis of the large Irish family, the locus was placed in an 11.50 cM region between D5S2507 and D5S2042. Marker D5S2507 is located at 66.81 cM and marker D5S2042 is located at 78.31 cM. This locus had been previously examined for linkage for our RLS3002 Irish pedigree; however, linkage was not excluded from the entire locus. These previous results almost excluded the chromosome 5 locus but a small 3.08 cM gap remained between 72.53 cM and 75.61 cM. When this initial study was being carried out, only 11 family members were available. A number of other family members became available at a later date; therefore, it was necessary to genotype these members for the markers already examined. This was done in the hope that the additional family members would give more power to the study, giving a greater LOD score and thereby reduce or remove the remaining gap. Table 3.3 lists all the markers examined and outlines their positions (cM), the maximum LOD scores obtained at theta equal to zero and the corresponding maximum excluded distances in cM. Five of the six markers had been previously examined (Abdulrahim 2008). These markers are shown in blue. The results of the original data is shown in italics and the updated data is shown in regular text underneath.

Table 3.3: Markers used to examine chromosome 5 locus.

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from (cM)**	**Area Excluded to (cM)**
D5S418	58.55	-11.04	13.63	44.92	72.18
D5S2500	69.23	*-5.51* -6.89	*3.30* 6.94	*65.93* 62.29	*72.53* 76.17
D5S2072	73.35	*0.17* 0.57	*0.00* 0.00	- -	- -
D5S647	74.07	*0.78* 1.38	*0.00* 0.00	- -	- -
D5S2036	74.68	*-1.98* -1.94	*0.00* 0.00	- -	- -
D5S2003	78.31	*-5.51* -5.44	*4.70* 4.92	*73.61* 73.39	*83.01* 83.23

As mentioned, five markers which had been examined previously were genotyped for the remaining members of the RLS3002 family (PATH185, PATH186, PATH187, PATH199, PATH200, PATH207 and PATH208) which had been unavailable at the initial investigation of this locus. An additional marker, D5S418 was also examined for this locus. Following investigation with the additional family members the 3.08 cM gap was removed. Figure 3.2 illustrates the positions of the markers, and the corresponding regions of exclusion on chromosome 5. The total area excluded spans from 44.92 cM to 83.23 cM.

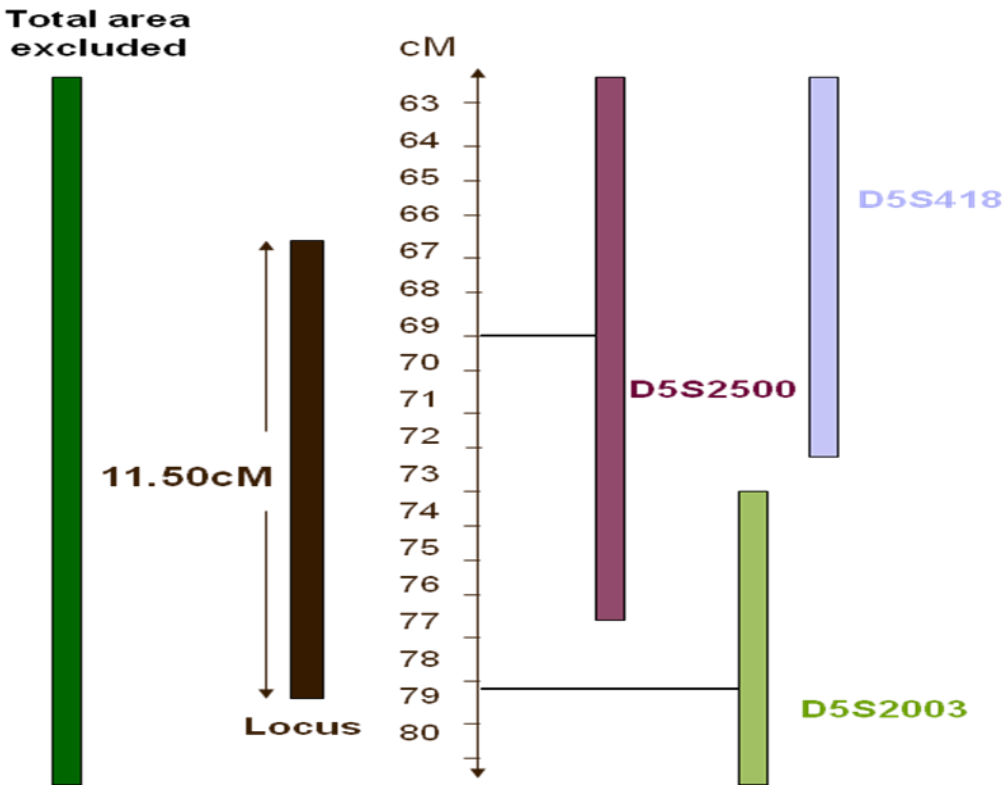


Figure 3.2: Illustration of areas of exclusion for chromosome 5 locus
Black arrow with scale represents a portion of chromosome 5. The black bar to the left of chromosome 5 represents the RLS locus spanning 11.50 cM. The regions excluded by the markers D5S418, D2S2500 and D2S2003 are represented by the coloured bars to the right of chromosome 5. The dark green bar on the far left illustrates the total area excluded combining results from all three markers.

3.3.2 Chromosome 12 locus (RLS1; MIM 102300)

Chromosome 12 locus is flanked by markers D12S1044 and D12S78, located at 97.16 cM and 111.87 cM respectively. The distance between these markers is 14.17 cM (Desautels, Turecki et al. 2001). This locus was already examined for family RLS3002; however these results did not totally exclude the locus. There was a gap of 0.10 cM which remained to be excluded. Seven markers were previously examined for the chromosome 12 locus. During this study an additional four markers were examined at this locus. Table 3.4 lists all the markers examined during the initial study (blue text) and the four additional markers examined, the LOD scores obtained at theta equal to zero, the distance of excluded regions, and the corresponding region of exclusion. Marker D12S1300 was repeated for the seven additional markers, similar to the chromosome 5 markers. The results of the original data is shown in italics and the updated data is shown in regular text underneath.

Table 3.4: Markers used to examine chromosome 12 RLS1 locus.
(Original data is shown in italics and updated data is shown in regular text.)

Marker	Position (cM)	Max LOD (θ=0)	Excluded (cM)	Area Excluded from (cM)	Area Excluded to (cM)
D12S1052	83.19	*-3.50*	*0.00*	*83.19*	*83.19*
D12S326	86.40	-6.75	3.20	83.20	89.60
D12S1064	95.03	*-3.22*	*1.20*	*93.83*	*96.23*
D12S351	95.56	-4.51	9.72	85.84	105.28
D12S1044	103.90	*-6.10*	*4.92*	*98.98*	*108.82*
D12S1300	104.12	*-1.70* -2.82	*0.00* 2.20	- 101.92	- 106.32
D12S346	104.65	-10.90	11.82	92.83	116.47
D12S1030	109.47	*0.01*	*0.00*	-	-
D12S318	109.47	*-2.78*	*0.90*	*108.57*	*110.37*
D12S78	111.87	*-2.78*	*-1.40*	*105.67*	*118.07*

In the previous study five markers obtained statistically significant LOD scores and excluded various regions of the chromosome 12 locus. The marker D12S1052 showed a significant LOD score however it was only significant at θ=0.00. Hence there was no area of exclusion except at the position of the marker itself. Four additional markers D12S326, D12S351, D12S1300 and D12S346 were examined for this locus, to attempt to exclude the remaining 0.10 cM gap which was positioned between 110.37 cM and 110.47 cM. Marker D12S346 was successful at excluding this gap, excluding from 92.83 cM to 116.47 cM. Figure 3.3 illustrates the positions of the markers, and the corresponding regions of exclusion on chromosome 12. The total area excluded expands from to 83.19 cM to 118.07 cM.

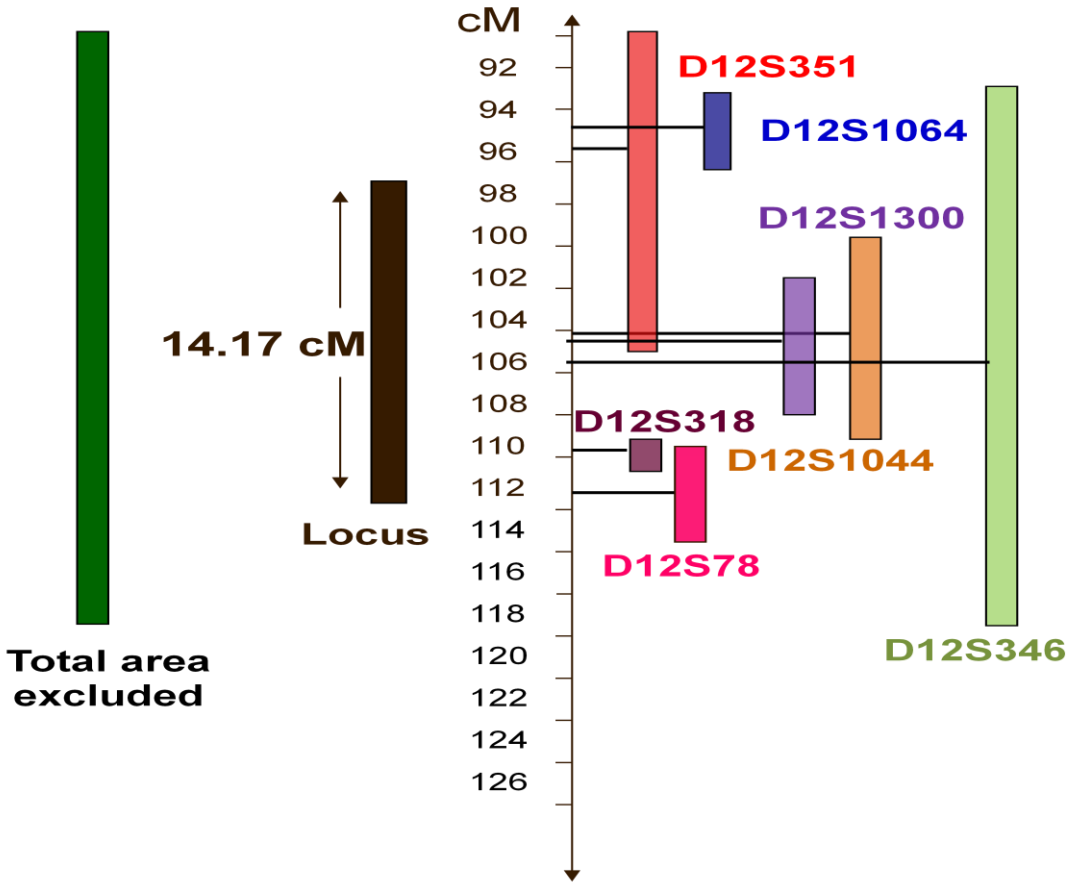


Figure 3.3: Illustration of areas of exclusion on chromosome 12 locus
Black arrow with scales represents a portion of chromosome 12 . The black bar to the left of chromosome 12 represents the chromosome 12 locus spanning 14.17 cM. The regions excluded by the markers D12S1064, D12S351, D12S1044, D12S1300 D12S346, D12S318, and D12S78 are represented by the coloured bars to the right of chromosome 12. The dark green bar on the far left illustrates the total area excluded combining results from all markers.

3.3.3 Chromosome 9 locus (RLS3; MIM 610438)

The chromosome 9 RLS locus is the largest found to date, and is located on the small arm of chromosome 9. The candidate region is defined by markers D9S1779 (0.00 cM) and D9S162 (34.42 cM) in a region covering a 34.42 cM. Five markers had been previously examined, of which four obtained a statistically significant LOD score. However, two gaps remained to be excluded on the chromosome 9 locus. The first gap was located at the telomeric end of chromosome 9 and was 15.00 cM in length, and the second was located between 21.36 cM and 24.81 cM, 3.4 cM in length. Two new markers, D9S1813 and D9S1810, located at the telomeric end were ordered for the 15.00 cM gap. Marker D9S274 was re-examined with the remaining members of the RLS3002 family which had been unavailable at the initial investigation of this locus. Table 3.5 lists the markers investigated and summarizes, their positions, the LOD scores obtained at theta equal to zero, the distance of excluded regions, and the corresponding region of exclusion.

Table 3.5: Markers used to examine chromosome 9 locus.
(Original data is shown in italics and updated data is shown in regular text.)

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from (cM)**	**Area Excluded to (cM)**
D9S917	0.00	*-0.11*	*0.00*	-	-
D9S1779	0.00	*-2.20*	*0.00*	-	-
D9S1871	8.37	*0.43*	*0.00*	-	-
D9S1813	9.83	-5.80	6.30	3.50	16.16
D9S288	9.83	-8.35	7.45	2.38	17.28
D9S1810	12.78	-3.4	4.90	7.88	17.68
D9S286	18.06	*-3.23*	*3.30*	-	-
D9S274	28.42	*-3.10* -5.60	*3.61* 8.89	- 19.53	- 37.31
D9S162	34.42	*-3.30*	*5.02*	-	-

The results for each marker on the initial examination are highlighted in blue in table 3.5 above. The results which were achieved after genotyping the additional family members are in the unshaded lines below their corresponding marker. The two additional markers examined are included in this family. Following investigation with the additional family members the 3.40 cM gap located between 21.36 cM and 24.81 cM was removed. The 14.76 cM gap was partially removed; however a 2.38 cM gap remains at the top of chromosome 9. Figure 3.4 illustrates the positions of the markers, and the corresponding regions of exclusion on chromosome 9.

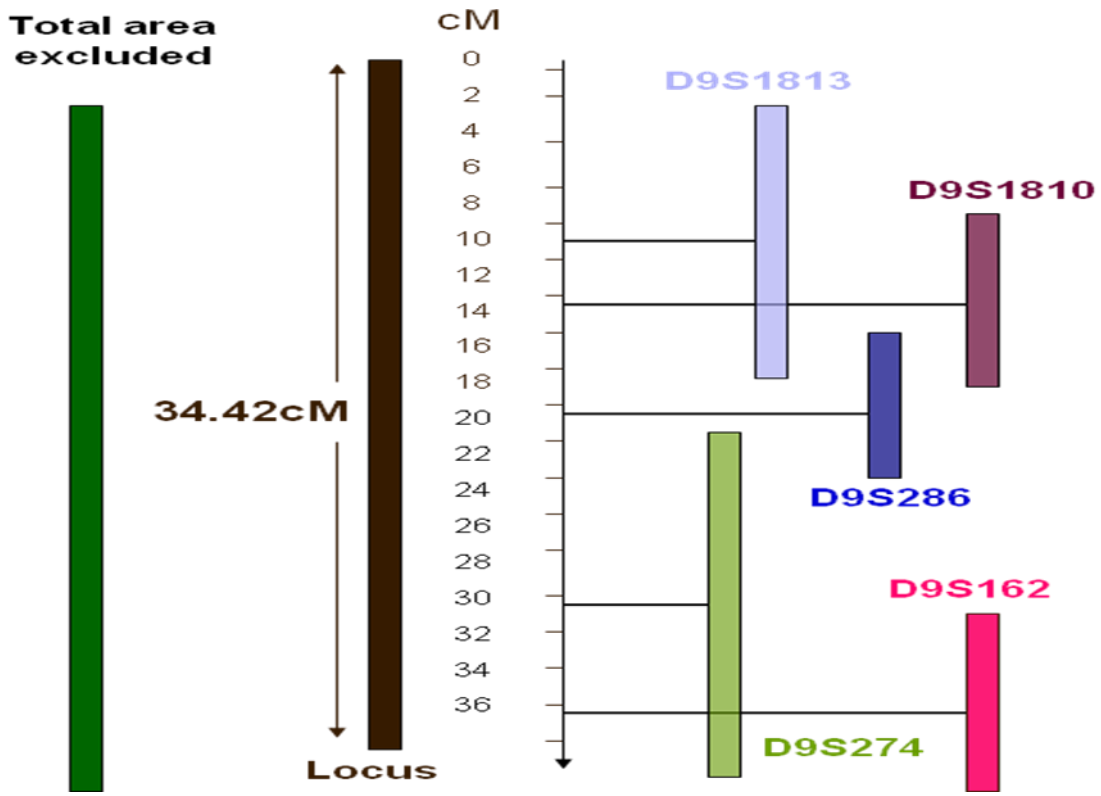


Figure 3.4: Illustration of areas of exclusion on chromosome 9 locus
Black arrow with scales represents chromosome 9 (scales in cM). The black bar to the left of chromosome 9 represents the chromosome 9 locus spanning 34.42 cM between markers D9S1779 and D9S162. The regions excluded by the markers D9S1813, D9S1810, D9S286, D9S274 and D9S162 are represented by the light blue, purple, dark blue, green and pink bars to the right of chromosome 9. The green bar on the far left illustrates the total area excludeds.

The results almost exclude the chromosome 9 locus, however a small 2.38 cM gap remains to be excluded at 0.00 cM to 2.38 cM. A haplotype analysis was carried out at this locus. It is clear from this analysis that there is no haplotype that is present in all the affected individuals and not present in the unaffected individuals. Therefore, due to the fact that the gap is small and that the haplotype analysis confirms exclusion of the locus, it can be assumed that the chromosome 9 locus is not linked to RLS in the pedigree.

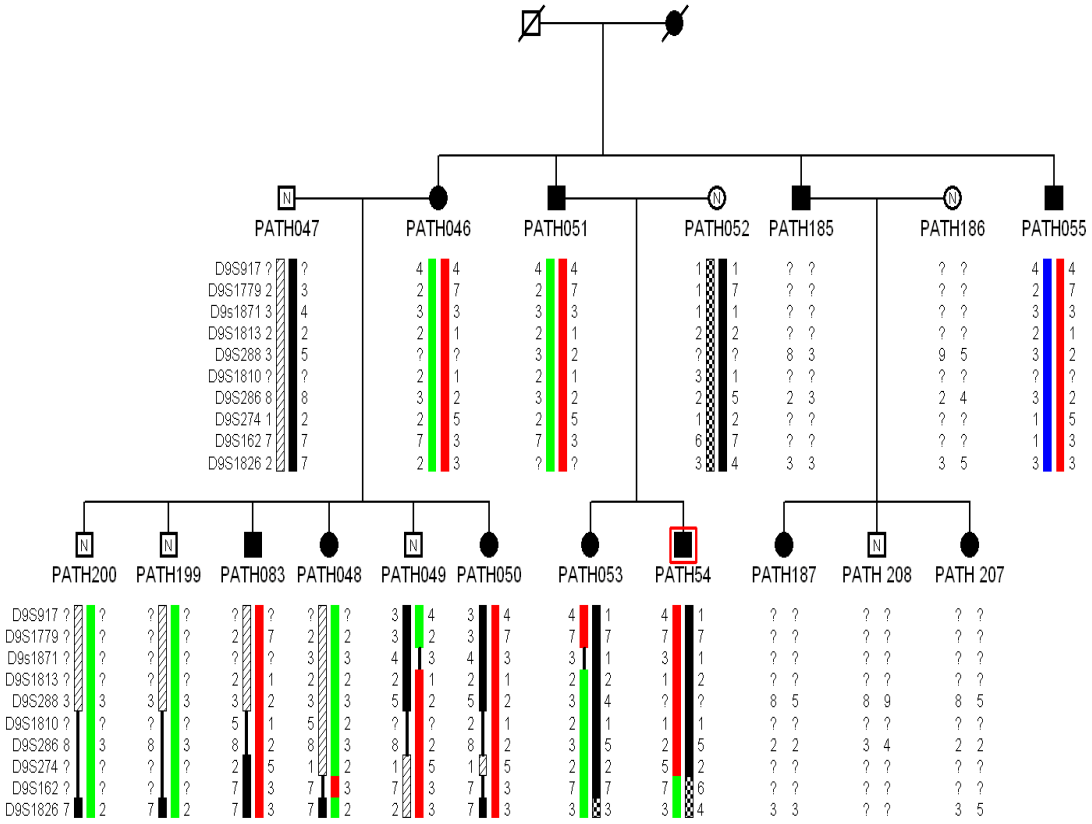


Figure 3.5: Haplotype analysis of RLS3002 pedigree for the chromosome 4 locus

A list of the examined markers is present on the left hand side. The alleles for each individual obtained from genotyping using these markers are listed below each individual. Each haplotype formed from the series of alleles is given a coloured bar so it can be recognised easily. All the coloured haplotypes are present in both affected (filled squares and circles) and unaffected (squares and circles containing the letter N) individuals. Hence there is no one shared haplotype between all the affected individuals that is absent in normal individuals.

This data, coupled with the LOD score results provides evidence that there is no linkage between RLS and the chromosome 9 locus in this family.

3.3.4 Chromosome 2 locus (RLS4; MIM 610439)

The chromosome 2 RLS locus is located on 2q33. The candidate region is located between markers D2S311 (196.85 cM) and D2S2208 (205.06 cM), with a distance of 8.21 cM between them. At this locus four markers were examined. Each of the markers showed negative LOD scores of <-2, all of which excluded variable distances. Table 3.6 lists the markers investigated and summarises their relative positions, the LOD scores obtained at theta equal to zero, the distance of excluded regions, and the corresponding region of exclusion.

Table 3.6: Markers used to examine chromosome 2 locus.

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from (cM)**	**Area Excluded to (cM)**
D2S311	196.85	-5.6	2.20	194.65	199.05
D2S2289	199.18	-3.4	2.60	196.58	201.78
D2S2358	203.46	-3.2	2.60	200.86	206.06
D2S2208	205.06	-5.6	9.90	195.16	214.96

Four markers were examined within and surrounding the chromosome 2 locus, each of which obtained statistically significant LOD scores and excluded distinct regions of the RLS4 locus. Figure 3.4 illustrates the positions of the markers, and the corresponding regions of exclusion on chromosome 2.

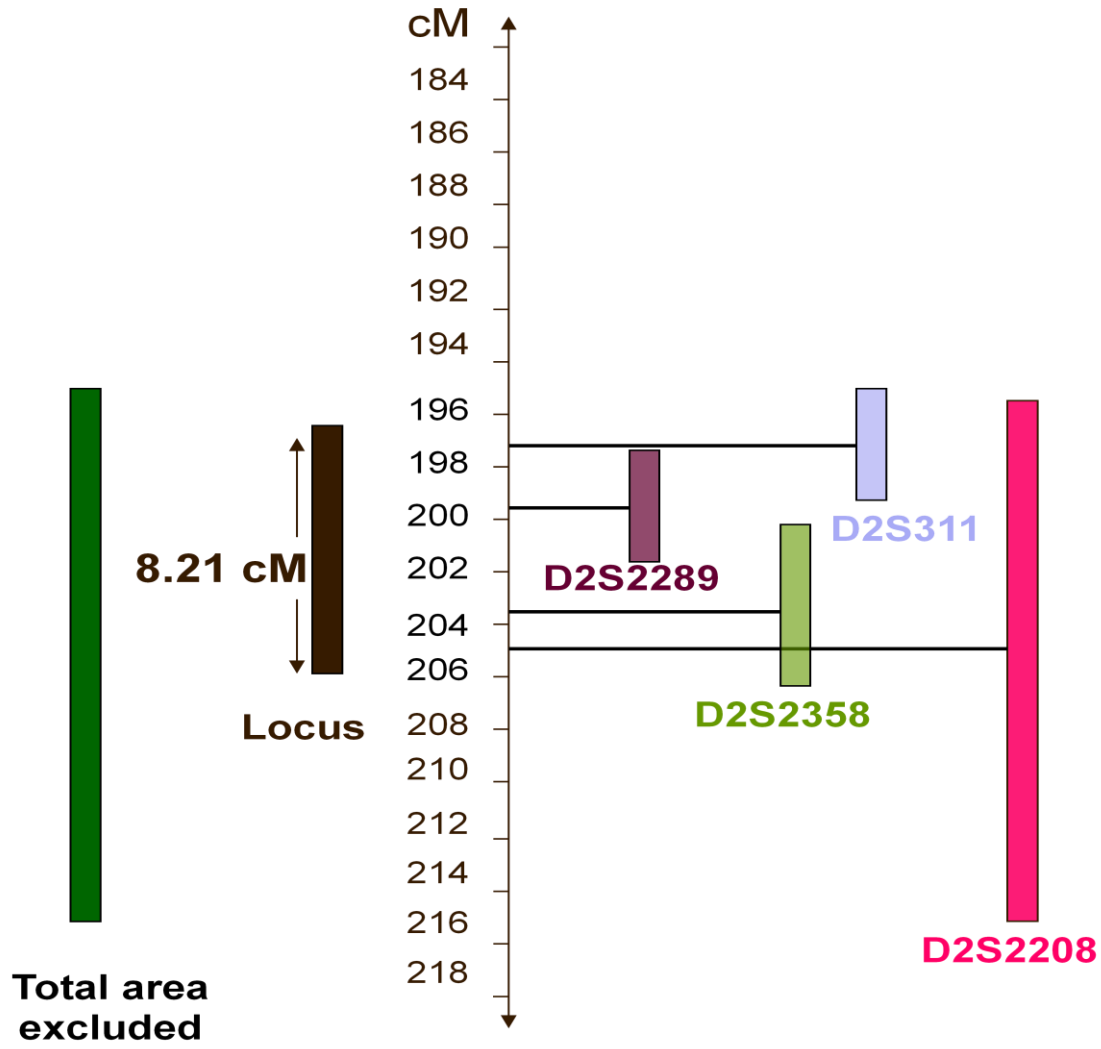


Figure 3.6: Illustration of areas of exclusion on chromosome 2 locus
Black arrow with scales represents a portion of chromosome 2. The black bar to the left of chromosome 2 represents the RLS4 locus spanning 8.21 cM between markers D2S311 and D2S2208. The regions excluded by the markers D2S311, D2S2289, D2S2358, and D2S2208 are represented by the coloured bars to the right of chromosome 2. The green bar on the far left illustrates the total area excluded combining all four markers.

3.3.5 Chromosome 20 locus (RLS5; MIM 611242)

The chromosome 20 locus, referred to as RLS5, is located at 20p13. The region is flanked by the telomeric end of chromosome 20p (above marker D20S1155 at 0.00 cM) and by a 1.07 cM interval between markers D20S849 (13.98 cM) and D20S835 (15.05 cM). According to the Marshfield map the candidate region spans approximately 15.05 cM (http://research.marshfieldclinic.org/genetics/home/index.asp). It is important to note that the RLS5 locus paper estimates a locus of 16.00 cM (Levchenko, Provost et al. 2006). However, Levchenko and associates used the Decode map to estimate the positions of the microsatellite markers (http://www.decode.com).

Six markers were examined at this locus, all of which showed negative LOD scores of <-2. Each excluded varying distances on the chromosome 20 locus. Table 3.7 lists all of the six markers investigated and describes, their positions, the LOD scores obtained at theta equal to zero, the distance of excluded regions, and the corresponding region of exclusion.

Table 3.7: Markers used to examine chromosome 20 locus.

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from (cM)**	**Area Excluded to (cM)**
D20S199	6.25	-5.75	7.66	0.00	13.91
D20S842	8.97	-5.61	9.33	2.79	21.45
D20S181	9.53	-3.02	0.20	9.33	9.73
D20S116	11.2	-2.8	2.20	9.00	13.40
D20S849	13.98	-3.08	0.70	13.28	14.68
D20S882	15.05	-3.28	3.10	11.95	18.15

Six markers obtained statistically significant LOD scores and excluded various regions of the chromosome 20 locus, RLS5. The marker D20S842 showed a significant LOD score, -5.61 at θ=0.00, and excluded from 0.00 cM to 21.45 cM, thereby excluding the entire chromosome 20 locus. Figure 3.7 illustrates the positions of the markers, and the corresponding regions of exclusion on chromosome 20.

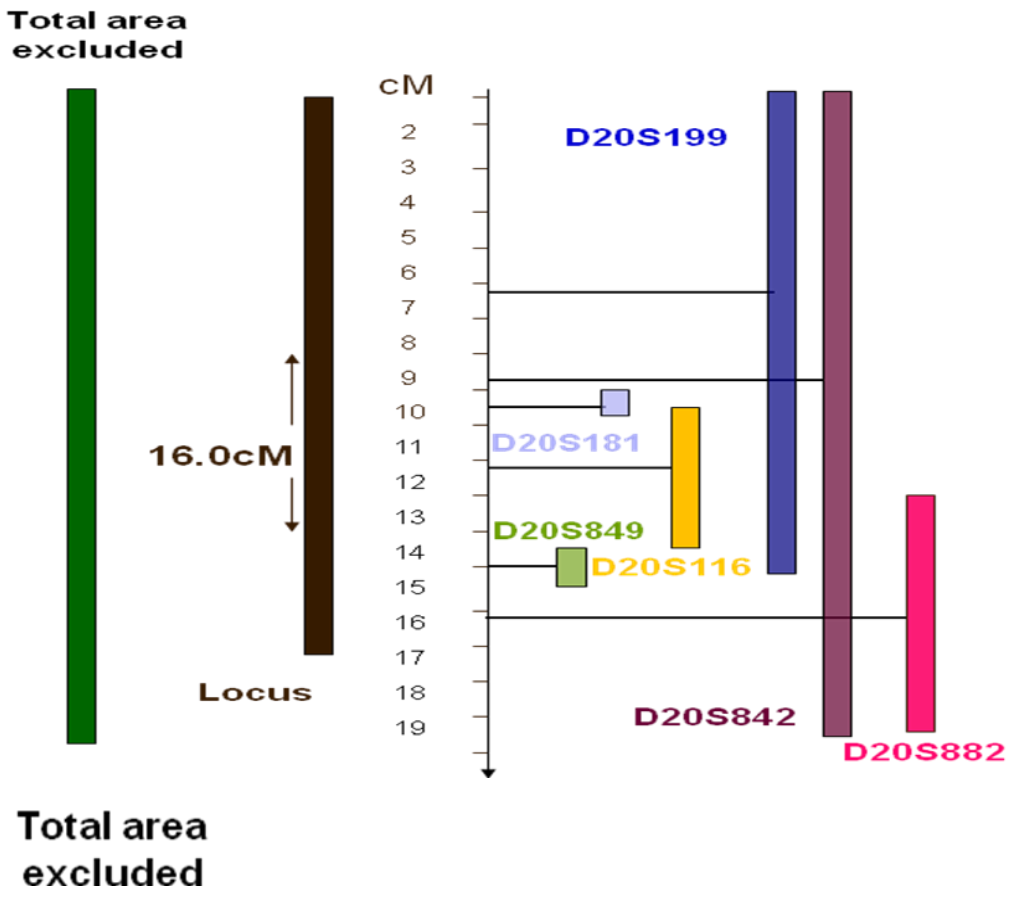


Figure 3.7: Illustration of areas of exclusion on chromosome 20 locus
Black arrow with scales represents a portion of chromosome 20. The black bar to the left of chromosome 20 represents the chromosome 20 locus spanning 8.20 cM between markers D20S311 and D20S2208. The regions excluded by the markers D20S199, D20S842, D20S181, D20S116, D20S849, and D20S882 are represented by the coloured bars to the right of chromosome 20. The green bar on the far left illustrates the total area excluded combining results of all markers.

3.3.6 Chromosome 16p locus

The chromosome 16 locus is flanked by markers 16p_m30 (43.21 cM) and 16p_m11 (44.36 cM), and 16p_m31 (47.01 cM) and D16S3068 (48.53 cM). This locus is split into two linked regions of ~830 and ~350 Kb. In genetic distance the locus is split into two regions; the first which is 1.16 cM, spanning from 43.21 cM to 44.36 cM, and the second which is 0.83cM spanning from 47.01 cM to 47.84 cM. Three markers (D16S3046, D16S403 and D16S3068), which were used in the initial study by Levchenko and associates, were examined for our pedigree (Levchenko, Montplaisir et al. 2008). Table 3.8 lists the three markers examined and outlines their positions, the LOD scores obtained at θ=0.00 and the equivalent excluded regions for the statistically significant scores.

Table 3.8: Markers used to examine chromosome 2 locus.

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from (cM)**	**Area Excluded to (cM)**
D16S3046	40.65	-8.37	6.03	34.62	46.68
D16S403	43.89	-2.05	0.00	-	-
D16S3068	48.53	-8.35	10.66	37.87	59.19

All three markers obtained statistically significant LOD scores. Markers D16S3046 and D16S3068 both excluded various regions of the chromosome 16 locus. The marker D16S403 showed a significant LOD score, however it was only significant at θ=0.00. Therefore, there was no distance of exclusion except at the position of the marker. Figure 3.8 illustrates the positions of the markers, and the corresponding regions of exclusion on chromosome 16. The total area excluded spans from 34.62 cM to 59.19 cM.

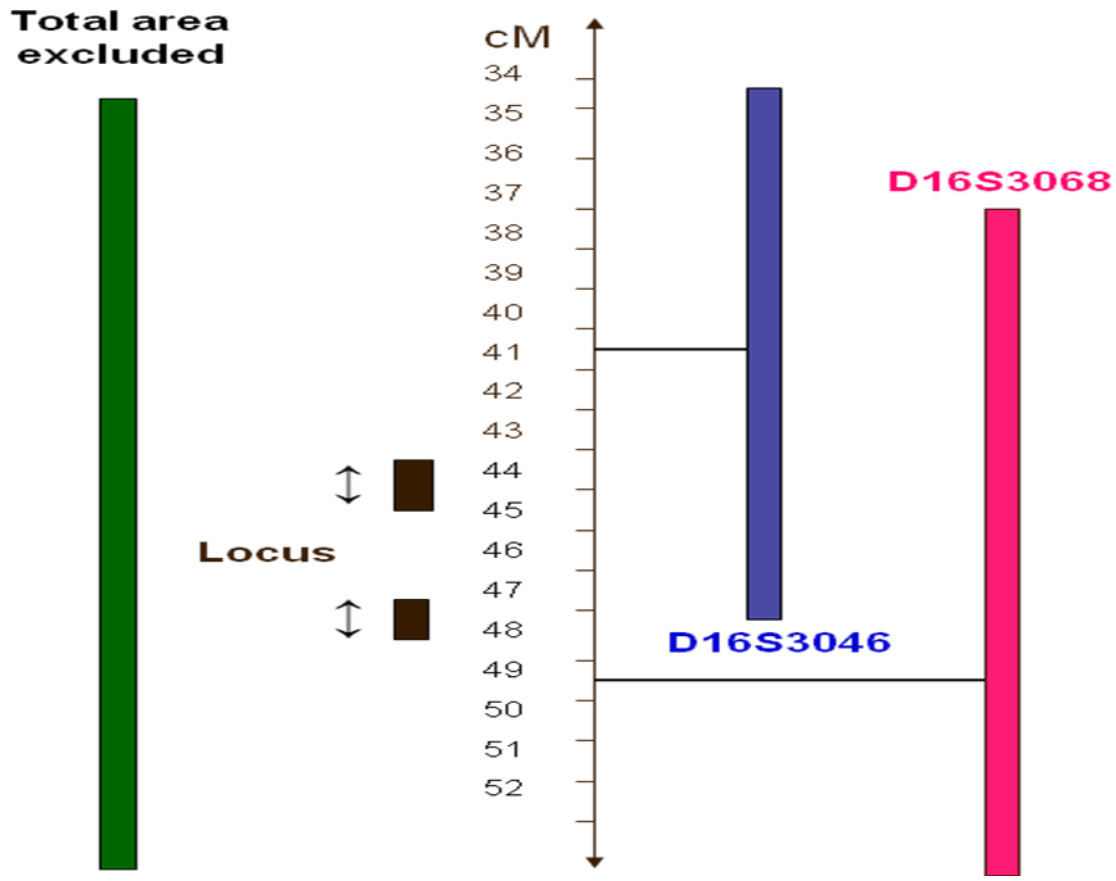


Figure 3.8: Illustration of areas of exclusion for the chromosome 16 locus
Black arrow with scales represents a portion of chromosome 16. The two black bars to the left of chromosome 16 represent the chromosome 16 locus spanning 1.16 cM and 0.83 cM respectively. The regions excluded by the markers D16S3046 and D16S3068 are represented by the coloured bars to the right of chromosome 16. The green bar on the far left illustrates the total area excluded.

3.3.7 Chromosome 19p locus

Suggestive evidence for linkage on chromosome 19p has been reported. The locus is flanked by SNP markers rs754292 and rs273265, and was further mapped within a 1.90 cM (0.30 Mb) interval between microsatellite markers D19S429 and D19S915. The locus is positioned at 45.38 cM to 47.31 cM on chromosome 19. Three markers were examined at this locus, and two of these markers, D19S226 and D19S414, were used in the initial study (Kemlink, Plazzi et al. 2008). All three markers showed negative LOD scores of <-2, each excluding variable distances. Table 3.9 lists the markers used to examine the chromosome 19 locus, their positions (cM), maximum obtained LOD scores at theta equal to zero, and the corresponding maximum excluded distances (cM).

Table 3.9: Markers used to examine the chromosome 19 locus.

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from (cM)**	**Area Excluded to (cM)**
D19S226	42.28	-7.44	1.80	40.48	44.08
D19S410	45.48	-5.02	2.40	43.08	47.88
D19S414	53.81	-7.66	8.89	44.92	62.70

All three markers examined obtained statistically significant LOD scores and excluded various regions of the chromosome 19p locus. The marker D19S414, which had been used in the initial study, showed a significant LOD score of -7.66 at θ=0.00, and excluded from 44.92 cM to 62.70 cM, thus excluding the entire chromosome 19p locus. Similarly for marker D19S410, it excluded the 1.90 cM locus as it excludes from 43.08 cM to 47.88 cM.

Figure 3.9 illustrates the positions of the three markers and the corresponding regions of exclusion on chromosome 19p.

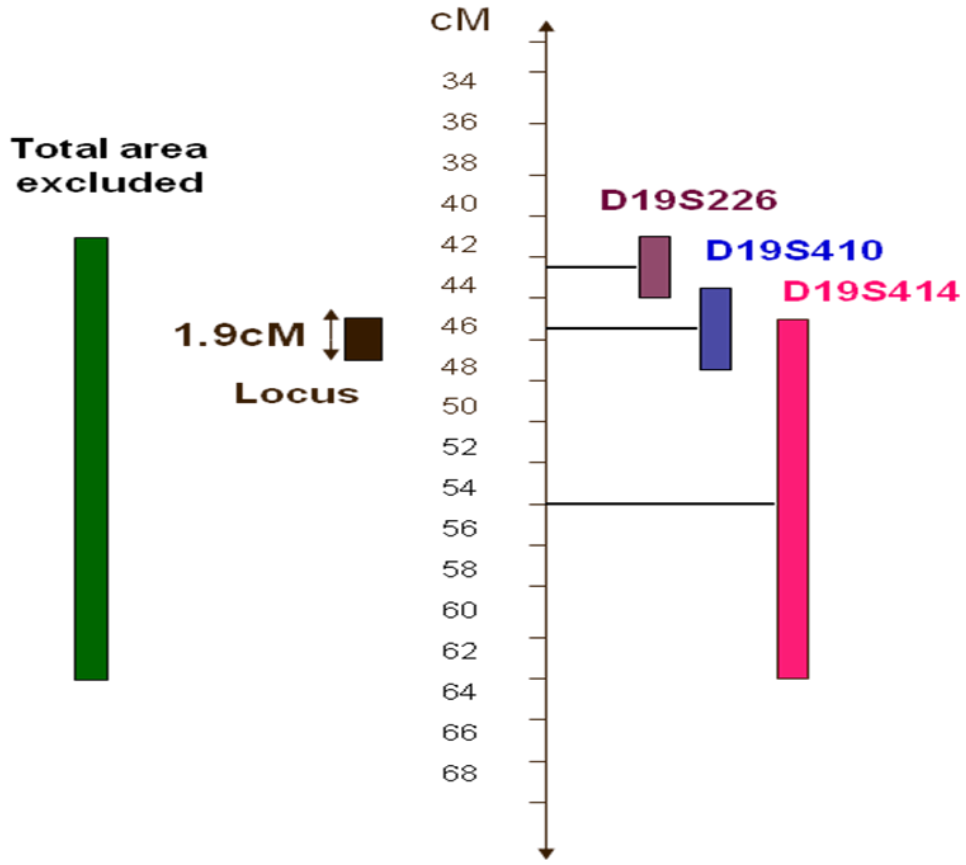


Figure 3.9: Illustration of areas of exclusion the chromosome 19 locus
Black arrow with scales represents a portion of chromosome 19. The black bar to the left of chromosome 19 represents the chromosome 19 locus spanning 1.90 cM. The regions excluded by the markers D19S226, D19S410, and D19S414 are represented by the coloured bars to the right of chromosome 19. The green bar on the far left illustrates the total area excluded combining all three markers.

3.3.8 Chromosome 4q locus

The Chromosome 4q locus is flanked by markers D4S406 and D4S402, located at 117.06 cM and 124.45 cM respectively. Three markers were examined at this locus, and all three markers showed negative LOD scores of <-2, each excluding variable distances.Table 3.10 lists the three markers examined and outlines, their positions, the LOD scores obtained at θ=0.00 and the equivalent excluded regions for the statistically significant scores.

Table 3.10: Markers used to examine chromosome 4 locus.

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from (cM)**	**Area Excluded fom to (cM)**
D4S2989	117.06	-8.38	3.61	113.45	120.67
D4S407	117.06	-8.68	4.51	112.55	121.57
D4S1575	132.05	-11.17	8.87	123.18	140.92

All three markers obtained statistically significant LOD scores. Markers D4S2989 and D4S407 excluded various regions of the chromosome 4 locus from 113.45 cM to 121.57 cM. Marker D4S1575 excluded from 123.18 cM to 140.92 cM. Therefore, a 1.62 cM gap remains to be excluded from the chromosome 4 locus. Figure 3.10 illustrates the positions of the markers, and the corresponding regions of exclusion on chromosome.

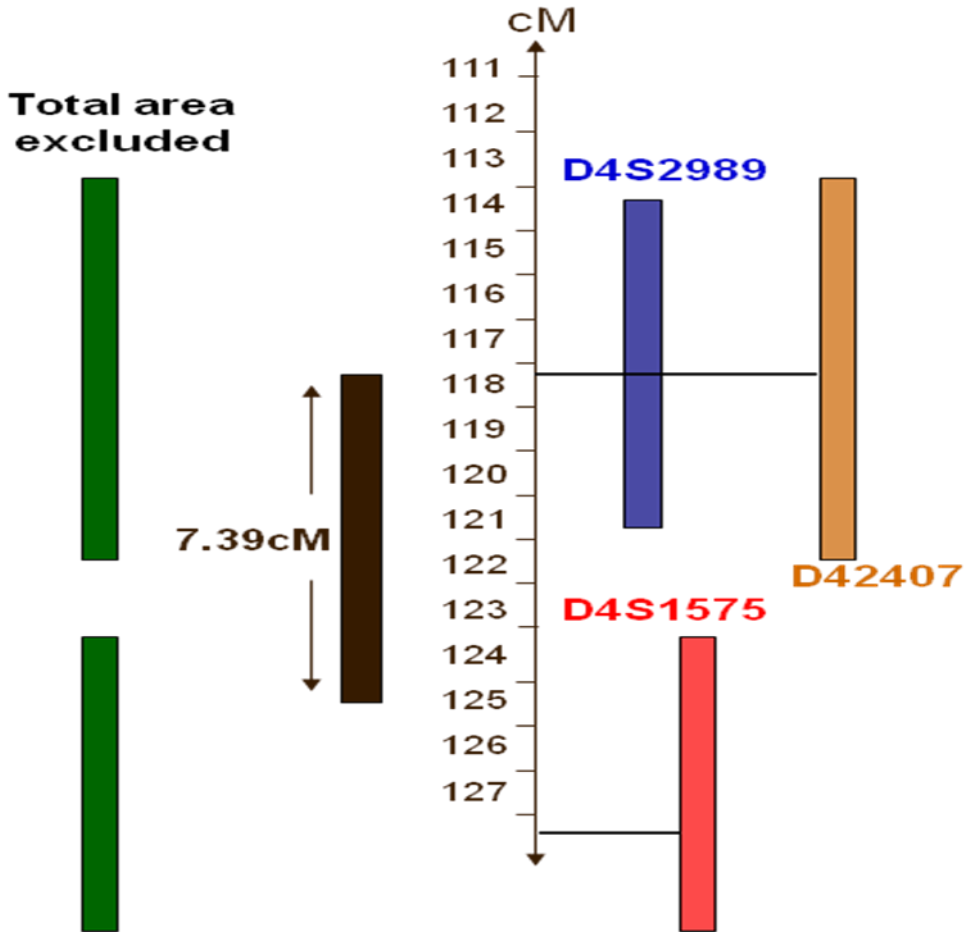


Figure 3.10: Illustration of areas of exclusion on chromosome 4 locus
Black arrow with scales represents a portion chromosome 4. The black bar to the left of chromosome 4 represents the chromosome 4 locus spanning 7.39 cM between markers D4S406 and D4S402. The regions excluded by the markers D4S2989, D4S407 and D4S1575 are represented by the coloured bars to the right of chromosome 4. The green bar on the far left illustrates the total area excluded combining all four markers.

The results almost exclude the chromosome 4 locus, however a small 1.62 cM gap remains to be excluded. A haplotype analysis was carried out at this locus. It is clear from this analysis that there is no haplotype that is present in all the affected individuals and not present in the unaffected individuals. Therefore, due to the fact that the gap is small and that the haplotype analysis confirms exclusion of the locus, it can be assumed that the chromosome 4 locus is not linked to RLS in the pedigree.

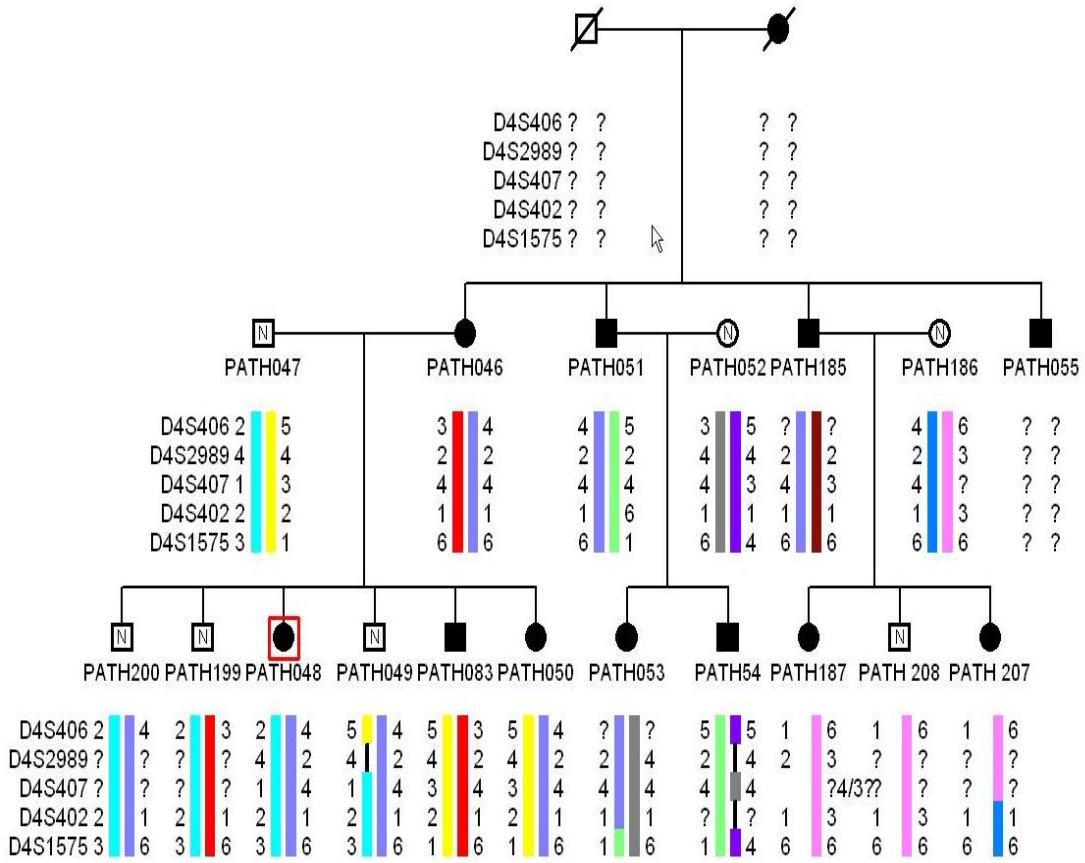


Figure 3.11: Haplotype analysis of RLS3002 pedigree for the chromosome 4 locus

A list of the examined markers is present on the left hand side. The alleles for each individual obtained from genotyping using these markers are listed below each individual. Each haplotype formed from the series of alleles is given a coloured bar so it can be recognised easily. Unfortunately PATH187, PATH207 and PATH208 could not be assigned a second bar as only one marker defined the two bars for PATH185 (their mother), and that marker information was not available for the three children. All the coloured haplotypes are present in both affected (filled squares and circles) and unaffected (squares and circles containing the letter N) individuals. Hence there is no one shared haplotype between all the affected individuals that is absent in normal individuals. It is therefore possible to conclude that there is no linkage between RLS and the chromosome 4 locus in this family.

3.3.9 Chromosome 17p locus

The chromosome 17p RLS locus extends from D17S1857 (43.01 cM) to D17S1294 (50.74 cM) spanning a distance of 7.73 cM (Winkelmann J 2006). Four markers were examined at this locus. Three of the four markers showed a negative LOD score of <-2, each excluding variable distances. Table 3.11 lists the markers that were examined, the LOD scores obtained at θ=0.00 and the corresponding excluded regions.

Table 3.11: Markers used to examine chromosome 17 locus.

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from (cM)**	**Area Excluded to (cM)**
D17S1857	43.01	-1.51	0.00	-	-
D17S2196	44.62	-8.52	7.76	36.86	52.38
D17S1294	50.74	-6.01	6.03	44.71	56.77
D17S798	53.41	-6.50	5.52	47.89	58.93

The area excluded by the three markers, D17S2196, D17S1294 and D17S798, extends from 36.86 cM to 58.93 cM. The chromosome 17p locus extends from 43.01 cM to 50.74 cM; therefore, it is possible to conclude that there is no linkage between RLS and the chromosome 17p locus in this family. Figure 3.12 illustrates the position of the three markers that obtained significant negative LOD scores and the corresponding regions of exclusion.

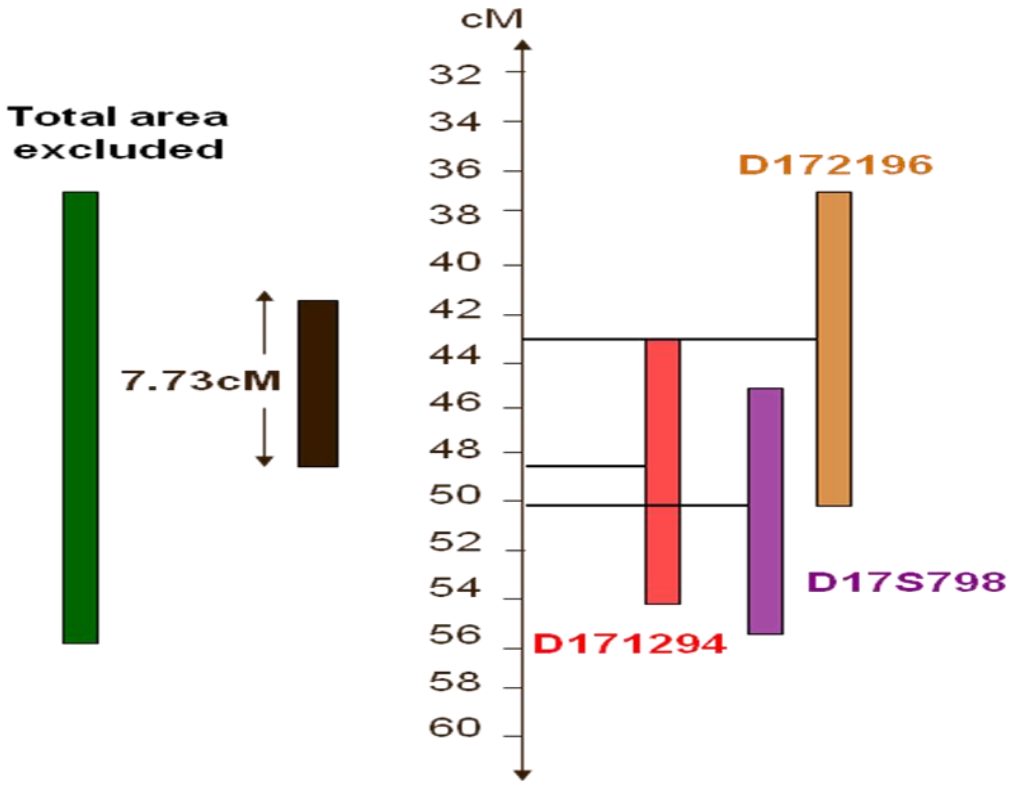


Figure 3.12: Illustration of areas of exclusion on chromosome 17 locus
Black arrow with scales represents a portion of chromosome 17. The black bar to the left of chromosome 17 represents the chromosome 17 locus spanning 7.73 cM between markers D17S1857 and D17S1294. The regions excluded by the markers D17S2196, D17S1294 and D17S798 are represented by the coloured bars to the right of chromosome 17. The green bar on the far left illustrates the total area excluded .

3.3.10 Association within the *BTBD9* gene (RLS6; MIM 611185)

Association with several SNPs in the *BTBD9* gene has been confirmed by a number of independent studies since 2007. Three SNPs in particular; rs9296249, rs9357271, and rs3922809, have been reported to be associated with susceptibility to RLS. These SNPs are located at 53.80-53.82 cM on chromosome 6. Two microsatellite markers were examined in this area; D6S1610 and D6S1607. Both showed significant LOD scores of <-2, excluding varied distances. Table 3.12 lists the two markers in questions, showing the LOD score obtained at θ=0.00 and the corresponding excluded regions.

Table 3.12: Markers used to examine SNPs within the *BTBD9* gene.

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from (cM)**	**Area Excluded to (cM)**
D6S1610	53.81	-2.71	1.20	52.61	55.01
D6S1607	59.34	-8.54	6.64	52.70	65.98

The two markers examined for this region both obtained significantly significant LOD scores, and excluded the area containing the associated SNPs. The markers D6S1610 showed a significant LOD score of -2.71 at θ=0.00, excluding 1.20 cM either side. The marker D6S1607 showed a significant LOD score of -8.54 at θ=0.00, excluding 6.64 cM either side, thereby overlapping with the area excluded by marker D6S1607. Figure 3.13 illustrates the positions of the two markers and the regions excluded on chromosome 6.

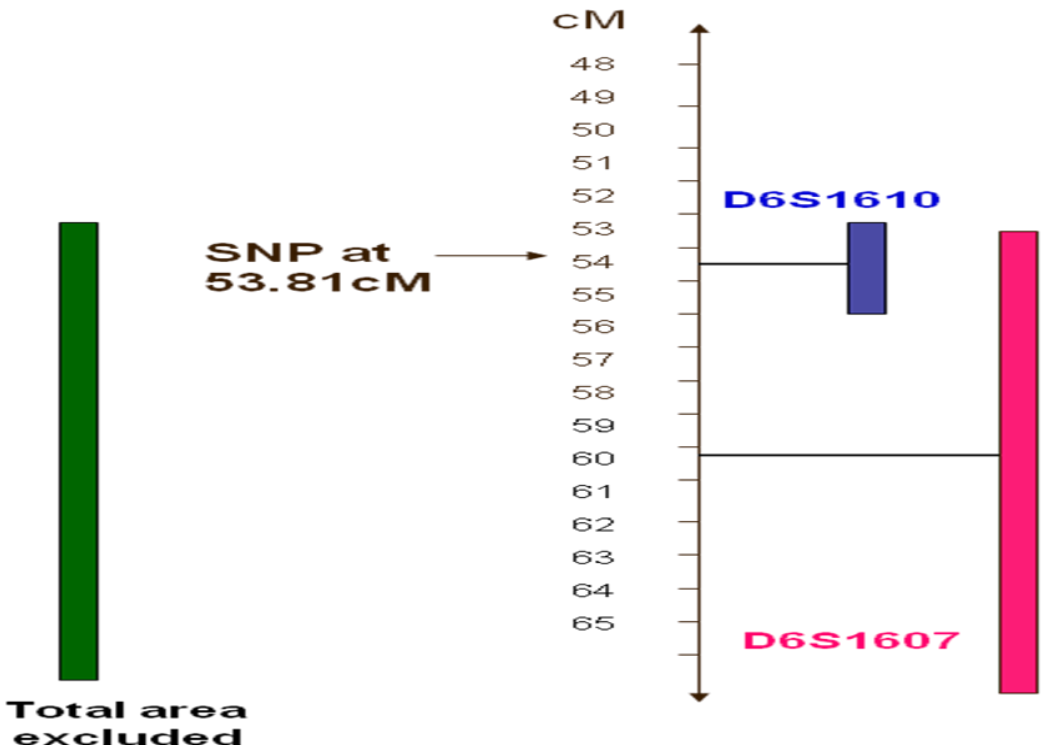


Figure 3.13: Illustration of areas of exclusion for variants within the *BTBD9* gene

Black arrow with scales represents a portion of chromosome 2. The black arrow at approximately 53.80 cM represents the area where the SNPs are located. The regions excluded by the markers D6S1610 and D6S1607 are represented by the coloured bars to the right of chromosome 6. The green bar on the far left illustrates the total area excluded combining the two markers.

3.3.11 Association within the *MEIS1* gene (RLS7; MIM 612853)

A significant association was found between RLS and rs2300478 in the *MEIS1* gene (601739) on the short arm of chromosome 2; 2p14-p13. This SNP is located at approximately 84.42 cM. Seven markers were selected and examined in this region. Six of the seven markers showed negative LOD scores of <-2. However, only three of these markers excluded variable distances. Table 3.13 lists all of the markers investigated and shows their positions (cM), the LOD scores obtained at θ=0.00 and the regions excluded for the markers with statistically significant LOD scores.

Table 3.13: Markers used to examine SNP within the *MEIS1* gene.

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from (cM)**	**Area Excluded to (cM)**
D2S2739	73.61	-5.59	7.56	66.05	81.17
D2S337	80.60	-5.59	3.00	77.60	83.60
D2S290	84.42	-2.17	0.00	-	-
D2S166	84.42	-3.23	0.00	-	-
D2S285	86.02	-2.41	0.00	-	-
D2S2113	88.15	0.22	0.00	-	-
D2S2110	90.82	-5.12	8.27	82.55	99.09

Seven markers were examined in this region, however only three of these obtained a statistically significant LOD score, and excluded various regions surrounding the SNP rs2300478. The markers D2S290, D2S166, and D2S285 showed a significant LOD score of <-2, however it was only significant at θ=0.00. Therefore, there was no distance of exclusion aside from the position of the marker itself. The marker D2S2113 did not show a significant LOD score, and yielded an inconclusive LOD score of 0.22 at θ=0.00.

Figure 3.14 illustrates the positions of the markers, and the corresponding regions of exclusion on chromosome 2. The total area excluded spans from 66.05 cM to 99.09 cM.

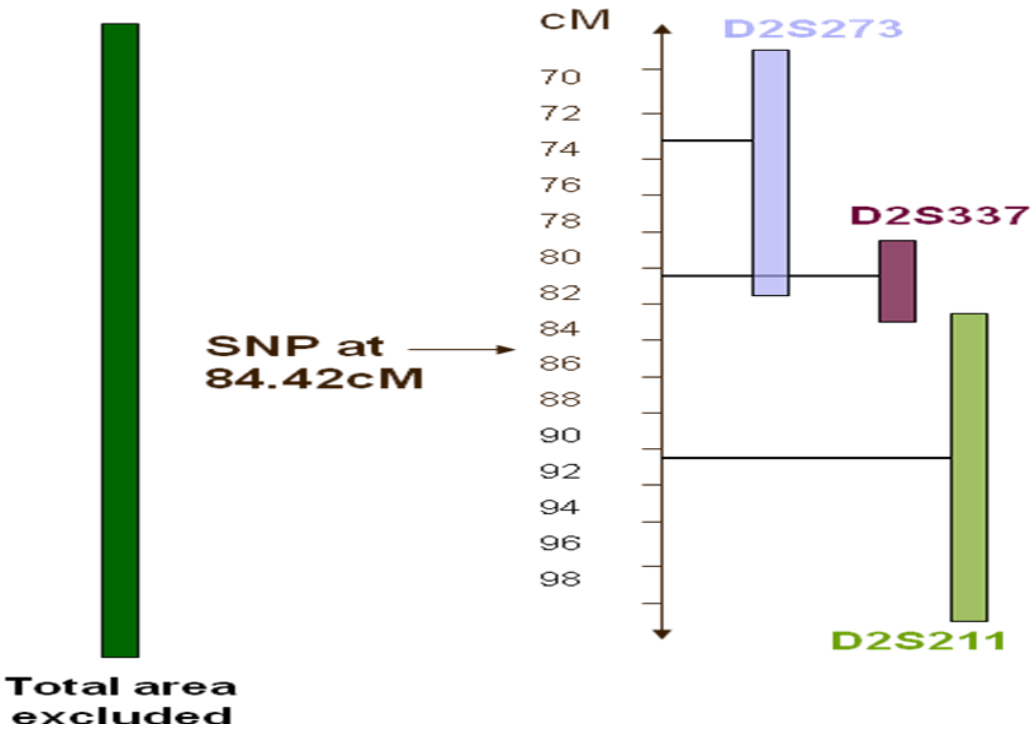


Figure 3.14: Illustration of areas of exclusion for the SNP within the *MEIS1* gene

Black arrow with scales represents a portion of chromosome 2. The black arrow at approximately 84.42cM to the left of chromosome 2 represents the region which the SNP is located. The regions excluded by the markers D2S273, D2S337, and D2S211 are represented by the coloured bars to the right of chromosome 2. The dark green bar on the far left illustrates the total area excluded.

3.3.12 Association between RLS and variants in the *MAP2K5/LBXCOR1* gene

Highly significant associations were found between RLS and a number of variants in a locus containing the genes *MAP2K5* and *LBXCOR1* on the long arm of chromosome 15. These associations were located at approximately 66.90 cM. Four markers were selected and examined in this region. All four of the markers showed negative LOD scores of <-2, each excluding variable distances, depending on the LOD scores obtained. Table 3.14 displays the markers investigated and outlines their positions (cM), the LOD score obtained at θ=0, and the corresponding excluded regions.

Table 3.14: Markers used to examine variants within the *MAP2K5* and *LBXCOR1* genes.

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from (cM)**	**Area Excluded to (cM)**
D15S1015	66.90	-3.91	1.10	65.80	68.00
D15S153	68.10	-5.51	3.71	64.39	71.81
D15S131	71.28	-9.82	12.03	59.25	83.31
D15S211	75.85	-5.92	7.45	68.40	83.30

Four markers were examined in this region, all of which obtained significantly significant LOD scores, each excluding varying distances on chromosome 15. The marker D15S131 obtained a LOD score of -9.82 at θ=0.00, excluding a large distance of 12.03 cM either side of the marker, thus overlapping with the regions excluded by the other three markers. Figure 3.15 illustrates the positions of the markers, and the corresponding regions of exclusion on chromosome 15. The total area excluded spans from 59.25 cM to 83.31 cM.

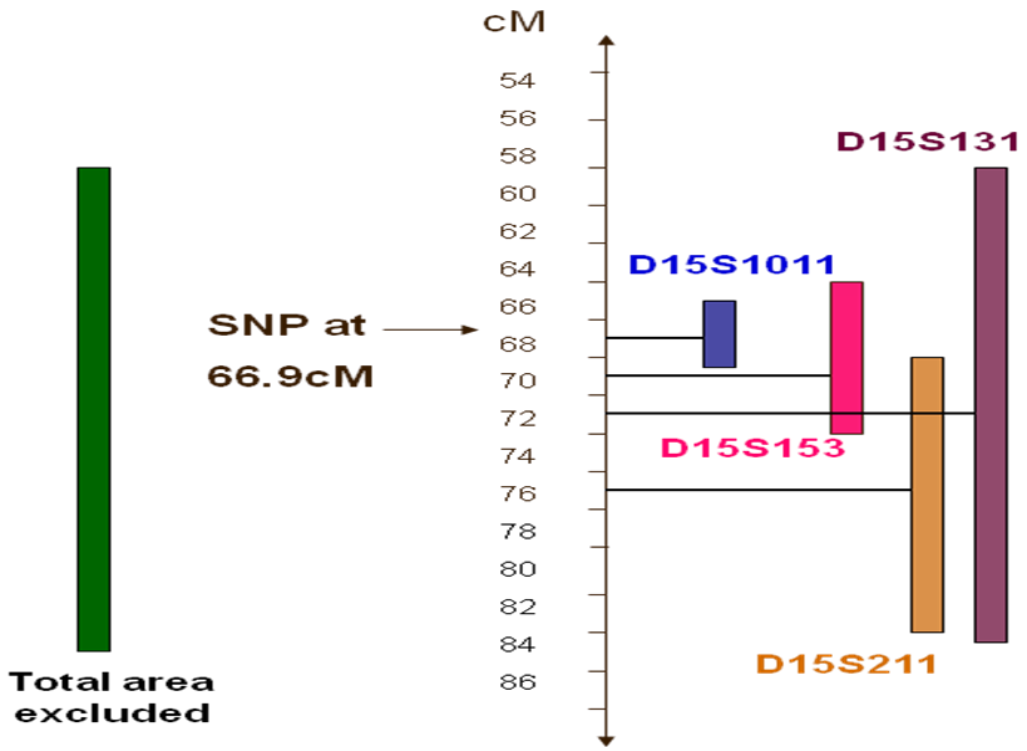


Figure 3.15: Illustration of areas of exclusion for variants within the *MAP2K5/LBXCOR1* genes

Black arrow with scales represents a portion of chromosome 15. The black arrow at approximately 66.90 cM to the left of chromosome 15 represents the region which the SNP is located. The regions excluded by the markers D15S1011, D15S153, D15S211, and D15S131 are represented by the coloured bars to the right of chromosome 15. The dark green bar on the far left illustrates the total area excluded combining the four markers.

3.3.13 Protein tyrosine phosphatase, receptor type, D (*PTPRD*)

Protein tyrosine phosphatase, receptor type, D (*PTPRD*) is a member of the protein tyrosine phosphatase (PTP) family. A significant association was found between RLS and two independent SNPs, rs1975917 and rs4626664, in the *PTPRD* gene (5789) on the short arm of chromosome 9; 9p23-p24.3 (Schormair, Kemlink et al. 2008). Two markers were selected and examined in this region. Both of these markers showed negative LOD scores of <-2, thereby excluding variable distances. Table 3.15 lists all of the markers investigated and shows their positions, the LOD scores obtained at θ=0.00 and the regions excluded for the markers with statistically significant LOD scores.

Table 3.15: Markers used to examine SNPs within the *PTPRD* gene.

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from**	**Area Excluded to**
D9S286	18.06	-5.06	7.56	10.50	25.62
D9S274	28.42	-5.61	3.00	19.53	37.31

Two markers were examined in this region, and both excluded the region surrounding the SNPs rs1975917 and rs4626664. Figure 3.16 illustrates the positions of the markers, and the corresponding regions of exclusion on chromosome 9. The total area excluded spans from 10.50 cM to 37.31 cM.

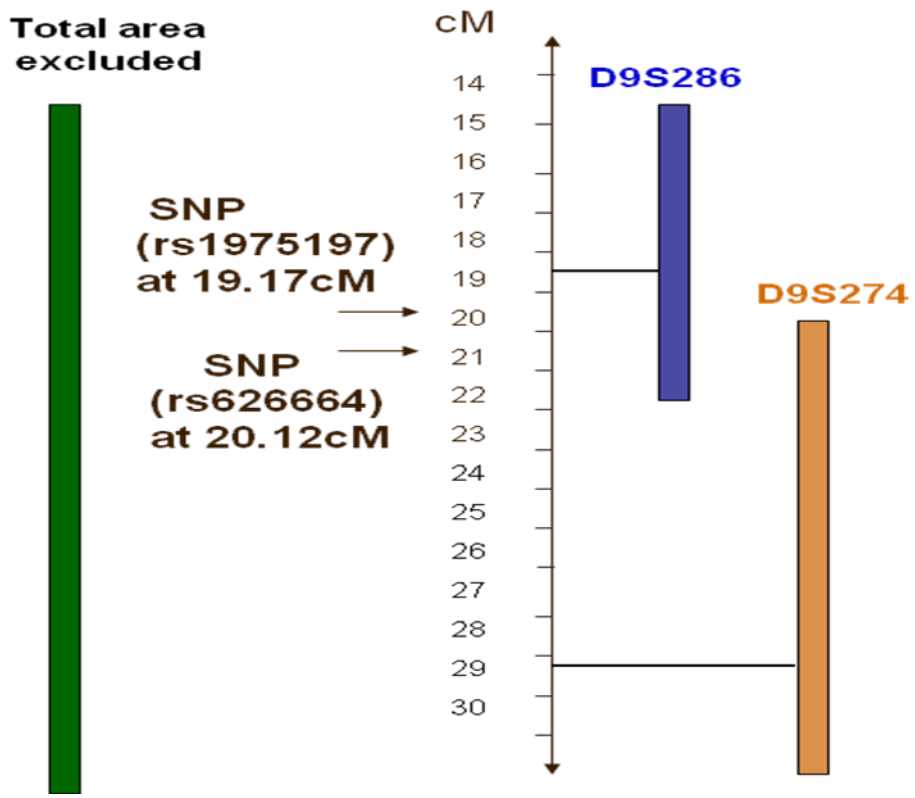


Figure 3.16: Illustration of areas of exclusion for SNPs within the *PTPRD* gene
Black arrow with scales represents a portion of chromosome 9. The two black arrows at approximately 19.17 cM and 20.12 cM to the left of chromosome 9 represent the regions which the SNPs are located. The regions excluded by the markers D9S286 and D9S274 are represented by the coloured bars to the right of chromosome 9. The dark green bar on the far left illustrates the total area excluded combining all three markers.

3.3.14 Association of variants in the *NOS1* gene and RLS

Highly significant associations were found between RLS and a number of variants in the neuronal nitric oxide synthase (*NOS1*) gene on the long arm of chromosome 12. Association was observed with SNP rs7977109 which is located at a genetic distance of 129.30 cM on chromosome 12 (Winkelmann, Lichtner et al. 2008). Two markers were selected and examined in this region. The two markers, D12S2082 and D12S366, both showed negative LOD scores of <-2, each excluding variable distances, depending on the LOD scores obtained. Table 3.16 displays the markers investigated and outlines their positions (cM), the LOD score obtained at θ=0.00 and the corresponding excluded regions.

Table 3.16: Markers used to examine variants within the *NOS1* gene.

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from (cM)**	**Area Excluded to (cM)**
D12S2082	130.94	-6.63	2.20	128.74	133.14
D12S366	133.33	-8.56	2.20	131.13	135.53

Two markers were examined in this region, both of which obtained significant LOD scores, each excluding varying distances on chromosome 12. Marker D12S2082 obtained a LOD score of -6.63 at θ=0.00 and marker D12S366 obtained a LOD score of -8.56 at θ=0.00, however both excluded 2.20 cM either side of the position in which it is located. Figure 3.17 illustrates the positions of the markers, and the corresponding regions of exclusion on chromosome 12. The total area excluded spans from 128.74 cM to 135.53 cM.

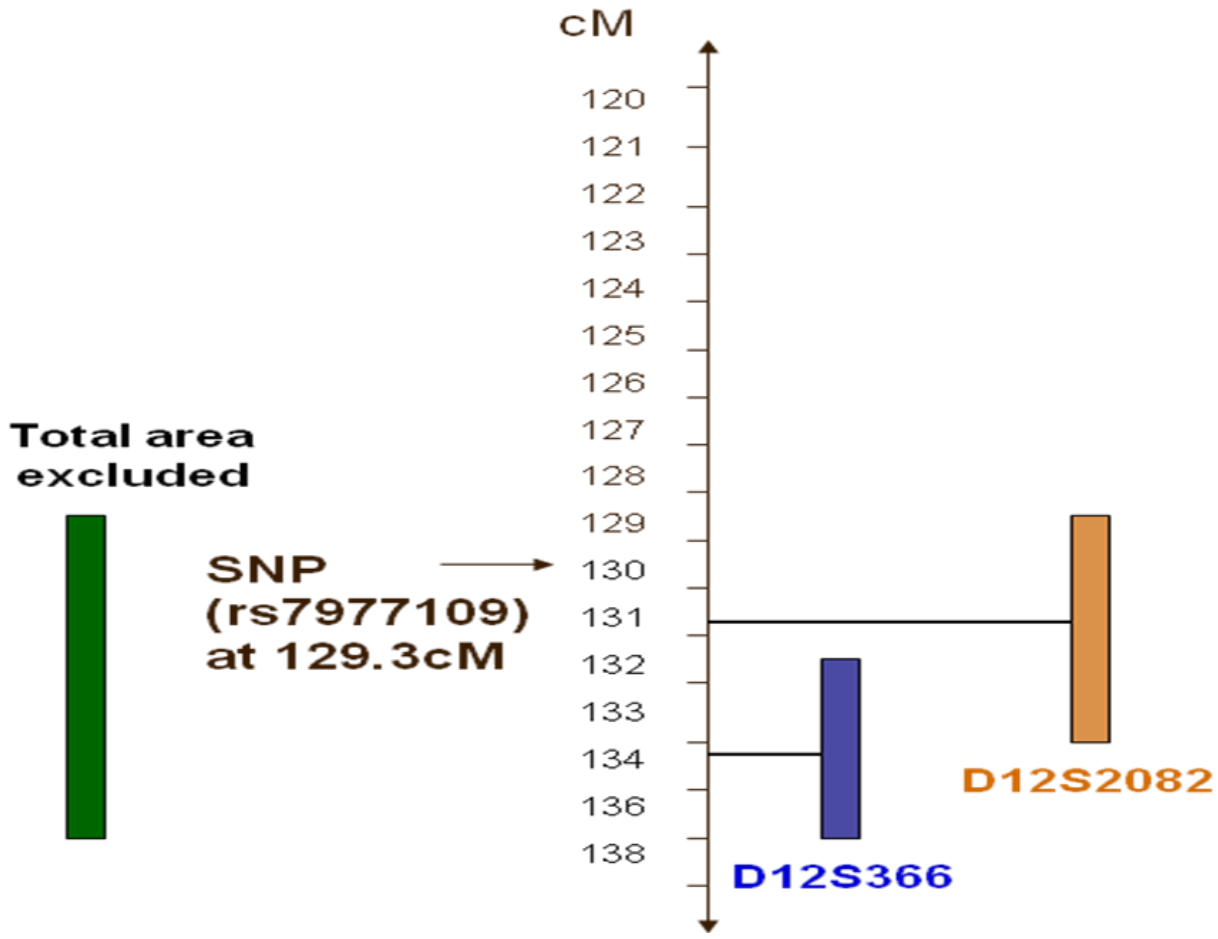


Figure 3.17: Illustration of areas of exclusion for variants within the *NOS1* gene
Black arrow with scales represents a portion of chromosome 12. The black arrow at 129.30 cM to the left of chromosome 12 represents the region which the SNP rs7977109 is located. The regions excluded by the markers D12S2082 and D12S366 are represented by the orange and blue bars to the right of chromosome 12. The dark green bar on the far left illustrates the total area excluded.

3.4 Conclusion

A new Irish family RLS3002 which consisted of 18 members had been previously recruited (Figure 2.1). This is a three generation family. Unfortunately the affected grandmother and unaffected grandfather were deceased at the time of the study, and DNA samples were unavailable. In the second and third generation, there are 11 affected individuals, three unaffected spouses and four unaffected children. Although the chromosome 12q locus mapped by Desautels and associates in a French-Canadian family displayed autosomal recessive inheritance (Desautels, Turecki et al. 2001), this Irish pedigree displayed and autosomal dominant pattern of inheritance as reported in all the other RLS loci. This is evident as all the affected individuals have only one parent who is affected.

At the time the previous study was carried out only four loci had been described, chromosome 12q, 14p, 9p and 5q. RLS linkage was successfully excluded for the entire RLS2 locus (chromosome 14q) in this family. Due to time constraints the other three loci were not fully excluded; therefore, further work had to be carried out for these loci. A further six RLS loci and five associated regions had been identified since the initial study was performed. It was therefore necessary to investigate if pedigree RLS3002 was linked to any of these regions.

When the previous study was carried out only 11 family members were available to participate in this study. However, over the years following, physical examination and questionnaires were administered for the remaining seven individuals, and subsequently these additional blood sample were collected. The addition of these seven members would add considerable power to the study. Therefore, this study began by re-investigating the markers used for the chromosome 5p, 12q and 9p loci,

and genotyping was performed for any individuals who hadn't been previously available. This was in the hope that the addition of the genotypes would increase the power of the study and generate stronger LOD scores for specific markers and therefore, exclude any gaps remaining in the loci.

The chromosome 5 locus was examined first. A 1.10 cM gap remained in this locus which was not excluded. Following investigation with the additional family member the LOD score of marker D5S2500 decreased from -5.51 at θ=0.00 to -6.89 at θ=0.00. Previously it had been excluded from 65.93 cM to 72.53 cM, however it now excluded from 62.29 cM to 76.17 cM, thereby excluding the gap which was positioned between 72.53 cM to 73.61 cM. The chromosome 5 locus was successfully excluded for RLS3002.

Chromosome 12 locus (RLS1) was next examined. A small gap of 0.10 cM remained to be excluded for this locus. Four additional markers were examined at this locus and marker D12S346 which gave a LOD score of 10.90 at θ=0.00 was successful at excluding the gap which was positioned between 110.37 cM and 110.47 cM. The chromosome 12 locus was successfully excluded for RLS3002.

The chromosome 9 locus, which was found in an Italian population, is a very large locus, and two gaps remained to be investigated for pedigree RLS3002. The two gaps spanned 14.76 cM at the telomeric end of chromosome 9 and the other 3.40 cM gap was located between 21.36 cM and 24.81 cM. Following investigation with the additional family members and marker D9S274, which was used in the previous study, the 3.40 cM gap was removed. Two additional markers were investigated at the telomeric end, D9S1813 and D9S1810. The 14.76 cM gap was partially excluded for linkage, however there is still a small area where the markers failed to cover and

a 3.53 cM gap remains. The haplotye analysis provides evidence to confirm exclusion of linkage to this locus. Examination of further markers in the region of the area not excluded would confirm this further.

The chromosome 2 locus (RLS4) was next examined for linkage in this Irish pedigree. Four evenly spaced markers were chosen and examined, each of which obtained statistically significant LOD scores and successfully excluded the RLS4 locus for linkage to this pedigree. The markers D2S311 and D2S2208 which flank the locus and were used in the initial study by Pichler and associates were used in this study also. It is interesting that marker D2S2208 could solely exclude this locus for linkage to our Irish pedigree. It excluded 9.90 cM either side of where it was positioned, the locus only being 8.20 cM in length.

The RLS5 locus which was located in a French-Canadian population is located on the short arm of chromosome 20. Six markers were examined for this locus and each one excluded varying distances on the chromosome 20 locus, successfully excluding the entire chromosome 20 locus.

The chromosome 16p locus which was also found in a French-Canadian family is unusual as it is split into two linked regions of ~830 and ~350 Kb (Levchenko, Montplaisir et al. 2008). It is unusual to see a locus split into two linked regions caused by an unusual and rare double recombination in just one family member. Genotyping errors and DNA contamination was ruled out, so therefore the tight double recombination was either a consequence of a recombination event without crossover interference or due to the fact a very rare event occurred. Three markers were examined for this locus. All three gave a statistically significant LOD score,

however only two of the markers excluded various regions of the chromosome 16 locus. These markers were sufficient to fully exclude linkage for pedigree RLS3002.

There was suggestive linkage to chromosome 19p13. Three markers were examined at this locus, two of which were used in the initial study by Kemlink and associates (Kemlink, Plazzi et al. 2008). All three markers examined obtained a statistically significant LOD score, and RLS linkage was successfully excluded for the chromosome 19p13 locus in the family.

A relatively new approach used to find regions associated with RLS is the genome-wide association study or GWAS; this is a high-throughput approach which scans the entire genome in an unbiased manner, using statistical methods to determine associations between chromosomal loci and a given phenotype. It requires a large number of cases and a large number of controls which is often difficult to collect. Association studies don't always have to scan the entire genome and they can be used for specific regions, such as the explorative study carried out by Winkelmann and associates in a 21 Mb region encompassing the RLS1 critical region on chromosome 12 (Winkelmann, Lichtner et al. 2008).

Two recent studies used a large-scale high-density genome-wide approach. In the first study which was conducted in the German and Canadian population, three genomic regions were identified encoding the genes *MEIS1*, *BTBD9* and a third region encoding the genes *MAP2K5* and *LBXCOR1* (Winkelmann, Schormair et al. 2007). Coincidently a similar study was carried out in the Icelandic and US population, and an association was found in the identical variant in *BTBD9* (Stefansson, Rye et al. 2007).

Three SNPs were found to be associated in the BTBD9 gene, RLS6. Two markers were examined for linkage in this area on chromosome 6, both excluding varying distances, and specifically the region in which the variants are located. Therefore the SNPs associated with the *BTBD9* gene are not linked to our family and can be excluded.

The *MEIS1* gene is located on chromosome 2p14. Seven genes needed to be examined for this region. Four of the seven genes did not give any exclusion; therefore it was necessary to examine a further three markers. These markers excluded varying overlapping distances and subsequently excluded the region containing the associated SNP.

Highly significant associations were found between RLS and a number of variants in the locus containing the genes encoding *MAP2K5* and *LBXCOR1* on chromosome 15q. Four markers in this region were chosen and examined for linkage to RLS3002. All four markers obtained significant LOD scores, enabling the exclusion of this region.

Two independent signals within splice variants expressed in the CNS of *PTPRD* showed genome-wide significant association to RLS (Schormair, Kemlink et al. 2008). Two markers were chosen in this region and linkage analysis was performed for family RLS3002. Markers were successful in excluding the associated region for linkage to the family.

The association of variants in the *NOS1* gene and RLS implies an involvement of the NO/arginine pathway in the pathogenesis of RLS. Two markers were chosen in this region and following examination of our family with these markers, the associated region was excluded for linkage to our family.

Our analysis shows that the disease is not linked to any of the known RLS loci or risk-associated genes. Hence it can be assumed that members of the family may carry a gene mutation in a novel genetic locus. Therefore a new unidentified locus awaits discovery and provides further evidence for the genetic heterogeneity of RLS. In order to identify a novel-locus a genome-wide scan is required.

Chapter 4

Genome-wide Scan

4.1 Introduction

Initial work showed that pedigree RLS3002 is not linked to any of the known RLS loci or risk associated genes. Therefore it can be assumed that a new locus awaits identification in this pedigree, a locus which carries a gene mutation. In order to look for this novel locus a genome-wide scan was performed. This approach tests for linkage of the trait to anonymous polymorphic markers spread throughout the genome. A good genome-wide scan will attempt to cover the entire human genome using a set of markers evenly spaced across a given genetic map. The polymorphic markers used are generally chosen for their ease of genotyping and their position within a genetic map. An advantage of doing such a scan is that no previous knowledge of the function of any genes is required. Another huge advantage of this method, particularly for RLS, is that knowledge of the biology of the trait or disease in question is not a requirement. Genome-wide scans rely on a standard set of markers which are generally termed a "mapping set." For this study, two mapping set were used.

The first mapping set used was the Research Genetics Inc. mapping set (Appendix I). It comprised of 146 microsatellite markers, which were spaced at approximately 20 cM intervals throughout the genome. The markers were predominantly tetranucleotide repeats, although the set did contain some dinucleotide and trinucleotide markers also. The markers were fluorescently labelled with 6-FAM (blue), HEX (green) or TET (yellow) labels.

The second mapping set used was the Applied Biosystems Linkage mapping set version 2.5 (Appendix II). This mapping set comprises of 28 panels of fluorescently labeled primer pairs selected to amplify informative microsatellite loci from the

Généthon human linkage map. All markers in this set are dinucleotide repeats, and are fluorescently labeled with either 6-FAM (blue), VIC (green) or NED (yellow). There is exactly 400 microsatellite markers in this mapping set and the set has a resolution of 10 cM. Each of the 28 panels consists of a group of markers that can be loaded into one capillary or into one lane in a gel. Therefore no one marker in any panel has the same fluorescent dye or is in the same size range as another marker in that panel. Panels sometimes contain markers from more than one chromosome in order to gain the most efficiency. The allele size range for this mapping set is based on the range of sizes seen for a particular marker in the CEPH database. However, Applied Biosystems optimised and tailed the markers, and the primers used for this set are often different to those recommended by the standard databases e.g. NCBI and Ensembl. Therefore it is often necessary to correct the genotyping results by adding/subtracting values up to 150 bp in order to adjust them to CEPH values.

It was important to find the total length of each chromosome before starting the genome-wide scan. By knowing the total genetic length of a chromosome it was possible to tell whether or not the telomere at the end of the chromosome was totally excluded or if another marker was required to exclude this area. The total sex averaged genetic length of the 22 autosomes is 3488 cM (Broman, Murray et al. 1998). Table 4.1 lists the genetic length of each individual chromosome Each of the 22 autosomes and its corresponding genetic distance (in cM) is given.

Table 4.1: The genetic length of each chromosome

Chromosome	Length (cM)	Chromosome	Length (cM)
1	289.66	12	168.79
2	269.07	13	114.99
3	228.14	14	138.19
4	211.66	15	122.15
5	197.54	16	134.13
6	193.14	17	126.46
7	181.97	18	126.00
8	167.91	19	105.02
9	168.98	20	101.22
10	173.13	21	57.78
11	147.78	22	62.32

As previously discussed (section 1.12) LOD scores of 3.00 (1000:1 odds in favour of linkage) or more are evidence of linkage and -2.00 or less are indicative of non-linkage/exclusion (Haines J.L 1998). Values between -2.00 and 3.00 are inconclusive and require further examination. Power calculation for linkage analysis based on family RLS3002 was performed using SLINK (http:/*linkage.rockefeller.edu/soft/**slink**.html*). SLINK simulation analysis indicated that the maximum LOD score achievable for this family is 3.41 at $\theta = 0.01$. This value is very informative in that it allows the investigator to gain insight into the full information content of the available pedigree material. The true maximum LOD score is the highest potential LOD score when all meiosis are fully informative under the assumed model and allele frequencies (Haines J.L 1998). It is important to consider that when carrying out two-point linkage analysis the maximum LOD score may not be attained even if there is linkage. When considering the significance of a particular LOD score it is important to note whether all the available linkage information has been captured. The observed maximum LOD score may not match the attainable maximum LOD score for several reasons, including lack of informativeness of key individuals or a difference in allele frequencies from

simulated results. Therefore when performing linkage analysis for RLS3002 any microsatellite marker which resulted in a maximum LOD score of approximately 1.00 or more was investigated further.

4.2 Materials and Methods

DNA extraction

The QIAamp Blood Kit from Qiagen described in section 2.2 was used to extract DNA from peripheral venous blood samples.

Polymerase Chain Reaction (PCR)

For this section of the study, two different recipes for PCR were used, depending on the mapping set in question.

Research Genetics Inc. mapping set

The first mapping set used was the Research Genetics Inc. mapping set, and PCR was carried out in 15 µl reaction volumes. Table 4.2 provides details of the reagents used. The table contains the name of the reagent, the stock concentration, the concentration used, and the amount of each reagent in a 15 µl reaction volume.

Table 4.2 Reagents used for PCR in 15 µl reaction volumes.

Reagent	**[Stock]**	**[Final]**	**1 sample (µl)**
5x buffer Promega	5x	1x	1.50
$MgCl_2$ Promega	25 mM	1.5 mM	0.90
dNTP	10 mM	0.2 mM	0.30
Forward Primer	100 ng/µl	100 ng	0.50
Reverse Primer	100 ng/µl	100 ng	0.50
Taq polymerase Promega	5 U/µl	0.05 U	0.10
H_2O			6.20
DNA		50-100 ng	5.00
Total Volume			15.0

Once the samples were prepared, each 15 µl reaction was placed in a 0.2 ml capped PCR tube and placed on the GStorm Thermal Cycler PCR machine. A temperature gradient was initially used to discover the optimal annealing temperature for each individual marker (as outlined earlier in section 3.2). The success of PCR was verified by agarose gel electrophoresis (as outlined in section 2.4).

Applied Biosystems Linkage mapping set version 2.5

The second mapping set, the Applied Biosystems Linkage mapping set version 2.5, required a different recipe for PCR. For this mapping set True Allele® PCR Premix was used. This is a PCR master mix that contains AmpliTaq Gold® DNA polymerase, dNTPs, $MgCl_2$, and buffer. True Allele® PCR Premix had been optimized for strong, specific PCR amplification of microsatellite markers in the ABI PRISM® Linkage Mapping Set. The reaction consisted of premix, primers, DNA and water and was performed in 15 µl reaction volumes. Table 4.3 provides details of the reagents used. The table contains the name of the reagent, the concentration used, and the amount of each reagent in a 15µl reaction volume.

Table 4.3. Reagents used for PCR using the PCR premix from Applied Biosystems.

Reagent	**[Final]**	**1 sample (µl)**
True Allele® PCR Premix		9.00
Primer mix	5 pmol	0.60
H_2O		3.00
DNA	50-100 ng	2.40
Total Volume		15.00

PCR was carried out on a GStorm Thermal cycler, under temperature control using the following program:

95°C for 12 min (Denaturation)
94°C for 15 s
55°C for 15 s
72°C for 30 s } 10 cycles
89°C for 15 s
55°C for 15 s
72°C for 30 s } 32 cycles
72°C for 10 min (Extension)

The success of PCR was verified by agarose gel electrophoresis (as outlined in section 2.4).

Genotyping and Linkage analysis were successfully performed for all markers under investigation (as per section 2.5 to 2.7). Figure 4.2 below is an example of ABI analysis reading of genotyping for two parents and two children. The marker used in this example is D10S597, a marker which was used in the genome-wide scan, and part of the Applied Biosystems Linkage mapping set version 2.5.

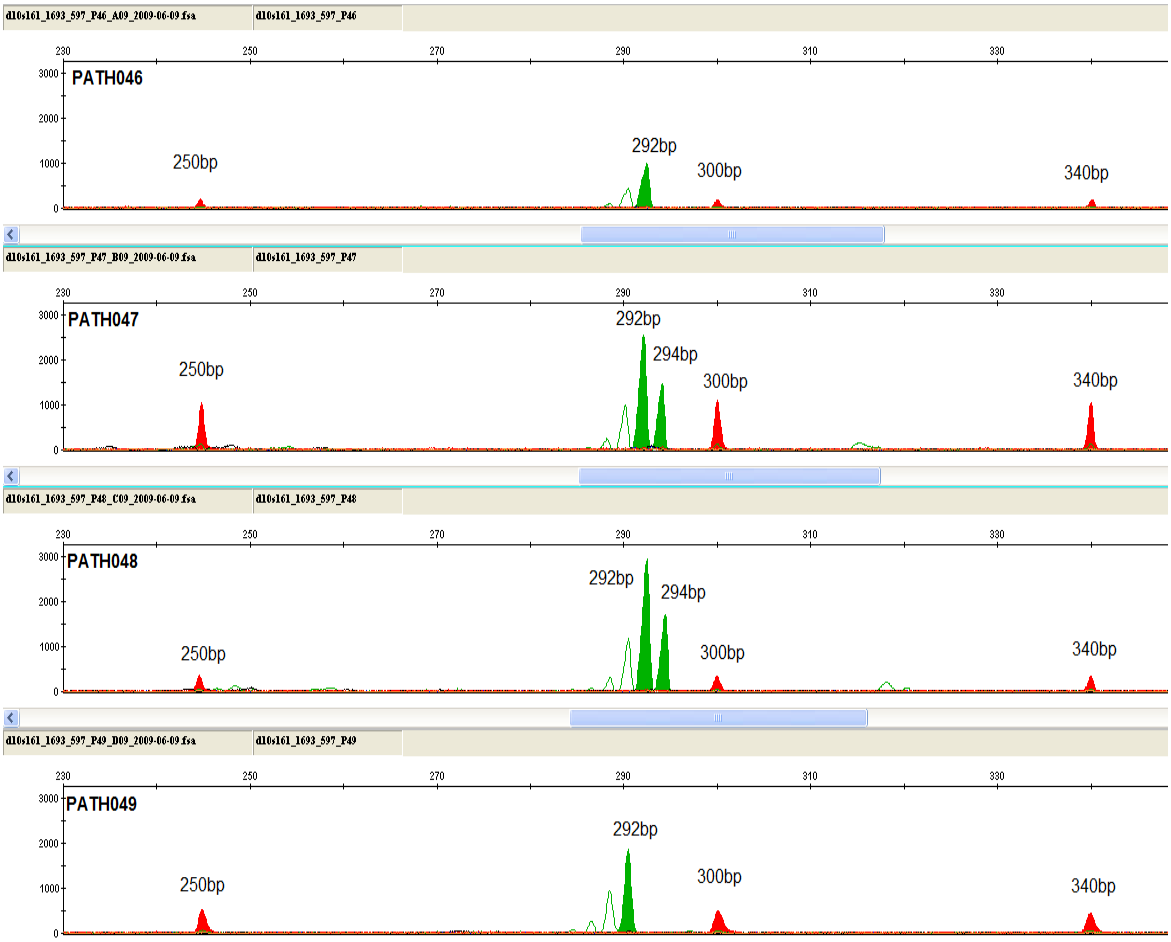


Figure 4.1: An example of ABI genotyping of related individuals

Marker D10S597 using samples PATH046 (mother), PATH047 (father), PATH048 (child1) and PATH049 (child 2). The red peaks refer to the internal size standard used to determine allele sizes. The alleles are shown as green peaks. The colour of the peak corresponds to the colour of the fluorescent label, which in this example is HEX. The positions of the peaks correspond to the size of the allele which in this case is 273-297 bp. The mother (PATH046) is homozygous for this marker (one peak), and carries two copies of the 292 bp allele. The father (PATH047) is heterozygous (two peaks) and carries a copy of the 292 bp allele and a copy of the 294 bp allele. Child 1 (PATH048) is heterozygous and the alleles can be traced through the generations. The 292 bp allele is passed from the mother and the 294 bp allele is passed from the father. Child 2 (PATH049) is homozygous for this marker (one single peak) with the inheritance of one 292 bp allele from the mother and another 292 bp from the father.

4.3 Results

The results for each chromosome will be given individually, combining the results from the two mapping sets. Markers which had been previously ordered for examination of RLS loci and associated regions, and which have been previously discussed in chapter 3, have been included in these results also in order to identify regions that have been excluded.

4.3.1 Chromosome 1

The Research Genetics Inc. mapping set contained eight markers for chromosome one, five of which were tetranucleotide repeats and the remaining three were trinucleotide. The Applied Biosystems mapping set contained 31 markers for chromosome 1. Table 4.4 lists the markers that were examined from both mapping sets, their positions, the LOD scores obtained at θ=0.00 the corresponding maximum excluded distances in cM, and the regions excluded. Markers highlighted in blue represent markers from the Research Genetics Inc. mapping set. Markers highlighted in pink represent markers from the Applied Biosystems mapping set.

Table 4.4: Markers used to examine chromosome 1.

Marker	Position (cM)	Max LOD (θ=0)	Excluded (cM)	Area Excluded from (cM)	Area Excluded to (cM)
D1S468	4.22	-5.52	1.90	2.32	6.12
D1S214	14.04	-5.31	2.40	11.64	16.44
D1S450	20.61	-6.79	3.61	17.00	24.22
D1S2667	24.68	-5.49	1.70	22.98	26.38
D1S2697	37.05	-5.55	6.33	30.72	43.38
D1S199	45.33	-8.21	2.00	43.33	47.33
D1S234	55.10	-8.24	9.00	46.10	64.10
D1S1622	56.74	-5.31	2.50	54.24	59.24
D1S255	65.47	-5.09	0.40	65.07	65.87
D1S2797	75.66	-2.08	0.00	-	-
D1S2890	85.68	-6.61	3.10	82.57	88.78
D1S3728	89.49	-5.43	1.60	87.89	91.09
D1S230	95.31	-6.58	2.40	92.91	97.71
D1S2841	106.45	-8.36	12.13	94.32	118.58
D1S1728	109.04	-2.55	0.40	108.64	109.44
D1S2868	126.16	0.60	0.00	-	-
D1S206	134.20	-4.25	4.31	129.89	138.51
D1S3723	140.39	-5.59	7.35	133.04	147.74
D1S2726	144.28	-5.08	5.32	138.96	149.6
D1S498	155.89	-7.22	1.90	153.99	157.79
D1S484	169.69	1.69	0.00	-	-
D1S2878	177.86	-10.62	9.51	168.35	187.37
D1S196	181.49	-7.37	5.62	175.87	187.11
D1S1589	192.05	-3.24	3.10	188.95	195.15
D1S413	212.42	-3.34	4.81	207.61	217.23
D1S249	222.65	-1.86	0.00	-	-
GATA124F08	226.16	-8.57	10.14	216.02	236.30
D1S425	231.11	-1.79	0.00	-	-
D1S213	242.23	-7.65	9.31	232.92	251.54
D1S3462	247.23	-5.27	1.30	245.93	248.53
D1S2800	252.12	-7.64	7.45	244.67	259.57
D1S2785	266.27	-5.88	1.50	264.77	267.77
D1S547	267.51	0.34	0.00	-	-
D1S2842	273.96	-1.79	0.00	-	-
D1S2836	285.75	-5.24	1.50	284.25	287.25

Thirty-five markers from the two mapping sets were examined for chromosome 1. Twenty-eight of these markers obtained a statistically significant LOD score, and excluded various regions on the chromosome. Marker D1S2797 showed a significant LOD score of <-2, however it was only significant at θ=0.00. Therefore, there was

no distance of exclusion aside from the position of the marker itself. Markers D1S2868, D1S484, D1S249, D1S425, D1S547 and D1S2842 did not show significant LOD scores, and yielded inconclusive LOD scores of 0.60 at θ=0.00, 1.69 at θ=0.00, -1.86 at θ=0.00, -1.79 at θ=0.00, 0.34 at θ=0.00 and -1.79 at θ=0.00 respectively. Figure 4.1 illustrates the positions of the markers that obtained significant negative LOD scores and the corresponding regions of exclusion on chromosome 1.

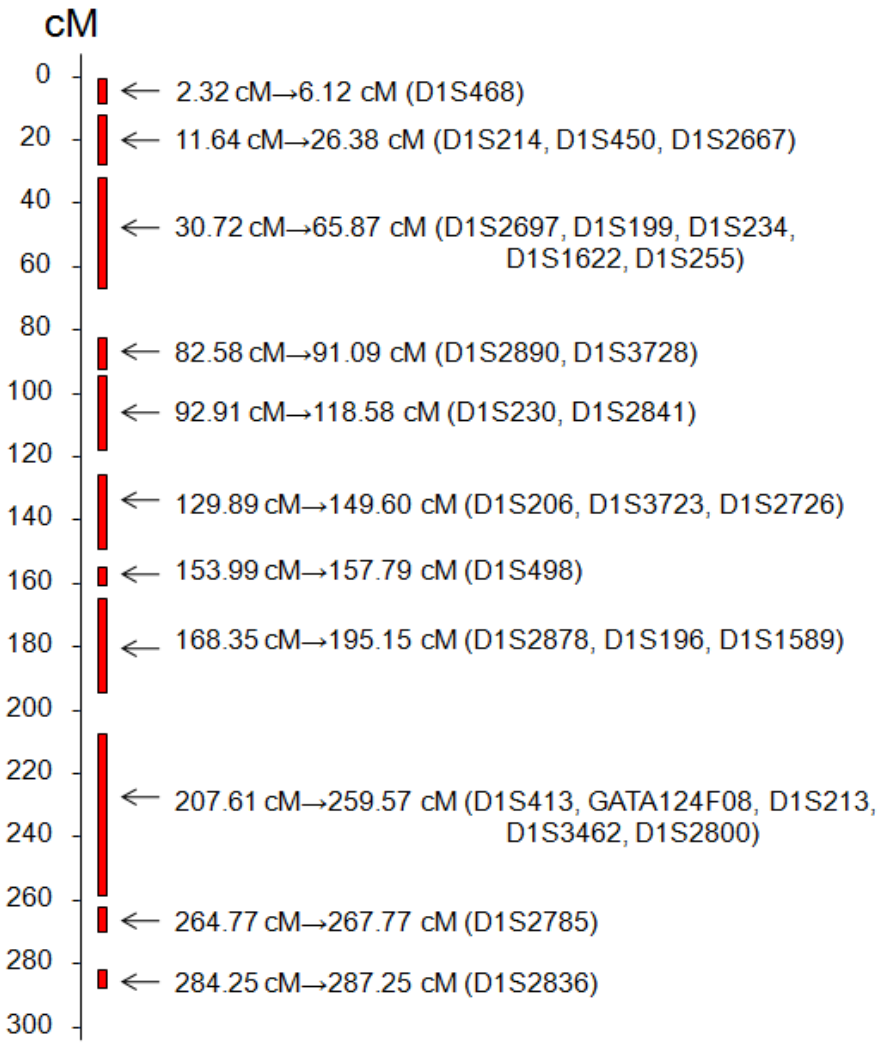


Figure 4.2: Illustration of areas of exclusion for Chromosome 1
Black vertical line with scales represents chromosome 1 (scale in cM). The red bars on the right illustrates the total areas excluded combining all markers from the two linkage sets. The marker(s) which contributed to these exclusion values are also indicated.

The results of the genome-wide scan exclude a substantial area of chromosome 1. A number of gaps remain on chromosome 1 however the majority of these gaps are small. The largest gap is seen between 65.87 cM and 82.58 cM. A total of 196.14 cM of chromosome 1 was excluded, which equates to 67.7% of the entire chromosome. The highest LOD score seen for this chromosome was for D1S484 at169.69 cM, which presented with a LOD score of 1.69 at θ=0.00. Although this LOD score was less than the +3 required for evidence of linkage, it was one of the highest LOD scores observed, therefore it required further investigation (section 4.4.1).

4.3.2 Chromosome 2

The Research Genetics Inc. mapping set contained eleven markers for chromosome 2, nine of which were tetranucleotide repeats. The Applied Biosystems mapping set contained 30 markers for chromosome 2. Table 4.5 lists the markers that were examined from both mapping sets, their positions, the LOD scores obtained at θ=0.00, the corresponding maximum excluded distances in cM, and the regions excluded. Markers highlighted in blue represent markers from the Research Genetics Inc. mapping set. Markers highlighted in pink represent markers from the Applied Biosystems mapping set.

Eight markers from the Research Genetics Inc mapping set were completed for chromosome two and eighteen makers from the Applied Biosystems Mapping set were completed. Eleven additional markers on chromosome 2 had been ordered previously to examine the chromosome 2 locus (Section 3.3.4) and to examine the association between RLS and a variation within the *MEIS1* gene (section 3.3.11). Figure 4.2 illustrates the total areas excluded from chromosome 2 when markers from both mapping sets and previously examined markers have been combined.

Table 4.5: Markers used to examine chromosome 2.

Marker	Position (cM)	Max LOD (θ=0)	Excluded (cM)	Area Excluded from (cM)	Area Excluded to (cM)
D2S2211	15.61	0.03	0.00	-	-
D2S2952	17.88	-3.08	6.84	11.04	24.72
D2S168	27.6	-5.56	3.10	23.96	30.16
D2S405	47.97	-1.78	0.00	-	-
D2S367	54.96	-8.28	11.71	43.25	66.67
D2S2259	64.29	-8.11	8.48	55.81	72.77
D2S1356	64.29	-5.86	3.10	61.19	67.39
D2S391	70.31	-7.05	8.89	61.42	79.20
D2S2739	73.61	-5.59	7.56	66.05	81.17
D2S337	80.6	-5.59	3.00	77.6	83.6
D2S290	84.42	-2.17	0.00	-	-
D2S166	82.42	-3.23	0.00	-	-
D2S285	86.02	-2.41	0.00	-	-
D2S327	88.15	-5.17	1.90	86.25	90.05
D2S2113	88.15	0.22	0.00	-	-
D2S2110	90.82	-5.12	8.27	82.55	99.09
D2S286	94.05	-6.07	10.55	83.50	104.60
D2S1777	99.41	0.22	0.00	-	-
D2S329	101.02	-2.79	2.20	98.82	103.22
D2S2333	103.16	-8.18	6.54	96.62	109.70
D2S160	122.96	-4.69	4.11	118.85	127.07
D2S112	141.62	-8.54	1.20	140.42	142.82
D2S1334	145.08	-5.62	9.93	135.15	155.01
D2S151	152.04	-8.36	7.35	144.69	159.39
D2S142	161.26	-6.51	1.80	159.46	163.06
D2S117	194.45	-7.66	6.64	187.81	201.09
D2S311	196.90	-5.61	4.40	192.50	201.30
D2S2289	199.20	-3.43	4.00	195.20	203.20
D2S2358	203.50	-3.16	5.20	198.30	208.70
D2S325	204.53	-5.49	7.97	196.56	212.50
D2S2208	205.10	-5.55	9.90	196.56	212.50
D2S2382	213.50	-7.67	5.22	208.28	218.72
D2S434	215.73	-3.57	0.60	215.13	216.33
D2S126	221.13	0.48	0.00	-	-
D2S206	240.79	-0.22	0.00	-	-
D2S2968	251.94	0.52	0.00	-	-
D2S125	260.63	-5.97	7.97	252.66	268.60

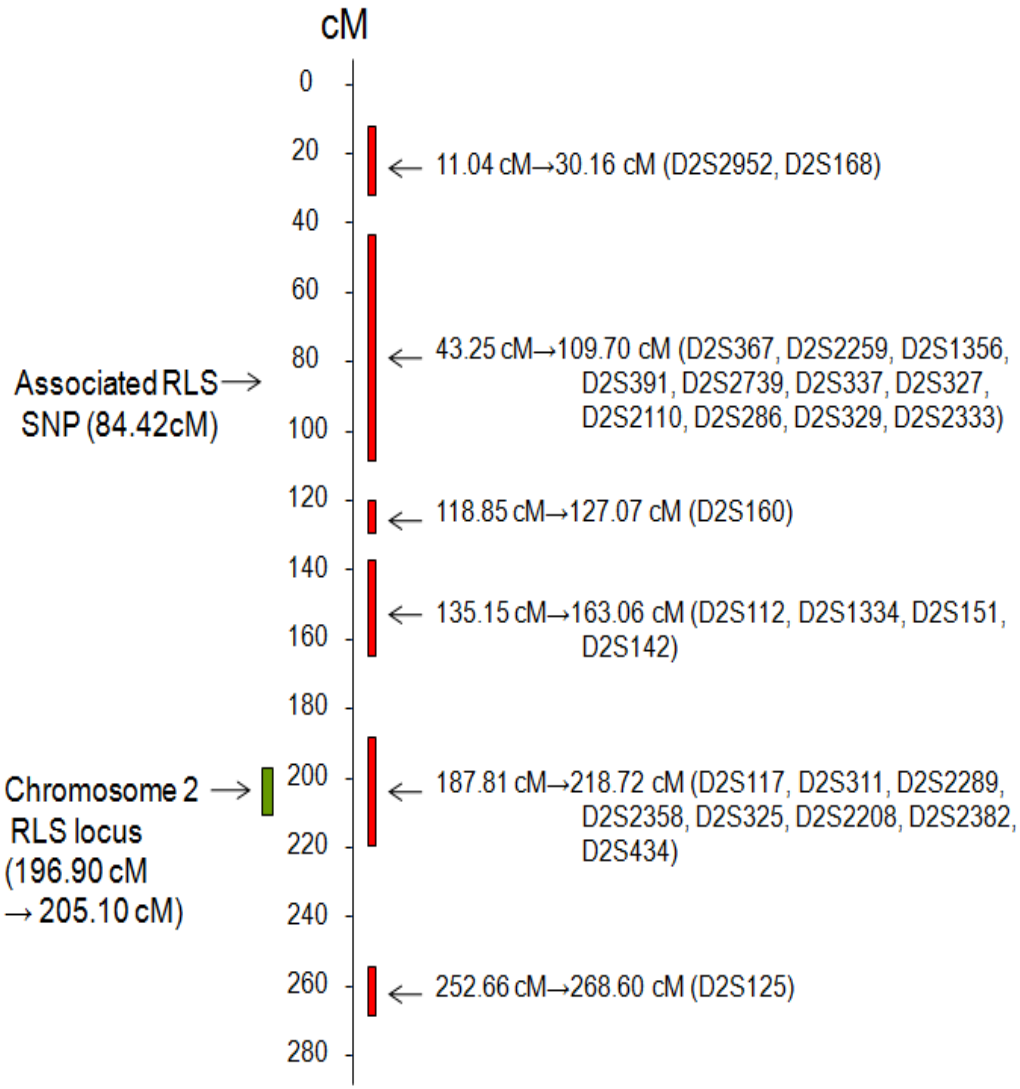


Figure 4.3: Illustration of areas of exclusion for Chromosome 2
Black vertical line with scales represents chromosome 2 (scales in cM). The red bars on the right illustrates the total areas excluded combining all markers from the two linkage set. The marker(s) which contributed to these exclusion values are also indicated.

The results of the genome-wide scan exclude a substantial area on chromosome 2. A number of gaps remain on chromosome 2; the largest gap present is between 218.72 cM and 252.66 cM. A total of 168.55 cM of chromosome 2 was excluded, which equates to 62.6% of the entire chromosome. The highest LOD score seen for this chromosome was for D1S2968 at 251.94 cM, which presented with a LOD score of 0.52 at θ=0.00. This LOD score was not high enough to require further investigation.

4.3.3 Chromosome 3

The Research Genetics Inc. mapping set contained nine markers for chromosome 3, five of which were tetranucleotide repaeats and the remaining three were trinucleotide. The Applied Biosystems mapping set contained 16 markers for chromosome one. Table 4.6 lists the markers that were examined from both mapping sets, their positions, the LOD scores obtained at θ=0.00, the corresponding maximum excluded distances in cM, and the regions excluded. Markers highlighted in blue represent markers from the Research Genetics Inc. mapping set. Markers highlighted in pink represent markers from the Applied Biosystems mapping set. Markers highlighted in green represent markers which were found in both mapping sets.

Table 4.6: Markers used to examine chromosome 3.

Marker	Position (cM)	Max LOD (θ=0)	Excluded (cM)	Area Excluded from (cM)	Area Excluded to (cM)
D3S1304	22.33	-2.07	0.00	-	-
D3S1263	36.10	-8.58	8.89	27.21	44.99
D3S2338	42.10	-3.69	0.20	41.90	42.30
D3S3038	44.81	-5.64	9.00	35.81	53.81
D3S1266	52.60	-3.12	0.20	52.40	52.80
D3S1277	61.52	0.49	0.00	-	-
D3S1289	71.41	-11.08	7.15	64.26	78.56
D3S1300	80.32	-6.91	3.10	77.22	83.42
D3S1285	91.18	-6.34	3.91	87.27	95.09
D3S2406	102.64	-5.45	2.70	99.94	105.34
D3S1271	117.76	-3.98	1.30	116.46	119.06
D3S3045	124.16	-5.75	2.70	121.46	126.86
D3S1278	129.73	-8.21	6.84	122.89	136.57
D3S1303	136.32	-5.84	3.40	132.92	139.72
D3S1267	139.12	-7.92	10.55	128.57	149.67
D3S1292	146.60	-4.08	11.18	135.42	157.78
D3S1569	158.38	-5.85	10.45	147.93	168.83
D3S3053	181.87	-8.54	11.30	170.57	193.17
D3S1565	186.04	-6.02	1.80	184.24	187.84
D3S1262	201.14	-3.04	1.00	200.14	202.14
D3S1580	207.73	-4.96	9.10	198.63	216.83
D3S3023	(deCode)	0.26	0.00	-	-

Twenty-two markers from the two mapping sets were examined for chromosome 3. Nineteen of these markers obtained a statistically significant LOD score, and excluded various regions on the chromosome. Marker D3S1304 showed a significant LOD score of <-2, however it was only significant at θ=0.00. Therefore there was no distance of exclusion aside from the position of the marker itself. Markers D3S1277 and D3S3023 did not show significant LOD scores, and yielded inconclusive LOD scores of 0.49 at θ=0.00 and 0.26 at θ=0.00 respectively. Marker D3S3023 was part of the Research Genetics Inc mapping set, however the position of the marker on the Marshfield map was not available. A genetic position was only available from the deCODE map (www.**decode**.com/*)*. The marker did not give a significant LOD score and therefore the position on the Marshfield map was not needed per se. Figure 4.3 illustrates the positions of the markers that obtained significant negative LOD scores and the corresponding regions of exclusion on chromosome 3.

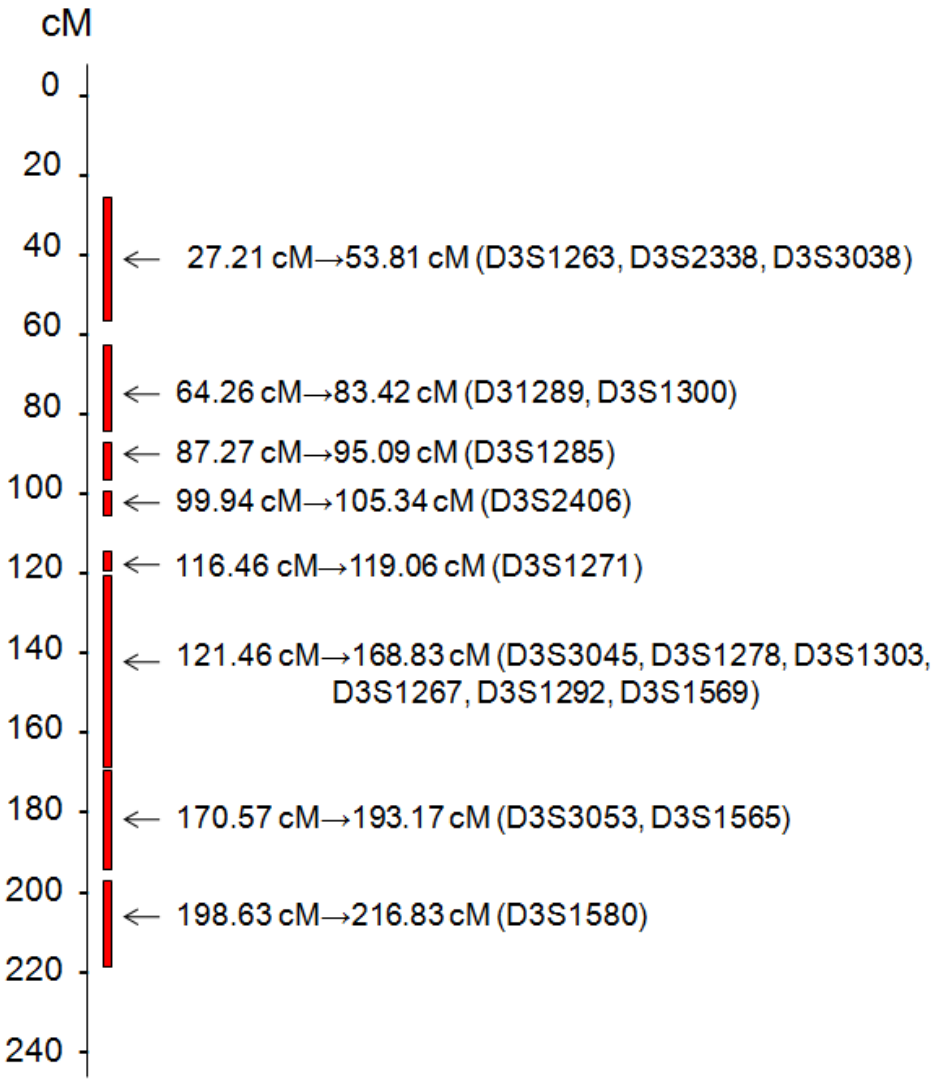


Figure 4.4: Illustration of areas of exclusion for Chromosome 3
Black vertical line with scales represents chromosome 3 (scales in cM). The red bars on the right illustrates the total areas excluded combining all markers from the two linkage set. The marker(s) which contributed to these exclusion values are also indicated.

These results exclude a large area of chromosome 3. A number of gaps remain on chromosome 3, the largest gap present at the telomere between 0.00 cM and 27.21 cM. A total of 154.25 cM of chromosome three was excluded, which equates to 67.6% of the entire chromosome. The highest LOD score seen for this chromosome was for D3S1277 at 61.52 cM, which presented with a LOD score of 0.49 at θ=0.00. This LOD score was not high enough to deserve further investigation.

4.3.4 Chromosome 4

The Research Genetics Inc. mapping set contained eight markers for chromosome 4, six of which were tetranucleotide repeats. The Applied Biosystems mapping set contained 29 markers for chromosome four. Table 4.7 lists the markers that were examined from both mapping sets. The positions (cM), maximum obtained LOD scores at theta equal to zero, the corresponding maximum excluded distances in cM, and the regions excluded are listed here. Markers highlighted in blue represent markers from the Research Genetics Inc. mapping set. Markers highlighted in pink represent markers from the Applied Biosystems mapping set. Markers highlighted in green represent markers which were found in both mapping sets. Markers which have not been highlighted were ordered previously for investigation of linkage to the chromosome 4 locus (section 3.3.7).

Table 4.7: Markers used to examine chromosome 4.

Marker	Position (cM)	Max LOD (θ=0)	Excluded (cM)	Area Excluded from (cM)	Area Excluded to (cM)
D4S412	4.74	0.33	0.00	-	-
D4S403	25.9	-8.64	12.24	13.66	38.14
D4S2632	50.53	-5.61	10.35	40.18	60.88
D4S3248	72.52	-3.21	0.90	71.62	73.42
D4S3243	88.35	-1.75	0.00	-	-
D4S1534	95.09	-5.94	6.13	88.96	101.22
D4S414	100.75	-8.57	3.41	97.34	104.16
D4S1647	104.94	-5.08	3.00	101.94	107.94
D4S1572	107.95	-1.78	0.00	-	-
D4S406	117.06	-8.38	2.90	114.16	119.96
D4S2989	117.06	-8.38	3.61	113.45	120.67
D4S407	117.06	-8.68	4.51	112.55	121.57
D4S402	124.45	-5.69	0.00	-	-
D4S1575	132.05	-11.18	8.87	123.18	140.92
D4S424	144.56	-5.79	7.45	137.11	152.01
D4S1629	157.99	-5.31	3.00	154.99	160.99
D4S413	157.99	-5.32	0.20	157.79	158.19
D4S1539	176.19	-2.51	0.80	175.39	176.99
D4S415	181.36	-5.85	4.31	177.05	185.67
D4S2417	181.93	-2.06	0.00	-	-
D4S408	195.06	-2.05	0.70	194.36	195.76
D4S1535	195.06	-2.85	0.20	194.86	195.26
D4S426	206.98	-10.98	13.63	193.35	220.61

Twenty-one markers from the two mapping sets were examined for chromosome 4. Two markers had been ordered previously for investigation of the chromosome 4 locus (included in the table above). Eighteen of the markers from the mapping sets obtained a statistically significant LOD score, and excluded various regions on the chromosome. Markers D4S402 and D4S2417 showed significant LOD scores of <-2, however they were only significant at θ=0.00. There was no distance of exclusion aside from the position of the marker itself. Markers D4S412, D4S3243 and D4S1572 did not show significant LOD scores, and yielded inconclusive LOD scores of 0.33 at θ=0.00, -1.75 at θ=0.00 and -1.78 at θ=0.00 respectively.

Figure 4.4 illustrates the positions of the markers that obtained significant negative LOD scores and the corresponding regions of exclusion on chromosome 4.

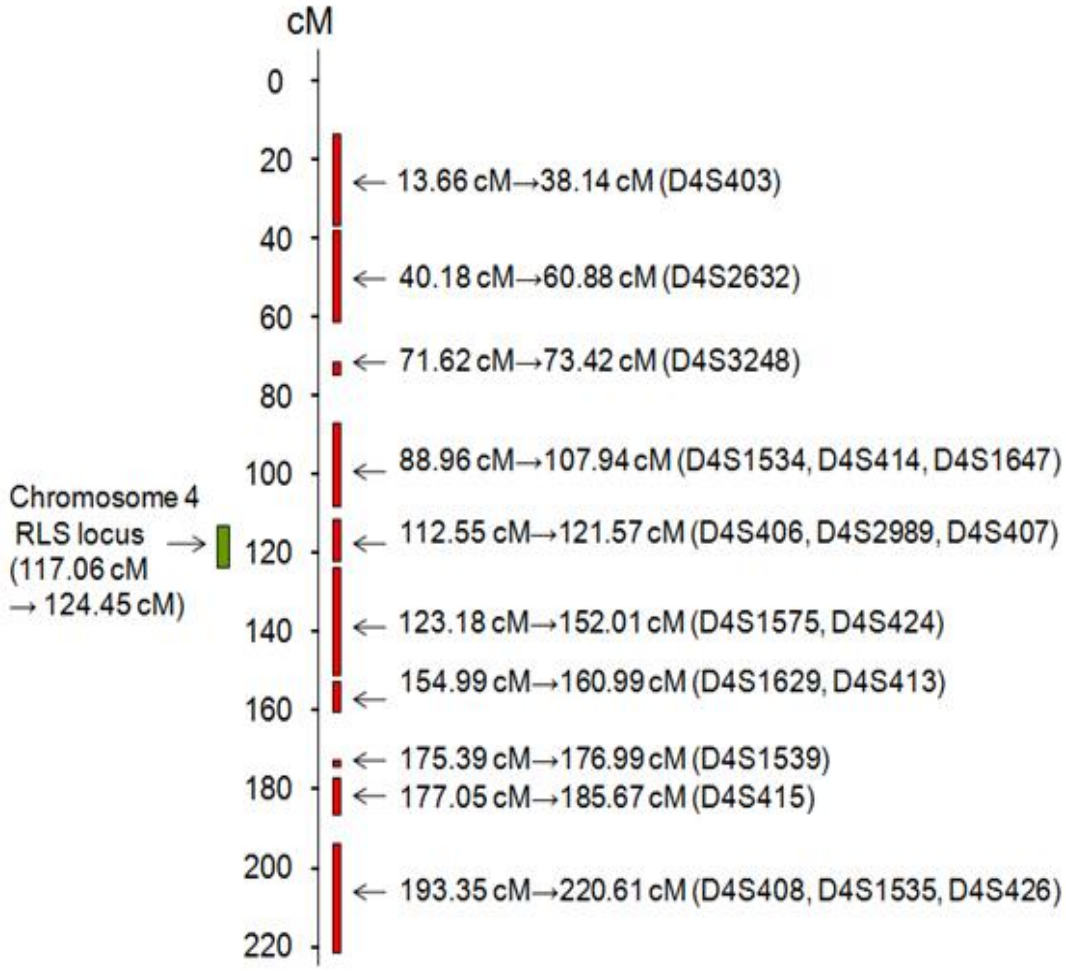


Figure 4.5: Illustration of areas of exclusion for Chromosome 4
Black vertical line with scales represents chromosome 4 (scales in cM). The red bars on the right illustrates the total areas excluded combining all markers from the two linkage set. The marker(s) which contributed to these exclusion values are also indicated.

These results exclude a large area of chromosome 4. A number of gaps remain on chromosome 4, the largest gap present at the telomere between 0.00 cM and 13.66 cM. A total of 147.29 cM of chromosome four was excluded, which equates to 69.6% of the entire chromosome. The highest LOD score seen for this chromosome was for D4S412 at 4.74 cM, which presented with a LOD score of 0.33 at θ=0.00. This LOD score was not high enough to deserve further investigation.

4.3.5 Chromosome 5

The Research Genetics Inc. mapping set contained seven markers for chromosome 5, five of which were tetranucleotide repeats. The Applied Biosystems mapping set contained 22 markers for chromosome 5. Table 4.8 lists the markers that were examined from both mapping sets, their positions (cM), maximum obtained LOD scores at theta equal to zero, the corresponding maximum excluded distances in cM, and the regions excluded. Markers highlighted in blue represent markers from the Research Genetics Inc. mapping set. Markers highlighted in pink represent markers from the Applied Biosystems mapping set. Markers which have not been highlighted were ordered previously for investigation of linkage to the chromosome 5 locus (section 3.3.1).

Table 4.8: Markers used to examine chromosome 5.

Marker	Position (cM)	Max LOD (θ=0)	Excluded (cM)	Area Excluded from (cM)	Area Excluded to (cM)
D5S1981	1.72	-8.37	8.38	0.00	10.10
D5S406	11.85	-5.53	2.10	9.75	13.95
D5S630	19.67	-5.59	6.13	13.94	25.80
D5S817	22.88	-2.87	0.20	22.68	23.08
D5S416	28.76	-5.32	3.61	25.15	32.37
D5S419	39.99	-7.19	10.55	29.44	50.54
D5S1470	45.34	-5.43	5.02	40.32	50.36
D5S426	51.99	-13.46	10.55	41.44	62.54
D5S418	58.55	-11.04	13.63	44.92	72.18
D5S2500	69.23	-6.86	6.94	62.29	76.17
D5S2122	78.31	-5.23	2.70	75.60	81.01
D5S2003	78.31	-5.44	4.92	73.39	83.23
D5S424	81.95	-4.09	1.50	80.45	83.45
D5S641	92.38	-11.19	4.41	87.97	96.79
D5S428	95.40	-11.18	3.61	91.79	99.01
D5S1725	97.82	-3.31	6.74	91.08	104.56
D5S644	104.76	-8.35	11.29	93.47	116.05
D5S2027	119.50	-6.11	2.60	116.90	122.10
D5S1505	129.83	-5.63	4.31	125.52	134.14
D5S471	129.83	-7.76	7.66	122.17	137.49
D5S2115	138.64	-10.70	7.35	131.29	145.99
D5S436	147.49	-8.37	12.03	135.46	159.52
D5S1480	147.49	-3.16	4.31	143.18	151.80
D5S410	156.47	-5.09	4.51	151.96	160.98
D5S400	174.80	-7.91	4.11	170.69	178.91
D5S211	182.89	-5.78	4.41	178.48	187.30
D5S408	195.49	-1.51	0.00	-	-

Twenty-five markers from the two mapping sets were examined for chromosome 5. Two additional markers, which have been included in table 4.8, had been ordered previously for investigation to the chromosome 5 locus. Twenty-four of the markers from the mapping sets obtained a statistically significant LOD score, and excluded various regions on the chromosome. Marker D5S408 did not show a significant LOD score, and presented with an inconclusive LOD score of -1.51 at θ=0.00.

Figure 4.5 illustrates the positions of the markers that obtained significant negative LOD scores and the corresponding regions of exclusion on chromosome 5.

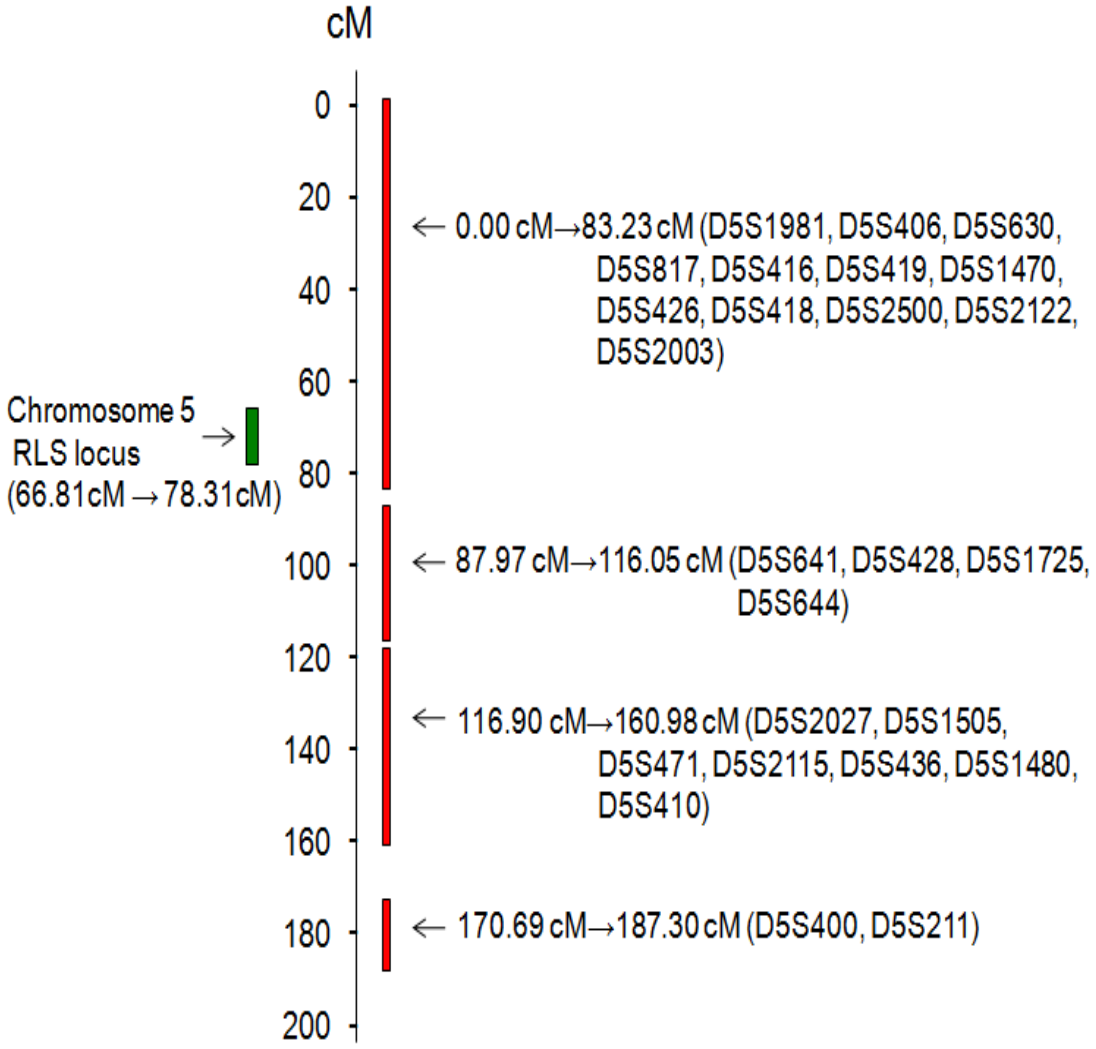


Figure 4.6: Illustration of areas of exclusion for Chromosome five
Black vertical line with scales represents chromosome 5 (scales in cM). The red bars on the right illustrates the total areas excluded combining all markers from the two linkage set. The marker(s) which contributed to these exclusion values are also indicated.

These results exclude a large area of chromosome 5. Only four gaps remain on chromosome five. These are positioned at 83.23 cM to 87.97 cM, 116.05 cM to 116.90 cM, 160.98 cM to 170.69 cM and 187.30 cM to 197.54 cM. A total of 172 cM of chromosome five was excluded, which equates to 87.1% of the entire chromosome. The highest LOD score seen for this chromosome was for D5S408 at 195.49 cM, which presented with a LOD score of -1.51 at θ=0.00. This LOD score did not need any warrant further investigation.

4.3.6 Chromosome 6

The Research Genetics Inc. mapping set contained five markers for chromosome 6, five which were tetranucleotide repeats and the remaining one a trinucleotide. The Applied Biosystems mapping set contained 20 markers for chromosome six. Table 4.9 lists the markers that were examined from both mapping sets, the LOD scores obtained at θ=0.00 and the corresponding excluded regions. Markers highlighted in blue represent markers from the Research Genetics Inc. mapping set. Markers highlighted in pink represent markers from the Applied Biosystems mapping set. Markers which have not been highlighted were ordered previously for investigation of linkage to the chromosome 6 locus (section 3.3.10).

Table 4.9: Markers used to examine chromosome 6.

Marker	Position (cM)	Max LOD (θ=0)	Excluded (cM)	Area Excluded from (cM)	Area Excluded to (cM)
D6S1574	9.81	-6.02	3.41	6.40	13.22
D6S470	18.22	-11.02	11.71	6.51	29.93
D6S2434	25.08	-5.32	1.80	23.28	26.88
D6S289	29.93	-8.10	6.64	23.29	36.57
D6S422	35.66	-8.64	9.72	25.94	45.38
D6S2439	42.27	-2.05	0.08	42.19	42.35
D6S1051	50.75	-3.12	0.20	50.55	50.95
D6S1610	53.81	-3.69	3.71	50.10	57.52
D6S1607	59.34	-8.54	6.64	52.70	65.98
D6S2410	73.13	-2.77	0.00	-	-
D6S257	79.92	-8.27	1.80	78.12	81.72
D6S460	89.93	-5.01	0.70	89.13	90.53
D6S462	99.01	-5.32	1.60	97.41	100.61
D6S287	121.97	-8.62	10.66	111.31	132.63
D6S1040	128.93	-8.54	3.81	125.12	132.74
D6S262	130.00	-5.43	3.20	126.80	133.20
D6S292	136.97	-6.40	4.71	132.26	141.68
D6S308	144.46	0.35	0.00	-	-
D6S441	154.1	-6.23	1.00	153.10	155.10
D6S2436	154.64	-5.84	5.22	149.42	159.86
D6S1581	164.78	0.29	0.00	-	-
D6S264	179.07	-5.96	5.62	173.45	184.69
D6S446	189.00	0.16	0.00	-	-
D6S281	190.14	-4.19	0.10	190.04	190.24

Twenty-two markers from the two mapping sets were examined for chromosome 6. Two additional markers, which have been included in table 4.8, had been ordered previously for investigation to the chromosome 6 locus. Eighteen of the markers from the mapping sets obtained a statistically significant LOD score, and excluded various regions on the chromosome. Marker D6S2410 showed a significant LOD scores of <-2, however it was only significant at θ=0.00. Therefore there was no

distance of exclusion aside from the position of the marker itself. Markers D6S308, D6S1581, and D6S446 did not show significant LOD scores, and produced inconclusive LOD scores of 0.35 at θ=0.00, 0.29 at θ=0.00 and 0.16 at θ=0.00 respectively. Figure 4.6 illustrates the positions of the markers that obtained significant negative LOD scores and the corresponding regions of exclusion on chromosome 6.

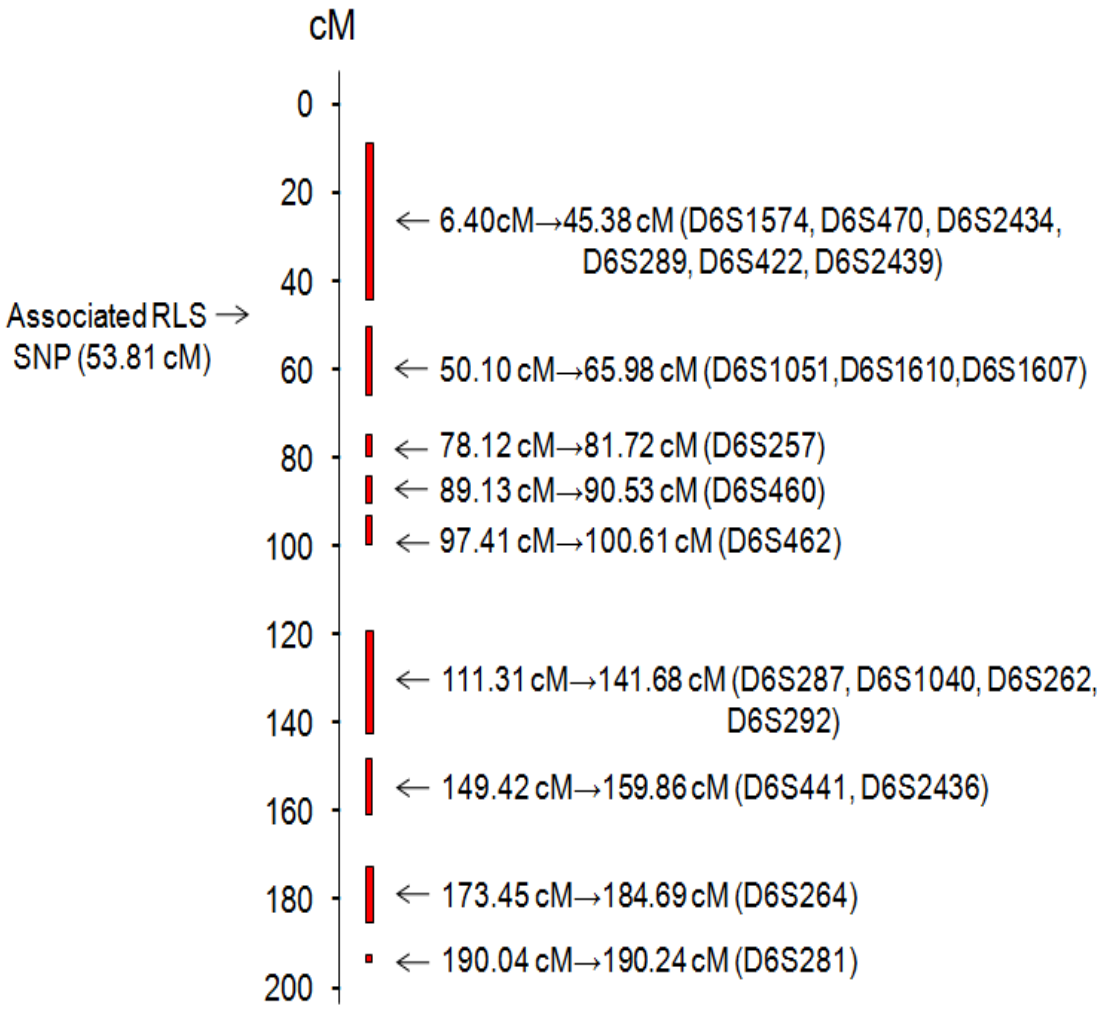


Figure 4.7: Illustration of areas of exclusion for Chromosome 6
Black vertical line with scales represents chromosome 6 (scales in cM). The red bars on the right illustrates the total areas excluded combining all markers from the two linkage set. The marker(s) which contributed to these exclusion values are also indicated.

These results exclude a large area of chromosome 6. A number of gaps remain on chromosome 6, the largest gap of 13.59 cM present between 159.86 cM and 173.45 cM. A total of 115.31 cM of chromosome 6 was excluded, which equates to 59.7% of the entire chromosome. The highest LOD score seen for this chromosome was for D6S308 at 144.46 cM, which presented with a LOD score of 0.35 at θ=0.00. This LOD score was not high enough to require further investigation.

4.3.7 Chromosome 7

The Research Genetics Inc. mapping set contained eight markers for chromosome 7, five of which were tetranucleotide repeats. The Applied Biosystems mapping set contained 22 markers for chromosome 7. Table 4.10 lists the markers that were examined from both mapping sets, the LOD scores obtained at θ=0.00 and the corresponding excluded regions. Markers highlighted in blue represent markers from the Research Genetics Inc. mapping set. Markers highlighted in pink represent markers from the Applied Biosystems mapping set. Markers highlighted in green represent markers which were found in both mapping sets.

Table 4.10: Markers used to examine chromosome 7.

Marker	Position (cM)	Max LOD (θ=0)	Excluded (cM)	Area Excluded from (cM)	Area Excluded to (cM)
D7S517	7.44	-5.61	1.20	6.24	8.64
D7S513	17.74	-5.59	7.54	10.20	25.28
D7S3051	29.28	-3.47	0.50	28.78	29.78
D7S493	34.69	-8.38	3.81	30.88	38.50
D7S516	41.69	-1.79	0.00	-	-
D7S484	53.70	-1.92	0.00	-	-
D7S2846	57.59	-3.31	1.10	56.49	58.69
D7S519	69.03	-5.77	7.56	61.47	76.59
D7S3046	78.65	-2.69	0.10	78.55	78.75
D7S669	90.42	-3.51	2.90	87.52	93.32
D7S821	109.12	-5.61	11.30	97.82	120.42
D7S530	134.55	-6.41	6.74	127.81	141.29
D7S1804	136.95	-1.31	0.00	-	-
D7S640	137.83	-7.21	8.07	129.76	145.90
D7S684	147.22	-5.43	4.71	142.51	151.93
D7S661	155.10	-8.01	9.31	145.79	164.41
D7S1823/ D7S3058	173.71	-5.35	0.40	173.31	174.11

Seventeen markers from the two mapping sets were examined for chromosome 7. Fourteen of the markers from the mapping sets obtained a statistically significant LOD score, and excluded various regions on the chromosome. Markers D7S516, D7S484, and D7S1804 did not show significant LOD scores, and produced inconclusive LOD scores of -1.79 at θ=0.00, -1.92 at θ=0.00 and -1.31 at θ=0.00 respectively. Figure 4.7 illustrates the positions of the markers that obtained significant negative LOD scores and the corresponding regions of exclusion on chromosome 7.

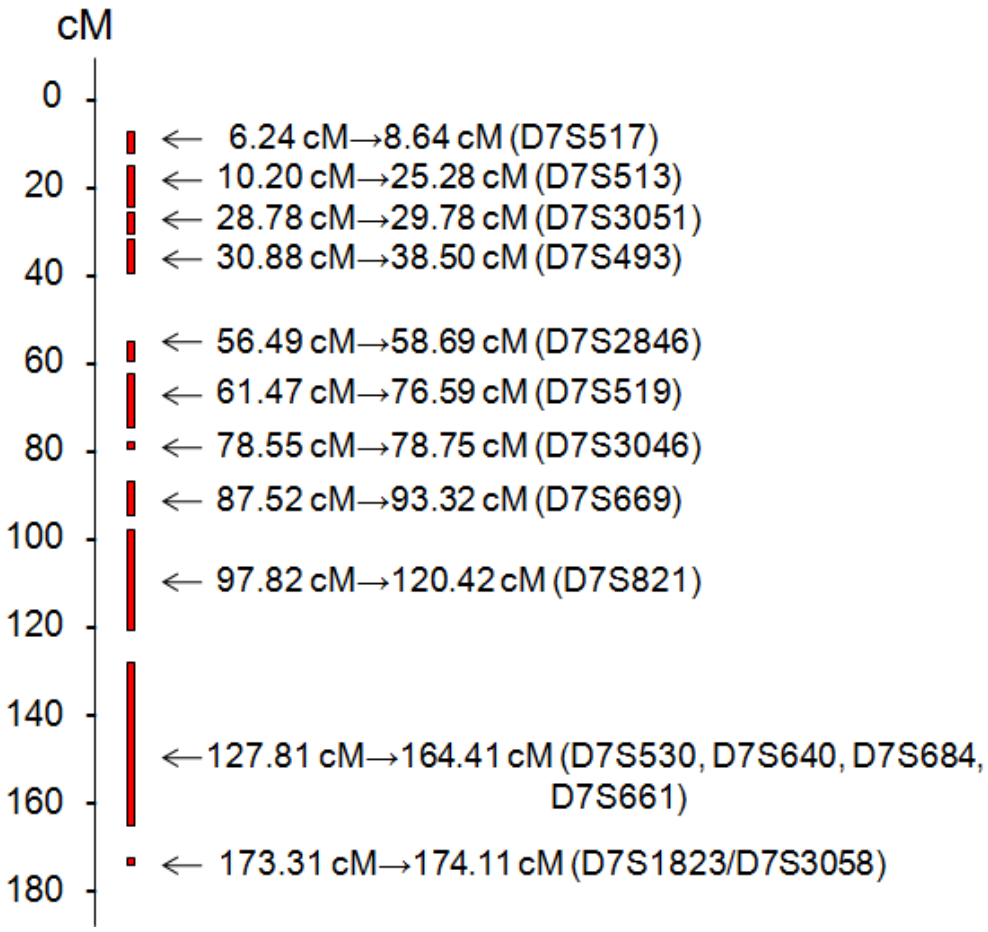


Figure 4.8: Illustration of areas of exclusion for Chromosome 7
Black vertical line with scales represents chromosome 7 (scales in cM). A number of gaps remain on chromosome 7, the largest which is seen between 38.50 cM and 56.49 cM. The red bars on the right illustrates the total areas excluded combining all markers from the two linkage set. The marker(s) which contributed to these exclusion values are also indicated.

These results of the genome-wide scan exclude a large area of chromosome 7. A total of 109.42 cM of chromosome 7 was excluded, which equals 60.1% of the entire chromosome. There were three markers which resulted in an inconclusive LOD score none of which were high enough to require further investigation and the marker(s) which contributed to these exclusion values.

4.3.8 Chromosome 8

The Research Genetics Inc. mapping set contained ten markers for chromosome 8, five of which were tetranucleotide repeats. The Applied Biosystems mapping set contained 14 markers for chromosome 8. Table 4.11 lists the markers that were examined from both mapping sets, the LOD scores obtained at θ=0.00 and the corresponding excluded regions. Markers highlighted in blue represent markers from the Research Genetics Inc. mapping set. Markers highlighted in pink represent markers from the Applied Biosystems mapping set. Markers highlighted in green represent markers which were found in both mapping sets.

Table 4.11: Markers used to examine chromosome 8.

Marker	Position (cM)	Max LOD (θ=0)	Excluded (cM)	Area Excluded from (cM)	Area Excluded to (cM)
D8S264	0.73	-0.25	0.00	-	-
D8S1130	22.41	-3.24	5.83	16.58	28.24
D8S549	31.73	-4.37	5.42	26.31	37.15
D8S258	41.55	-6.19	4.51	37.04	46.06
D8S136	43.96	0.58	0.00	-	-
D8S1771	50.05	-6.62	5.93	44.12	55.98
D8S505	60.87	-4.67	5.42	55.45	66.29
D8S532	64.75	-8.19	10.35	54.40	75.10
D8S285	71.00	-5.69	2.80	68.20	73.80
D8S260	79.36	-4.57	7.25	72.11	86.61
D8S2324	94.08	-5.91	11.08	83.00	105.16
GAAT1A4	110.20	0.33	0.00	-	-
D8S1784	118.15	0.37	0.00	-	-
D8S514	130.00	-5.95	0.40	129.6	130.4
D8S1179	135.08	-5.89	3.81	131.27	138.89
D8S284	143.82	-6.03	3.61	140.21	147.43
D8S256	148.12	-0.02	0.00	-	-
D8S272	154.02	-6.21	4.71	149.31	158.73

Eighteen markers from the two mapping sets were examined for chromosome 8. Two of these markers were found in both mapping sets, D8S185 and D8S260. Thirteen of the markers from the mapping sets obtained a statistically significant LOD score, and excluded various regions on the chromosome. Markers D8S264, D8S136, GAAT1A4, D8S1784 and D8S256 did not show significant LOD scores, and produced inconclusive LOD scores of -0.25 at θ=0.00, 0.58 at θ=0.00, 0.33 at θ=0.00, 0.37 at θ=0.00 and -0.02 at θ=0.00 respectively. Figure 4.8 illustrates the positions of the markers that obtained significant negative LOD scores and the corresponding regions of exclusion on chromosome 8.

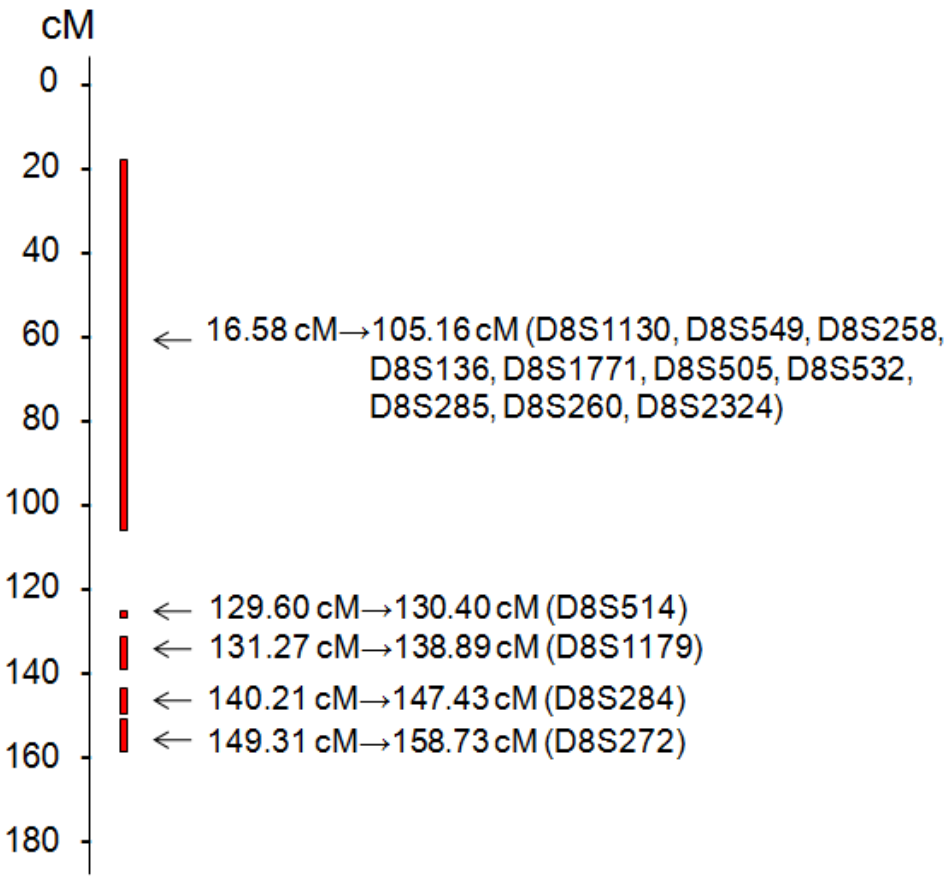


Figure 4.9: Illustration of areas of exclusion for Chromosome 8
Black vertical line with scales represents chromosome 8 (scales in cM). The red bars on the right illustrates the total areas excluded combining all markers from the two linkage set. The marker(s) which contributed to these exclusion values are also indicated.

The results of the genome-wide scan exclude a substantial area of chromosome 8. The largest gap which remains is between 105.16 cM and 129.60 cM. A large area of exclusion was observed between 16.58 cM and 105.16 cM. A total of 113.62 cM of chromosome 8 was excluded, which equates to 67.7% of the entire chromosome. The highest LOD score seen for this chromosome was for D8S136 at 43.96 cM, which presented with a LOD score of 0.58 at θ=0.00. This LOD score was not high enough to deserve further investigation and the marker(s) which contributed to these exclusion values.

4.3.9 Chromosome 9

The Research Genetics Inc. mapping set contained seven markers for chromosome 9, five of which were tetranucleotide repeats, one a trinucleotide, and the remaining two markers were dinucleotide. The Applied Biosystems mapping set contained 20 markers for chromosome nine. Table 4.12 lists the markers that were examined from both mapping sets, the LOD scores obtained at θ=0.00 and the corresponding excluded regions. Markers highlighted in blue represent markers from the Research Genetics Inc. mapping set. Markers highlighted in pink represent markers from the Applied Biosystems mapping set. Markers which have not been highlighted were ordered previously for investigation of linkage to the chromosome 9 locus (section 3.3.3).

Table 4.12: Markers used to examine chromosome 9.

Marker	Position (cM)	Max LOD (θ=0)	Excluded (cM)	Area Excluded from (cM)	Area Excluded to (cM)
D9S917	0	-0.11	0.00	-	-
D9S1779	0	-2.20	0.00	-	-
D9S1871	8.37	0.43	0.00	-	-
D9S288	9.83	-8.35	7.45	2.38	17.28
D9S1813	9.83	-5.81	6.33	3.50	16.16
D9S1810	12.78	-3.39	4.90	7.88	17.68
D9S286	18.06	-3.23	3.30	14.76	21.36
D9S274	28.42	-5.61	8.89	19.53	37.31
D9S285	29.52	-8.37	9.72	19.80	39.24
D9S157	32.24	-8.37	12.24	20.00	44.48
D9S162	34.42	-3.32	5.02	29.40	39.44
D9S171	42.73	-3.35	4.31	38.42	47.04
D9S1679	44.28	-7.66	9.72	34.56	54.00
D9S1121	44.28	-0.05	0.00	-	-
D9S169	49.20	-7.65	6.94	42.26	56.14
D9S161	51.87	-5.72	6.94	44.93	58.81
D9S1118	58.26	-5.31	2.70	55.56	60.96
D9S273	65.79	-2.06	0.70	65.09	66.49
D9S175	70.33	-5.31	2.70	67.63	73.03
D9S1122	75.88	0.32	0.00	-	-
D9S167	83.41	-4.98	9.51	73.90	92.92
D9S257	91.87	0.00	0.00	-	-
D9S287	103.42	-8.37	9.82	93.60	113.24
D9S1690	106.63	-7.79	6.23	100.40	112.60
D9S1677	117.37	-5.42	0.60	116.77	117.97
D9S1776	123.33	-5.43	4.21	119.12	127.54
D9S1682	132.09	-1.77	0.00	-	-
D9S282	136.47	-1.79	0.00	-	-
D9S290	140.86	-3.49	2.80	138.06	143.66
D9S1826	159.61	-5.84	0.20	159.41	159.81

Twenty-one markers from the two mapping sets were examined for chromosome 9. Nine additional markers, which have been included in table 4.12, had been ordered previously for investigation to the chromosome 9 locus. Sixteen of the markers from

the mapping sets obtained a statistically significant LOD score, and excluded various regions on the chromosome. Markers D9S1121, D9S1122, D9S257, D9S1682 and D9S282 did not show significant LOD scores, and yielded inconclusive LOD scores of -0.05 at θ=0.00, 0.32 at θ=0.00, 0.00 at θ=0.00, -1.77 at θ=0.00 and -1.79 at θ=0.00 respectively. Figure 4.9 illustrates the positions of the markers that obtained significant negative LOD scores and the corresponding regions of exclusion on chromosome 9.

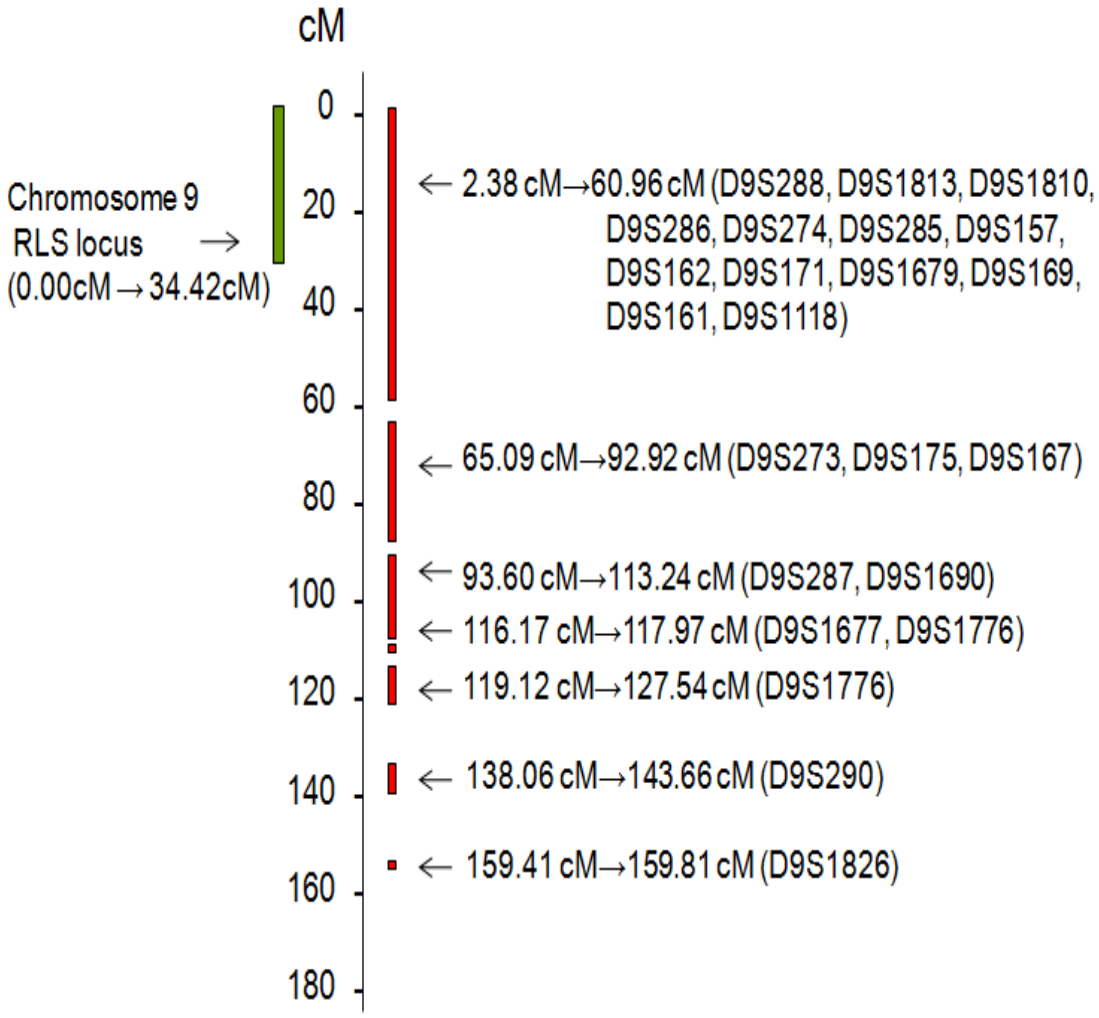


Figure 4.10: Illustration of areas of exclusion for Chromosome 9
Black vertical line with scales represents chromosome 9 (scales in cM). The red bars on the right illustrates the total areas excluded combining all markers from the two linkage set. The marker(s) which contributed to these exclusion values are also indicated.

These results exclude a large area of chromosome 9. A number of gaps remain on chromosome 9, the largest gap of 15.75 cM present between 143.66 cM and 159.41 cM. A total of 122.27 cM of chromosome 9 was excluded, which equates to 72.4% of the entire chromosome. The highest LOD score seen for this chromosome was for D9S1122 at 75.88cM, which presented with a LOD score of 0.32 at θ=0.00. This LOD score was not high enough to warrant further investigation.

4.3.10 Chromosome 10

The Research Genetics Inc. mapping set contained eight markers for chromosome 10, four of which were tetranucleotide repeats, two were trinucleotide repeats, and the remaining two markers were dinucleotide. The Applied Biosystems mapping set contained 19 markers for chromosome 10. Table 4.13 lists the markers that were examined from both mapping sets, the LOD scores obtained at θ=0.00 and the corresponding excluded regions. Markers highlighted in blue represent markers from the Research Genetics Inc. mapping set. Markers highlighted in pink represent markers from the Applied Biosystems mapping set. Markers highlighted in green represent markers which were found in both mapping sets.

Table 4.13: Markers used to examine chromosome 10.

Marker	Position (cM)	Max LOD (θ=0)	Excluded (cM)	Area Excluded from (cM)	Area Excluded to (cM)
D10S249	2.13	-5.39	0.20	1.93	2.33
D10S189	19.00	-5.61	11.18	7.82	30.18
D10S547	29.15	-5.56	6.54	22.61	35.69
D10S1653	40.36	-5.84	0.30	40.06	40.66
D10S548	45.70	1.71	0.00	-	-
D10S1423	46.23	-5.67	6.84	39.39	53.07
D10S197	52.10	-7.18	3.81	48.29	55.91
D10S1208	63.30	0.45	0.00	-	-
D10S196	70.23	-6.82	6.84	63.39	77.07
GATA121A08	88.41	-3.11	4.31	84.10	92.72
D10S537	91.13	-5.92	7.90	83.23	99.03
D10S2327	100.92	-2.86	1.70	99.22	102.62
D10S1686	105.04	-7.71	6.23	98.81	111.27
D10S185	116.34	-6.65	2.50	113.84	118.84
D10S192	124.27	0.24	0.00	-	-
D10S1239	125.41	-2.61	6.13	119.28	131.54
D10S597	128.73	-5.98	1.73	127	130.46
D10S1693	137.39	-5.57	4.21	133.18	141.6
D10S1230	142.78	-5.86	5.83	136.95	148.61
D10S587	147.57	-5.97	8.69	138.88	156.26
D10S217	157.89	-5.71	0.20	157.69	158.09
D10S1651	168.77	-3.94	2.40	166.37	171.17
D10S212	170.94	-11.34	14.71	156.23	185.65

Twenty-three markers from the two mapping sets were examined for chromosome 10. Twenty of the markers from the mapping sets obtained a statistically significant LOD score, and excluded various regions on the chromosome. Markers D10S548, D10S1208 and D10S192 did not show significant LOD scores, and yielded inconclusive LOD scores of 1.71 at θ=0.00, 0.48 at θ=0.00 and 0.24 at θ=0.00 respectively. Figure 4.10 illustrates the positions of the markers that obtained

significant negative LOD scores and the corresponding regions of exclusion on chromosome 10.

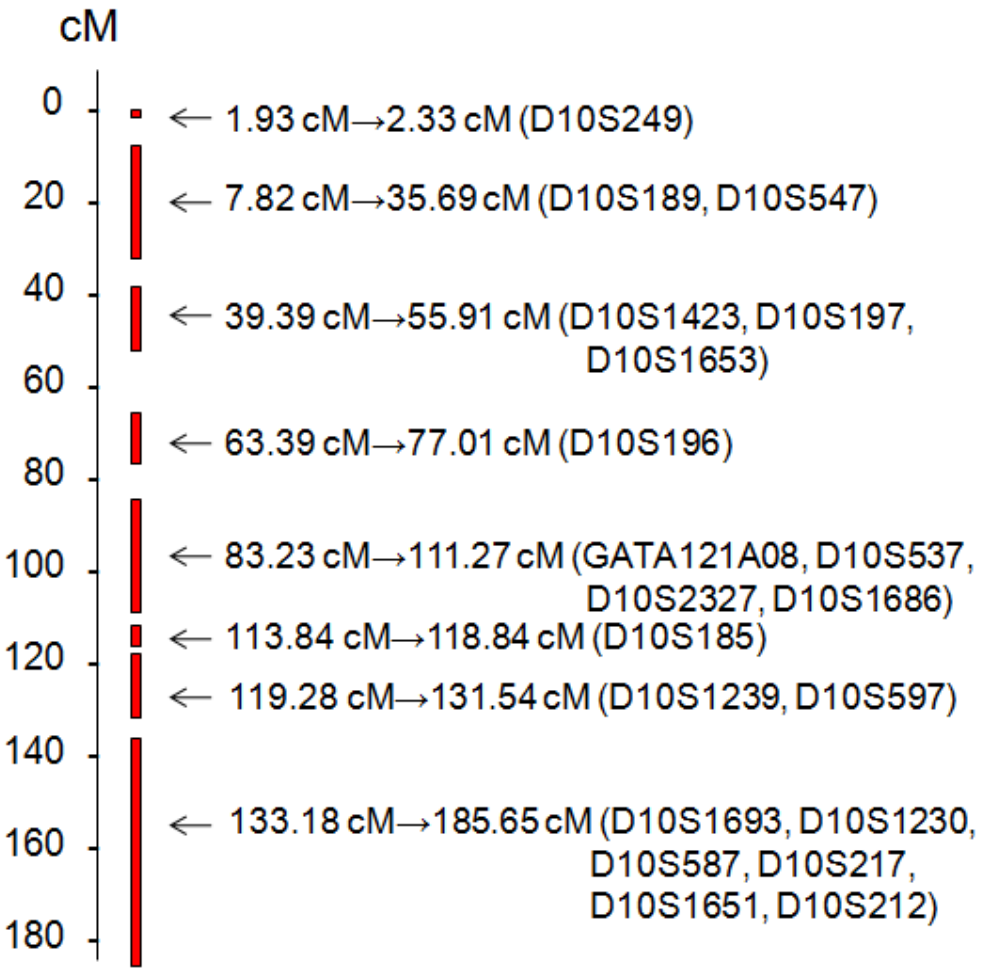


Figure 4.11: Illustration of areas of exclusion for Chromosome 10
Black vertical line with scales represents chromosome 10 (scales in cM). The red bars on the right illustrates the total areas excluded combining all markers from the two linkage set. The marker(s) which contributed to these exclusion values are also indicated.

These results exclude a substantial area of chromosome 10. A number of small gaps remain on chromosome 10, the largest gap only 7.48 cM, located between 55.91 cM and 63.39 cM. A total of 143.66 cM of chromosome 10 was excluded, which equates to 83% of the entire chromosome. The highest LOD score seen for this chromosome was for D10S548 at 45.70 cM, which presented with a LOD score of 1.71 at θ=0.00. This LOD score required further investigation (section 4.4.2).

4.3.11 Chromosome 11

The Research Genetics Inc. mapping set contained seven markers for chromosome 11, five of which were tetranucleotide repeats, one a trinucleotide, and the remaining two markers were dinucleotide. The Applied Biosystems mapping set contained 20 markers for chromosome 11. Table 4.14 lists the markers that were examined from both mapping sets, the LOD scores obtained at θ=0.00 and the corresponding excluded regions. Markers highlighted in blue represent markers from the Research Genetics Inc. mapping set. Markers highlighted in pink represent markers from the Applied Biosystems mapping set. Markers highlighted in green represent markers which were found in both mapping sets.

Table 4.14: Markers used to examine chromosome 11.

Marker	Position (cM)	Max LOD (θ=0)	Excluded (cM)	Area Excluded from (cM)	Area Excluded to (cM)
D11S4046	2.79	-6.22	8.48	0.00	11.27
D11S1338	12.92	-5.57	5.22	7.70	18.14
D11S902	21.47	-4.29	0.10	21.37	21.57
D11S1981	21.47	-1.46	0.00	-	-
D11S904	33.57	-7.08	3.41	30.16	36.98
D11S935	45.94	-5.54	5.11	40.83	51.05
D11S905	51.95	-1.77	0.00	-	-
D11S4191	60.09	-8.06	4.81	55.28	64.90
D11S987	67.48	-7.66	2.50	64.98	69.98
D11S1314	73.64	-2.06	0.00	-	-
D11S2371	76.13	-2.48	0.00	-	-
D11S937	79.98	-4.89	0.60	79.38	80.58
D11S901	85.48	-2.05	0.00	-	-
D11S4175	91.47	-6.56	0.60	90.87	92.07
D11S898	98.98	-5.83	3.10	95.88	102.08
D11S2000	100.62	-3.51	4.41	96.21	105.03
D11S4090	105.74	-5.42	2.90	102.84	108.64
D11S908	108.59	-5.31	5.62	102.97	114.21
D11S1998	113.13	-2.05	0.00	-	-
D11S925	118.47	-4.29	5.83	112.64	124.30
D11S912	131.26	-5.74	8.38	122.88	139.64
D11S1320	141.91	-4.42	3.61	138.30	145.52
D11S968	147.77	0.44	0.00	-	-

Twenty-three markers from the two mapping sets were examined for chromosome 11. Sixteen of the markers from the mapping sets obtained a statistically significant LOD score, and excluded various regions on the chromosome. Markers D11S1314, D11S2371, D11S901 and D11S1998 showed significant LOD scores of <-2, however they were only significant at θ=0.00. Therefore there was no distance of exclusion aside from the position of the marker itself. Markers D11S1981, D11S905 and D11S968 did not show significant LOD scores, and yielded inconclusive LOD

scores of -1.46 at θ=0.00, -1.77 at θ=0.00 and 0.44 at θ=0.00 respectively. Figure 4.11 illustrates the positions of the markers that obtained significant negative LOD scores and the corresponding regions of exclusion on chromosome 11.

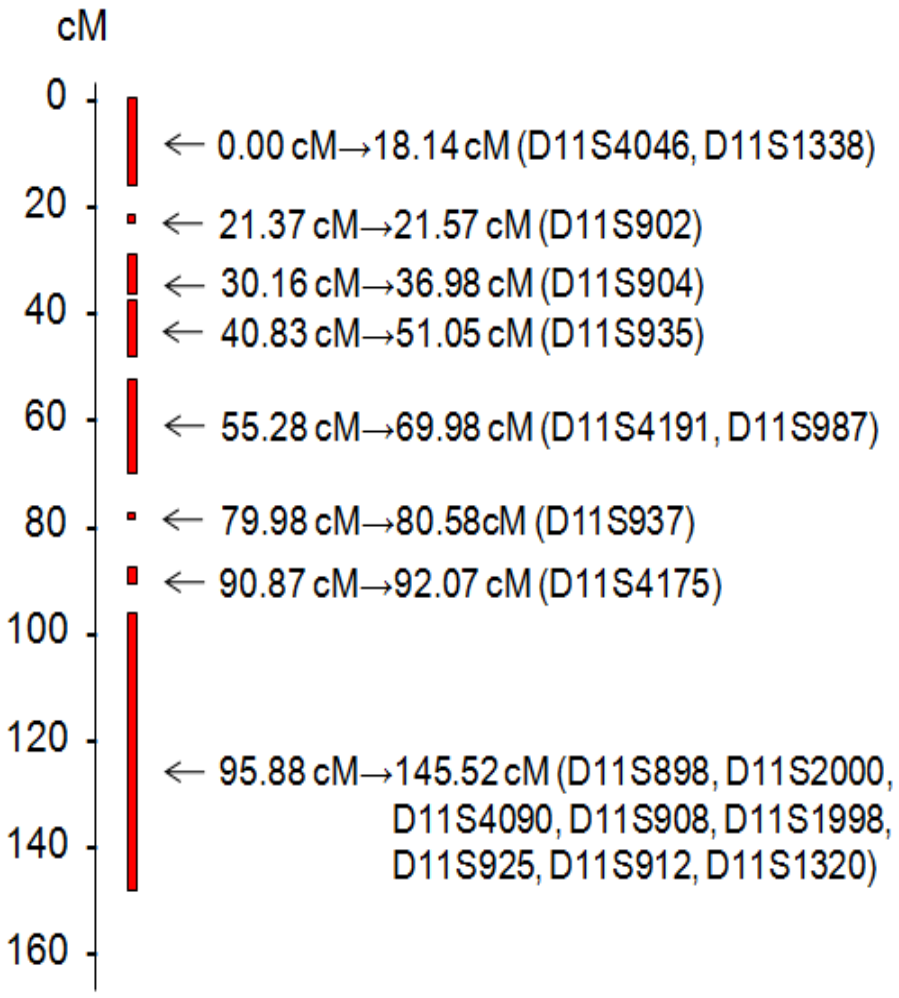


Figure 4.12: Illustration of areas of exclusion for Chromosome 11
Black vertical line with scales represents chromosome eleven (scales in cM). The red bars on the right illustrates the total areas excluded combining all markers from the two linkage set. The marker(s) which contributed to these exclusion values are also indicated.

These results exclude a considerable area of chromosome 11. A number of gaps remain on chromosome 11, the largest gap being 10.29 cM, located between 80.58 cM and 90.87 cM. A total of 101.52 cM of chromosome 11 was excluded, which equates to 68.7% of the entire chromosome. The highest LOD score seen for this chromosome was for marker D11S968 located at 147.77 cM, which presented with a LOD score of 0.44 at θ=0.00. This LOD score is inconclusive and is not high enough to require further investigation.

4.3.12 Chromosome 12

The Research Genetics Inc. mapping set contained four markers for chromosome 12, three of which were tetranucleotide repeats, and the remaining marker a trinucleotide repeat. The Applied Biosystems mapping set contained 19 markers for chromosome 12. Table 4.15 lists the markers that were examined from both mapping sets, the LOD scores obtained at θ=0.00 and the corresponding excluded regions. Markers highlighted in blue represent markers from the Research Genetics Inc. mapping set. Markers highlighted in pink represent markers from the Applied Biosystems mapping set. Markers which have not been highlighted were ordered previously for investigation of linkage to the chromosome 12 locus (section 3.3.2) and the *NOS1* associated region (section 3.3.14).

Table 4.15: Markers used to examine chromosome 12.

Marker	Position (cM)	Max LOD (θ=0)	Excluded (cM)	Area Excluded from (cM)	Area Excluded to (cM)
D12S352	0.00	-1.38	0.00	-	-
D12S99	12.60	-1.75	0.00	-	-
D12S1617	44.03	-5.95	3.81	40.22	47.84
D12S345	53.09	-6.33	2.60	50.49	55.69
D12S368	66.03	-5.94	7.35	58.68	73.38
D12S398	68.16	-5.64	5.02	63.14	73.18
D12S83	75.17	-7.07	14.6	60.57	89.77
D12S326	86.40	-6.75	3.20	83.20	89.60
D12S1064	95.03	-3.22	1.20	93.83	96.23
D12S351	95.56	-4.51	9.72	85.84	105.28
D12S1044	103.90	-6.10	4.90	99.0 0	108.8
D12S1300	104.12	-2.82	2.20	101.92	106.32
D12S346	104.65	-10.91	11.82	92.83	116.47
D12S318	109.40	-2.80	0.90	108.5 0	110.3
D12S78	111.80	-6.65	6.20	105.67	118.07
D12S2070	125.30	0.24	0.00	-	-
D12S79	125.31	-4.51	5.32	119.99	130.63
D12S2082	130.94	-7.58	2.20	128.74	133.14
D12S366	133.33	-6.63	2.20	131.13	135.53
D13S86	134.54	-11.22	9.93	124.61	144.47
D12S324	149.17	-8.67	6.54	142.63	155.71
D12S2078	149.60	-8.56	7.45	142.15	157.05
D12S1659	155.94	-7.76	5.93	150.01	161.87
D12S1723	164.63	-3.60	3.10	161.53	167.73

Nineteen markers from the two mapping sets were examined for chromosome 12. Five markers had been ordered and investigated previously for investigation of the chromosome 12 locus and associated region. These markers are included in the table above. Sixteen of the markers from the mapping sets obtained a statistically significant LOD score, and excluded various regions on the chromosome. Markers D12S352, D12S99 and D12S2070 did not show significant LOD scores, and yielded inconclusive LOD scores of -1.38 at θ=0.00, -1.75 at θ=0.00 and 0.24 at θ=0.00

respectively. Figure 4.12 illustrates the positions of the markers that obtained significant negative LOD scores and the corresponding regions of exclusion on chromosome 12.

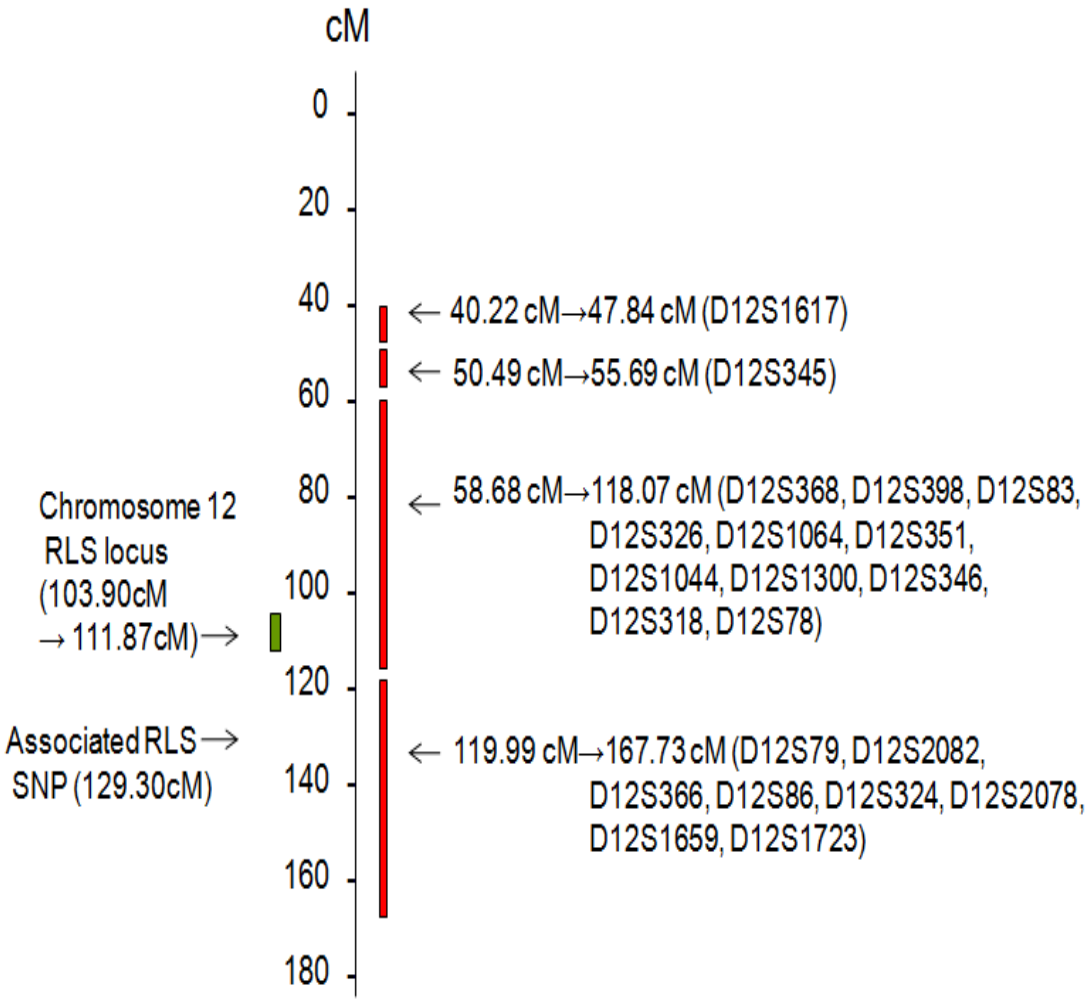


Figure 4.13: Illustration of areas of exclusion for Chromosome 12
Black vertical line with scales represents chromosome 12 (scales in cM). The red bars on the right illustrates the total areas excluded combining all markers from the two linkage set. The marker(s) which contributed to these exclusion values are also indicated.

These results exclude a considerable area of chromosome 12. A number of gaps remain on chromosome 12, the most substantial being that at the telomeric end of chromosome 12 where a gap of 40.22 cM remains between 0.00 cM and 40.22 cM. A total of 119.95 cM of chromosome ten was excluded, which equates to 71.1% of the entire chromosome. The highest LOD score seen for this chromosome was for marker D12S2070 located at 125.30 cM, which presented with a LOD score of 0.24 at θ=0.00. This LOD score is inconclusive and is not high enough to require further investigation.

4.3.13 Chromosome 13

The Research Genetics Inc. mapping set contained four markers for chromosome 13, three of which were tetranucleotide repeats and the remaining one a trinucleotide repeat. The Applied Biosystems mapping set contained 14 markers for chromosome 13. Table 4.16 lists the markers that were examined from both mapping sets, their positions (cM), the LOD scores obtained at θ=0.00, the corresponding maximum excluded distances in cM, and the regions excluded. Markers highlighted in blue represent markers from the Research Genetics Inc. mapping set. Markers highlighted in pink represent markers from the Applied Biosystems mapping set.

Table 4.16: Markers used to examine chromosome 13.

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from (cM)**	**Area Excluded to (cM)**
D13S175	6.03	-7.03	5.52	0.51	11.55
D13S217	17.21	-5.38	10.40	6.81	27.61
D13S171	25.08	0.38	0.00	-	-
D13S1493	25.80	-2.13	0.00	-	-
D13S218	32.90	0.00	0.00	-	-
D13S263	38.82	-4.73	0.10	38.72	38.92
D13S153	45.55	-8.13	2.90	42.65	48.45
D13S800	55.31	-5.52	0.60	54.71	55.91
D13S156	55.85	-3.22	5.52	50.33	61.37
D13S170	63.90	-10.16	9.62	54.28	73.52
D13S793	76.26	-5.74	4.01	72.25	80.27
D13S779	82.93	-5.52	4.61	78.32	87.54
D13S173	93.52	-5.51	7.76	85.76	101.28
D13S1265	98.82	-7.09	10.24	88.58	109.06

Fourteen markers from the two mapping sets were examined for chromosome 13. Eleven of the markers from the mapping sets obtained a statistically significant LOD score, and excluded various regions on the chromosome. Marker D13S1493

presented with a significant LOD score of <-2, however it was only significant at θ=0.00. Therefore there was no distance of exclusion aside from the position of the marker itself. Markers D13S171 and D13S218 did not show significant LOD scores, and yielded inconclusive LOD scores of 0.38 at θ=0.00 and 0.00 at θ=0.00 respectively. Figure 4.13 illustrates the positions of the markers that obtained significant negative LOD scores and the corresponding regions of exclusion on chromosome 13.

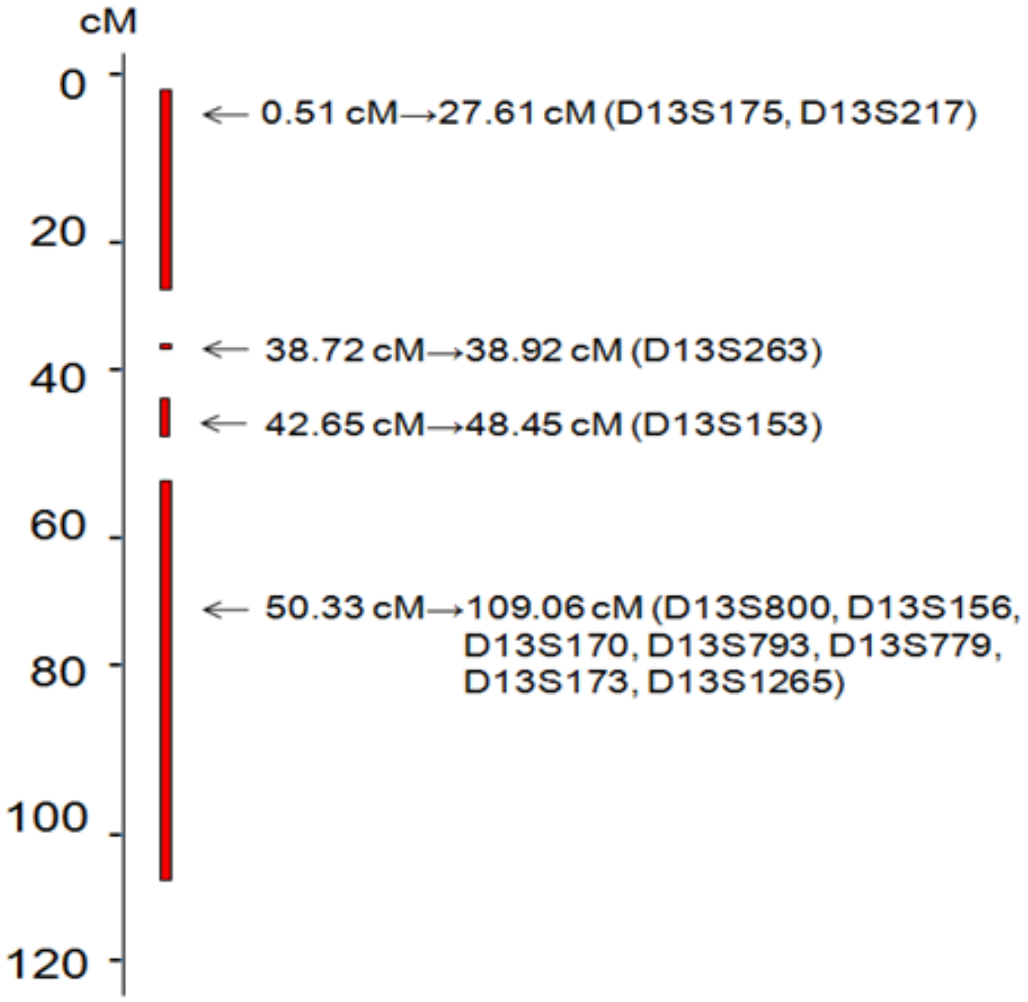


Figure 4.14: Illustration of areas of exclusion for Chromosome 13
Black vertical line with scales represents chromosome 13 (scales in cM). The red bars on the right illustrates the total areas excluded combining all markers from the two linkage set. The marker(s) which contributed to these exclusion values are also indicated.

These results exclude a considerable area of chromosome 13. A number of small gaps remain on chromosome 13, the largest being 11.11 cM located between 27.61 cM and 38.72 cM. A total of 91.83 cM of chromosome 13 was excluded, which equates to 79.9% of the entire chromosome. The highest LOD score seen for this chromosome was for marker D13S171 located at 25.08 cM, which presented with a LOD score of 0.38 at θ=0.00. This LOD score is inconclusive and is not high enough to require further investigation.

4.3.14 Chromosome 14

The Research Genetics Inc. mapping set contained eleven markers for chromosome 14, six of which were tetranucleotide repeats, two trinucleotides, and the remaining markers were dinucleotide. The Applied Biosystems mapping set contained 14 markers for chromosome 14. Table 4.17 lists the markers that were examined from both mapping sets, their positions (cM), the LOD scores obtained at θ=0.00, the corresponding maximum excluded distances in cM, and the regions excluded. Markers highlighted in blue represent markers from the Research Genetics Inc. mapping set. Markers highlighted in pink represent markers from the Applied Biosystems mapping set. Markers which have not been highlighted were ordered previously for investigation of linkage to the chromosome 14 locus (Abdulrahim 2008).

Table 4.17: Markers used to examine chromosome 14

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from (cM)**	**Area Excluded to (cM)**
D14S608	28.01	0.08	0.00	-	-
D14S283	13.89	-4.96	8.58	5.31	22.47
D14S70	40.11	-5.91	4.31	35.80	44.42
D14S599	40.68	0.39	0.00	-	-
D14S1049	40.94	-3.44	5.93	35.01	46.87
D14S1048	45.12	-3.23	1.10	44.02	46.22
D14S301	45.12	-2.95	0.10	45.02	45.22
D14S288	47.51	-3.17	0.90	46.61	48.41
D14S1013	47.51	-6.07	4.51	43.00	52.02
D14S1068	50.50	-6.14	4.61	45.89	55.11
D14S587	55.82	-1.75	0.00	-	-
D14S592	66.80	-5.43	1.90	64.9	68.7
D14S63	69.18	-11.33	12.88	56.3	82.06
D14S588	75.61	-8.57	7.76	67.85	83.37
D14S258	76.28	-7.96	10.55	65.73	86.83
D14S53	86.29	-2.92	1.20	85.09	87.49
D14S1279	95.89	-5.61	1.70	94.19	97.59
D14S65	117.30	-6.35	4.31	112.99	121.61
D14S985	126.61	-3.72	3.00	123.61	129.61

Fourteen markers from the two mapping sets were examined for chromosome 14. Five markers had been ordered, investigated and previously discussed for the investigation of the chromosome 14 locus. These markers are included in the table above. Eleven of the markers from the mapping sets obtained a statistically significant LOD score, and excluded various regions on the chromosome. Markers D14S608, D14S599 and D14S587 did not show significant LOD scores, and yielded inconclusive LOD scores of 0.08 at θ=0.00, 0.39 at θ=0.00 and -1.75 at θ=0.00 respectively. Figure 4.12 illustrates the positions of the markers that obtained significant negative LOD scores and the corresponding regions of exclusion on chromosome 14.

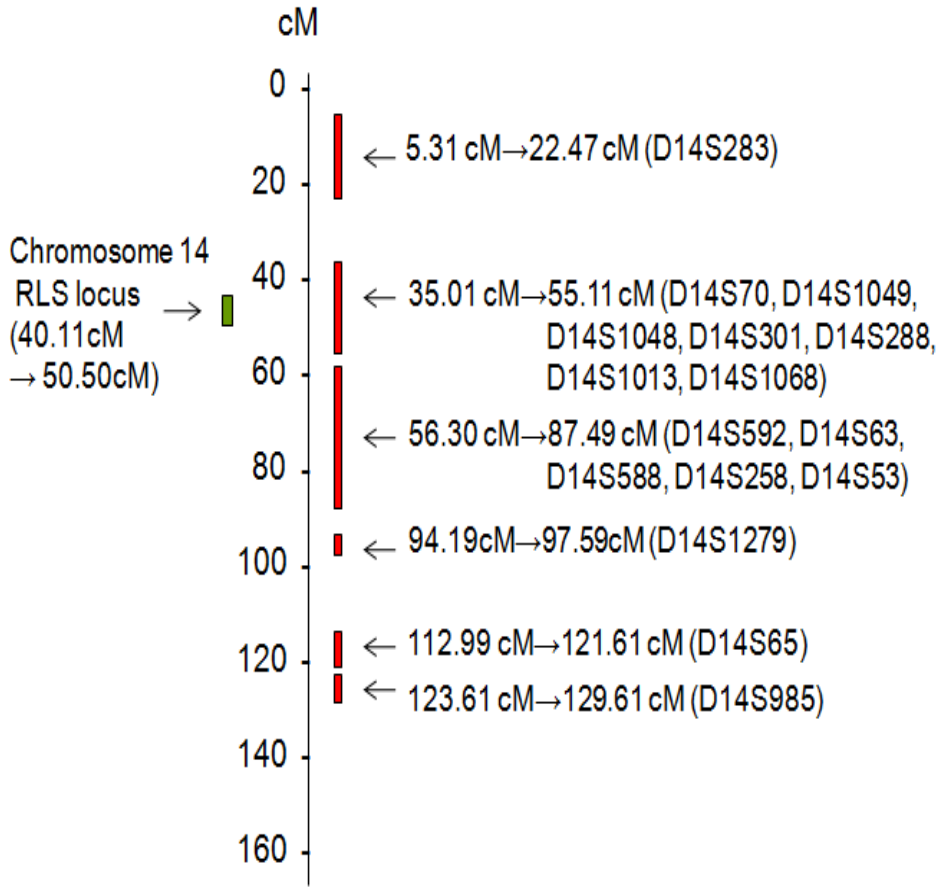


Figure 4.15: Illustration of areas of exclusion for Chromosome 14
Black vertical line with scales represents chromosome 14 (scales in cM). The red bars on the right illustrates the total areas excluded combining all markers from the two linkage set. The marker(s) which contributed to these exclusion values are also indicated.

These results exclude a substantial area of chromosome 14. A number of gaps remain on chromosome 14, the largest being 15.40 cM located between 97.59 cM and 112.99 cM. A total of 86.47 cM of chromosome 14 was excluded, which equates to 62.6% of the entire chromosome. The highest LOD score seen for this chromosome was for marker D14S599 located at 40.68 cM, which presented with a LOD score of 0.39 at θ=0.00. This LOD score is inconclusive and is not high enough to require further investigation.

4.3.15 Chromosome 15

The Research Genetics Inc. mapping set contained sixteen markers for chromosome 15; however three markers were only available at the time. Two of these markers were tetranucleotides, and the third marker a trinucleotide. The Applied Biosystems mapping set contained 13 markers for chromosome 15. Table 4.18 lists the markers that were examined from both mapping sets, their positions (cM), maximum obtained LOD scores at theta equal to zero, the corresponding maximum excluded distances in cM, and the regions excluded. Markers highlighted in blue represent markers from the Research Genetics Inc. mapping set. Markers highlighted in pink represent markers from the Applied Biosystems mapping set. The marker which has not been highlighted was ordered previously for investigation of linkage to the variants containing the genes encoding *MAP2K5* and *LBXCOR1* (section 3.3.12).

Table 4.18: Markers used to examine chromosome 15.

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from (cM)**	**Area Excluded to (cM)**
D15S128	6.11	-4.95	1.30	4.81	7.41
D15S1002	14.58	-6.21	1.40	13.18	15.98
D15S1007	25.86	-10.98	12.77	13.09	38.63
D15S994	40.25	-10.84	8.69	31.56	48.94
D15S978	45.62	-11.22	9.51	36.11	55.13
D15S117	51.21	-11.11	12.45	38.76	63.66
D15S1507	60.17	-5.59	3.00	57.17	63.17
D15S1015	66.9	-3.91	1.10	65.80	68.00
D15S153	68.1	-5.50	3.71	64.39	71.81
D15S131	71.28	-9.82	12.03	59.25	83.31
D15S211	75.85	-5.92	7.45	68.40	83.30
D15S205	78.92	-6.50	4.41	74.51	83.33
D15S127	86.81	-8.08	8.58	78.23	95.39
D15S816	100.59	-3.28	0.40	100.19	100.99
D15S130	100.59	-0.05	0.00	-	-
D15S120	112.58	-1.15	0.00	-	-

Fifteen markers from the two mapping sets were examined for chromosome 15. A separate marker had been ordered, investigated and previously discussed for the investigation of variants within chromosome 15. This marker is included in the table above. Thirteen of the markers from the mapping sets obtained a statistically significant LOD score, and excluded various regions on the chromosome. Markers D15S130 and D15S120 did not show significant LOD scores, and yielded inconclusive LOD scores of -0.05 at θ=0.00 and -1.15 at θ=0.00 respectively. Figure 4.12 illustrates the positions of the markers that obtained significant negative LOD scores and the corresponding regions of exclusion on chromosome 15.

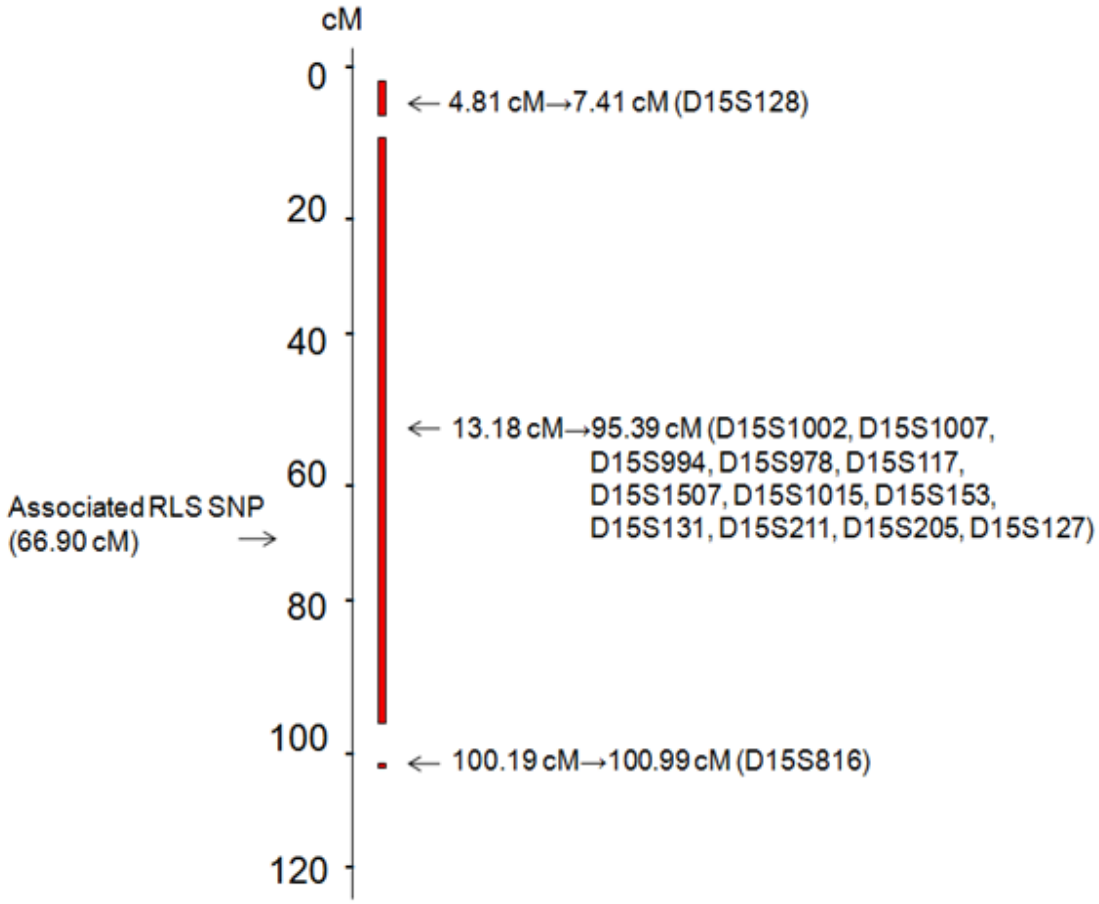


Figure 4.16: Illustration of areas of exclusion for Chromosome 15
Black vertical line with scales represents chromosome 15 (scales in cM). The red bars on the right illustrates the total areas excluded combining all markers from the two linkage set. The marker(s) which contributed to these exclusion values are also indicated.

These results exclude a large amount of chromosome 15. Only four gaps remain on chromosome 15. The largest and most significant of these gaps is seen between 100.99 cM and the telomere (122.15 cM). A substantial area of exclusion can be observed between 13.18 cM and 95.39 cM. A total of 85.61 cM of chromosome thirteen was excluded, which equals 70.1% of the entire chromosome. There was no considerably high LOD score observed for any of the markers on chromosome 15.

4.3.16 Chromosome 16

The Research Genetics Inc. mapping set contained two markers for chromosome 16, one which was a tetranucleotide repeat, and the other a trinucleotide repeat. The Applied Biosystems mapping set contained 13 markers for chromosome 16. Table 4.19 lists the markers that were examined from both mapping sets, their positions (cM), maximum obtained LOD scores at theta equal to zero, the corresponding maximum excluded distances in cM, and the regions excluded. Markers highlighted in blue represent markers from the Research Genetics Inc. mapping set. Markers highlighted in pink represent markers from the Applied Biosystems mapping set.

Table 4.19: Markers used to examine chromosome 16.

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from (cM)**	**Area Excluded to (cM)**
D16S404	18.07	-8.51	6.64	11.43	24.71
D16S3075	23.28	-6.21	1.80	21.48	25.08
D16S3046	40.65	-8.37	6.03	34.62	46.68
D16S403	43.89	-2.05	0.00	-	-
D16S3068	48.53	-8.36	10.66	37.87	59.19
D16S3136	62.11	-0.22	0.00	-	-
D16S415	67.62	-7.33	2.70	64.92	70.32
D16S3253	71.77	-0.96	0.00	-	-
D16S503	83.55	0.19	0.00	-	-
D16S515	92.1	-1.35	0.00	-	-
D16S516	100.39	-1.77	0.00	-	-
D16S3091	111.12	-5.71	2.00	109.12	113.12
D16S520	125.82	-2.98	0.60	125.22	126.42

Thirteen markers from the two mapping sets were examined for chromosome 16. Only seven of these markers from the mapping sets obtained a statistically significant LOD score, and excluded various regions on the chromosome. Marker D16S403 presented with a significant LOD scores of <-2, however it was only significant at θ=0.00. Therefore there was no distance of exclusion aside from the

position of the marker itself. Markers D16S3253, D16S503, D16S515 and D16S516 did not show significant LOD scores, and yielded inconclusive LOD scores of -0.96 at θ=0.00, 0.19 at θ=0.00, -1.35 at θ=0.00 and -1.77 at θ=0.00 respectively. Figure 4.16 illustrates the positions of the markers that obtained significant negative LOD scores and the corresponding regions of exclusion on chromosome 16.

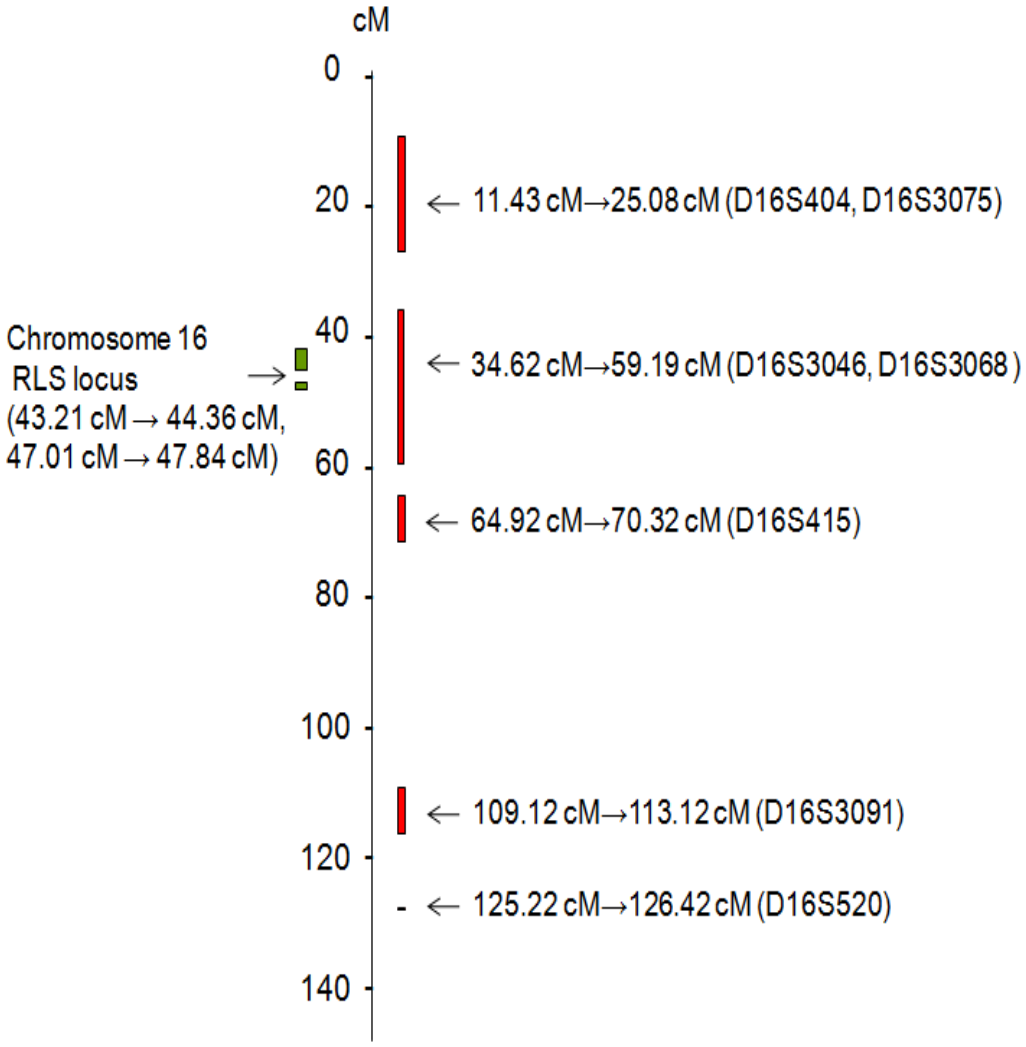


Figure 4.17: Illustration of areas of exclusion for Chromosome 16
Black vertical line with scales represents chromosome 16 (scales in cM). The red bars on the right illustrates the total areas excluded combining all markers from the two linkage set. The marker(s) which contributed to these exclusion values are also indicated.

This result for the scan of chromosome 16 was not as successful as the other chromosomes. A large number of substantial gaps remain on chromosome 16. The largest gap is 38.80 cM and located between 70.32 cM and 109.12 cM. Five of the markers obtained an inconclusive LOD score and the other markers showed quite small areas of exclusion. Therefore a large number of gaps remain to be investigated further. A total of 48.82 cM of chromosome 16 was excluded, which equals 36.4% of the entire chromosome. There was no considerably high LOD score observed for any of the markers on chromosome 16.

4.3.17 Chromosome 17

The Research Genetics Inc. mapping set contained six markers for chromosome 17, two which were tetranucleotide repeats, two which were trinucleotide repeats, and the remaining two markers were dinucleotide. The Applied Biosystems mapping set contained 15 markers for chromosome 17. Table 4.20 lists the markers that were examined from both mapping sets. Markers highlighted in blue represent markers from the Research Genetics Inc. mapping set. Markers highlighted in pink represent markers from the Applied Biosystems mapping set. Markers highlighted in green represent markers which were found in both mapping sets. The marker which has not been highlighted was ordered previously for investigation of linkage to the chromosome seventeen locus (section 3.3.9).

Table 4.20: Markers used to examine chromosome 17.

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from (cM)**	**Area Excluded to (cM)**
D17S849	0.63	-8.49	8.48	-7.85	9.11
D17S831	6.60	-5.96	5.52	1.08	12.12
D17S938	14.69	-7.93	5.42	9.27	20.11
D17S974	22.24	-5.61	6.23	16.01	28.47
D17S1852	22.24	-6.34	7.97	14.27	30.21
D17S799	31.96	-5.41	3.10	28.86	35.06
D17S921	36.14	-4.11	1.80	34.34	37.94
D17S1857	43.01	-1.51	0.00	-	-
D17S2196	44.62	-8.52	7.76	36.86	52.38
D17S1294	50.74	-6.01	6.03	44.71	56.77
D17S798	53.41	-6.50	5.52	47.89	58.93
D17S1868	64.16	-5.83	9.62	54.54	73.78
D17S2180	66.85	0.44	0.00	-	-
D17S787	74.99	-9.59	4.61	70.38	79.60
D17S944	82.56	1.36	0.00	-	-
D17S2193	89.32	-3.78	0.00	-	-
D17S949	93.27	-5.86	0.40	92.87	93.67
D17S785	103.53	-8.91	8.48	95.05	112.01
D17S784	116.86	-3.27	3.30	113.56	120.16
D17S928	126.46	-1.19	0.00	-	-

Nineteen markers from the two mapping sets were examined for chromosome 17. A separate marker had been ordered, investigated and previously discussed for the investigation of the chromosome 17 RLS locus. This marker is included in the table above. Fifteen of the markers from the mapping sets obtained a statistically significant LOD score, and excluded various regions on the chromosome. Marker D17S2193 presented with a significant LOD scores of <-2, however it was only significant at θ=0.00. Therefore there was no distance of exclusion aside from the position of the marker itself. Markers D17S1857, D17S2180, D17S944 and D17S928 did not show significant LOD scores, and yielded inconclusive LOD scores of -1.51 at θ=0.00, 0.44 at θ=0.00, 1.36 at θ=0.00 and -1.19 at θ=0.00 respectively. Figure 4.17 illustrates the positions of the markers that obtained significant negative LOD scores and the corresponding regions of exclusion on chromosome 17.

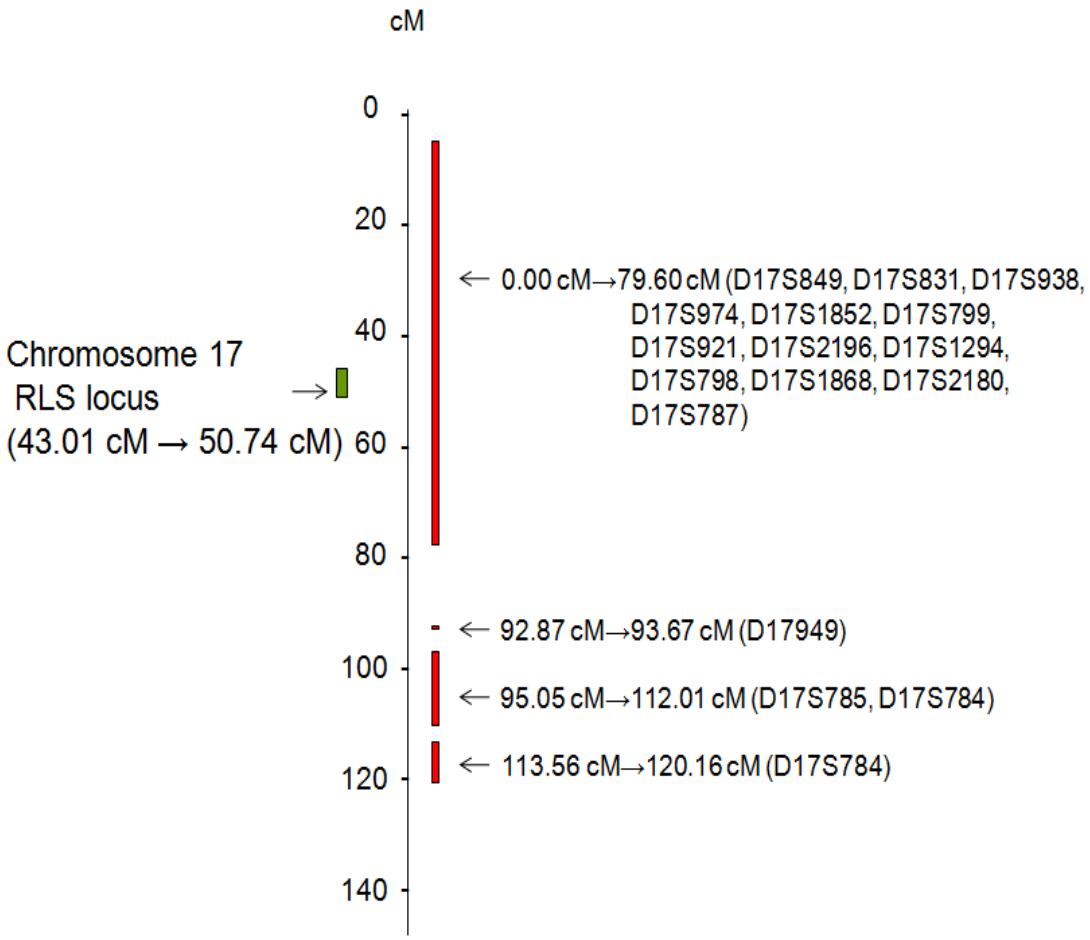


Figure 4.18: Illustration of areas of exclusion for Chromosome 17
Black vertical line with scales represents chromosome 17 (scales in cM). The red bars on the right illustrates the total areas excluded combining all markers from the two linkage set. The marker(s) which contributed to these exclusion values are also indicated.

These results exclude a large amount of chromosome 17. The largest gap which remains on chromosome 17 is located between 79.60 cM and 92.87 cM. A substantial area of exclusion can be observed between 0.00 cM and 79.60 cM. A total of 103.96 cM of chromosome seventeen was excluded, which equals 82.2% of the entire chromosome. The highest LOD score seen for this chromosome was for D17S944 at 82.56 cM, which presented with a LOD score of 1.36 at θ=0.00. Although this LOD score was less than the +3.00 required for evidence of linkage, it was one of the highest LOD scores observed, therefore it required further investigation (section 4.4.2).

4.3.18 Chromosome 18

The Research Genetics Inc. mapping set contained five markers for chromosome 18, three of which were tetranucleotide repeats, and the remaining two markers were dinucleotide. The Applied Biosystems mapping set contained 20 markers for chromosome 18. Table 4.21 lists the markers that were examined from both mapping sets, their positions (cM), maximum obtained LOD scores at theta equal to zero, the corresponding maximum excluded distances in cM, and the regions excluded. Markers highlighted in blue represent markers from the Research Genetics Inc. mapping set. Markers highlighted in pink represent markers from the Applied Biosystems mapping set. Markers highlighted in green represent markers which were found in both mapping sets. The marker which has not been highlighted was ordered previously for investigation of linkage to the chromosome 18 locus (section 3.3.9).

Table 4.21: Markers used to examine chromosome 18.

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from (cM)**	**Area Excluded to (cM)**
D18S59	0.00	-5.63	0.60	-0.60	0.60
D18S63	8.30	-5.57	1.50	6.80	9.80
D18S976	12.88	-5.81	4.11	8.77	16.99
D18S452	18.70	-2.07	0.00	-	-
D18S464	31.17	-4.81	7.45	23.72	38.62
D18S53	41.24	-1.79	0.00	-	-
D18S542	41.24	-1.82	0.00	-	-
D18S877	54.40	-1.49	0.00	-	-
D18S1102	62.84	-6.95	4.01	58.83	66.85
D18S64	84.80	-6.01	3.91	80.89	88.71
D18S1357	88.62	0.68	0.00	-	-
D18S68	96.48	-4.57	1.32	95.16	97.80
D18S61	105.03	-8.38	6.74	98.29	111.77
ATA82B04	106.81	-1.49	0.00	-	-
D18S1161	114.26	-8.60	6.94	107.32	121.20
D18S462	120.05	-8.58	10.66	109.39	130.71
D18S70	126.00	-5.79	8.27	117.73	134.27

Seventeen markers from the two mapping sets were examined for chromosome 18. Twelve of the markers from the mapping sets obtained a statistically significant LOD score, and excluded various regions on the chromosome. Markers D18S452 presented with a significant LOD scores of <-2, however it was only significant at θ=0.00. Therefore there was no distance of exclusion aside from the position of the marker itself. Marker D18S53, D18S542, D18S877, D18S1357 and ATA82B04 did not show significant LOD scores, and yielded inconclusive LOD scores of -1.79 at θ=0.00 -1.82 at θ=0.00 , -1.49 at θ=0.00, 0.68 at θ=0.00 and -1.49 at θ=0.00. Figure 4.18 illustrates the positions of the markers that obtained significant negative LOD scores and the corresponding regions of exclusion on chromosome 18.

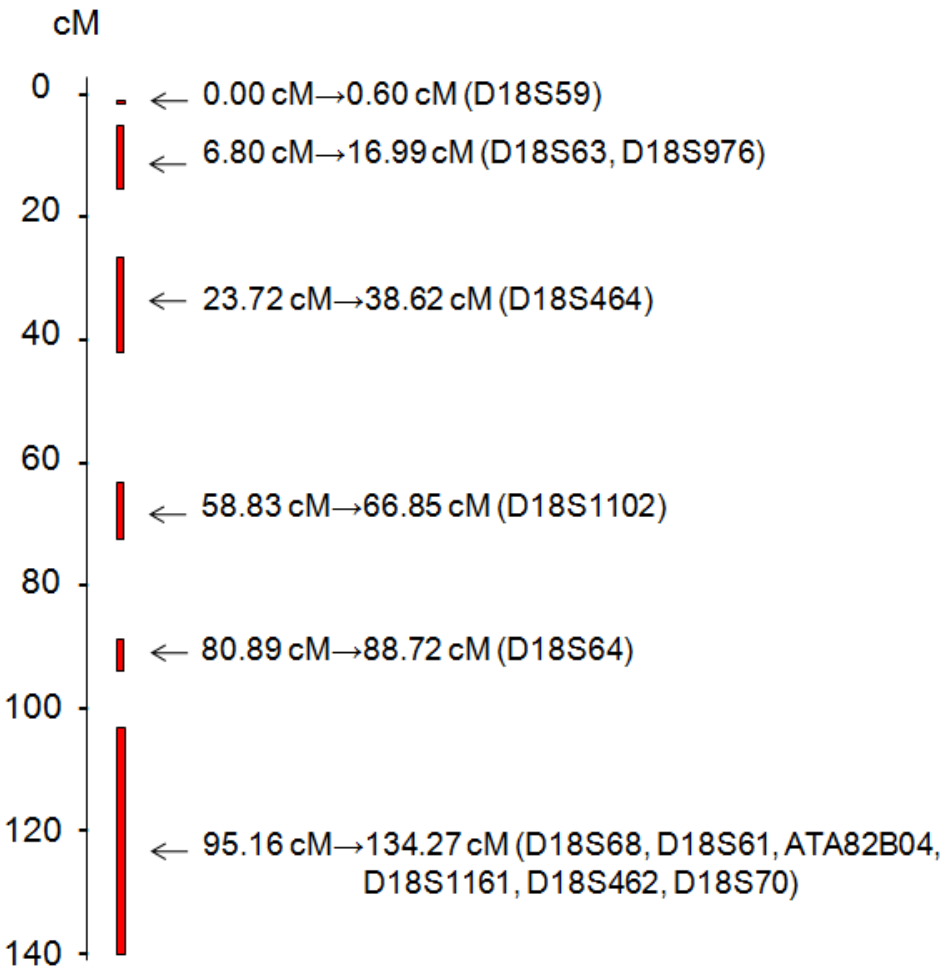


Figure 4.19: Illustration of areas of exclusion for Chromosome 18
Black vertical line with scales represents chromosome 18 (scales in cM). The red bars on the right illustrates the total areas excluded combining all markers from the two linkage set. The marker(s) which contributed to these exclusion values are also indicated.

These results exclude a substantial area of chromosome 18. A number of small gaps remain on chromosome 18, the largest gap only 7.48 cM, located between 38.62 cM and 58.83 cM. A total of 72.38 cM of chromosome 18 was excluded, which equates to 57.4% of the entire chromosome. The highest LOD score seen for this chromosome was for D18S1357 at 57.44 cM, which presented with a LOD score of 0.68 at θ=0.00. Although this LOD score was less than the +3.00 required for evidence of linkage, it was one of the highest LOD scores observed, therefore it required further investigation.

4.3.19 Chromosome 19

The Research Genetics Inc. mapping set contained seven markers for chromosome 19, five of which were tetranucleotide repeats, one a trinucleotide, and the remaining two markers were dinucleotide. The Applied Biosystems mapping set contained 20 markers for chromosome 19. Table 4.22 lists the markers that were examined from both mapping sets, their positions (cM), maximum obtained LOD scores at theta equal to zero, the corresponding maximum excluded distances in cM, and the regions excluded. Markers highlighted in blue represent markers from the Research Genetics Inc. mapping set. Markers highlighted in pink represent markers from the Applied Biosystems mapping set. The marker which has not been highlighted was ordered previously for investigation of linkage to the chromosome 19 locus (section 3.3.6).

Table 4.22: Markers used to examine chromosome 19.

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from (cM)**	**Area Excluded to (cM)**
D19S209	10.97	-0.71	0.00	-	-
D19S216	20.01	-2.62	0.00	-	-
D19S1034	20.75	-5.95	1.00	19.75	21.75
D19S884	26.37	-4.88	0.20	26.17	26.57
D19S586	32.94	-2.84	0.00	-	-
D19S221	36.22	0.48	0.00	-	-
D19S226	42.28	-7.44	1.80	40.48	44.08
D19S410	45.48	-5.03	2.40	43.08	47.88
D19S414	54.01	-5.44	8.89	45.12	62.9
D19S220	62.03	-5.68	9.10	52.93	71.13
D19S420	66.30	-8.36	14.06	52.24	80.36
D19S178	68.08	-5.59	6.64	61.44	74.72
D19S902	72.72	-9.16	7.86	64.86	80.58
D19S589	87.66	-2.56	0.40	87.26	88.06
D19S210	100.01	-6.406	6.60	93.41	106.61

Fourteen markers from the two mapping sets were examined for chromosome 19. A separate marker had been ordered, investigated and previously discussed for the

investigation of the chromosome 19 RLS locus. This marker is included in the table above. Ten of the markers from the mapping sets obtained a statistically significant LOD score, and excluded various regions on the chromosome. Markers D19S216 and D19S586 presented with a significant LOD scores of <-2; however they were only significant at θ=0.00. Therefore there was no distance of exclusion aside from the position of the marker itself. Marker D19S209 and D19S221 did not show significant LOD scores, and yielded inconclusive LOD scores of -0.71 at θ=0.00 and 0.47 at θ=0.00. Figure 4.19 illustrates the positions of the markers that obtained significant negative LOD scores and the corresponding regions of exclusion on chromosome 19.

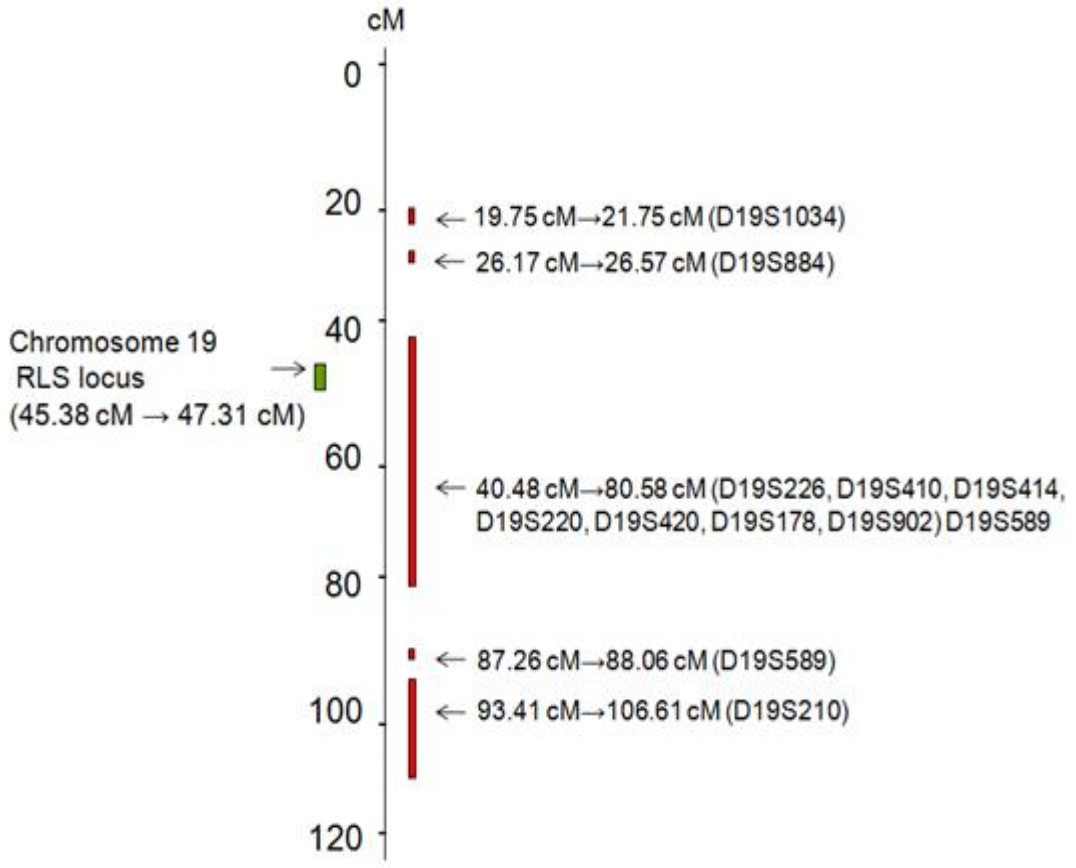


Figure 4.20: Illustration of areas of exclusion for Chromosome 19
Black vertical line with scales represents chromosome 19 (scales in cM). The red bars on the right illustrates the total areas excluded combining all markers from the two linkage set. The marker(s) which contributed to these exclusion values are also indicated.

These results exclude substantial areas on chromosome 19. A number of gaps remain on chromosome 19, the largest gap located between 0.00 cM and 19.75 cM. A total of 56.50 cM of chromosome 19 was excluded, which equates to 53.8% of the entire chromosome. The highest LOD score seen for this chromosome was for D19S209 at 10.97 cM, which presented with a LOD score of 2.01 at θ=0.67. Although this LOD score was less than the +3.00 required for evidence of linkage, it was the highest LOD score seen during the process of the genome-wide search and therefore further investigation of the region surrounding marker D19S209 was required.

4.3.20 Chromosome 20

The Research Genetics Inc. mapping set contained two markers for chromosome 20, one which was a tetranucleotide repeat, and one which was a dinucleotide repeat. The Applied Biosystems mapping set contained 20 markers for chromosome 20. Table 4.23 lists the markers that were examined from both mapping sets, their positions (cM), maximum obtained LOD scores at theta equal to zero, the corresponding maximum excluded distances in cM, and the regions excluded. Markers highlighted in blue represent markers from the Research Genetics Inc. mapping set. Markers highlighted in pink represent markers from the Applied Biosystems mapping set. The markers which have not been highlighted were ordered previously for investigation of linkage to the Chromosome 20 locus (section 3.3.5).

Table 4.23: Markers used to examine chromosome 20.

Marker	Position (cM)	Max LOD (θ=0)	Excluded (cM)	Area Excluded from (cM)	Area Excluded to (cM)
D20S864	0.00	-1.75	0.00	-	-
D20S117	2.83	-1.87	0.00	-	-
D20S199	6.25	-5.72	7.66	-1.41	13.91
D20S113	8.97	0.14	0.00	-	-
D20S842	8.97	-5.61	9.33	-0.36	18.30
D20S181	9.53	-3.02	0.20	9.33	9.73
D20S193	9.53	0.00	0.00	-	-
D20S116	11.20	-2.80	2.20	9.00	13.40
D20S889	11.20	-8.20	11.60	0.00	22.80
D20S482	12.12	-5.61	9.33	2.79	21.45
D20S849	13.98	-3.08	0.70	13.28	14.68
D20S895	13.98	0.00	0.00	-	-
D20S882	15.05	-3.28	3.10	11.95	18.15
D20S115	21.15	-6.19	4.41	16.74	25.56
D20S851	24.70	-3.12	0.30	24.4	25.00
D20S186	32.30	-8.52	10.24	22.06	42.54
D20S112	39.25	-8.63	8.07	31.18	47.32
D20S107	55.74	-5.68	7.76	47.98	63.50
D20S119	61.77	-8.53	8.17	53.6	69.94
D20S178	66.16	-6.19	4.01	62.15	70.17
D20S196	75.01	-11.33	8.07	66.94	83.08
D20S100	84.78	-2.07	0.00	-	-
D20S171	95.70	-8.97	5.62	90.08	101.32
D20S173	98.09	-2.99	1.40	96.69	99.49

Fourteen markers from the two mapping sets were examined for chromosome 20. Ten separate markers had been ordered, investigated and previously discussed for the investigation of the chromosome twenty RLS locus. These markers are included in the table above. Twelve of the markers from the mapping sets obtained a statistically significant LOD score, and excluded various regions on the chromosome. Marker D20S100 presented with a significant LOD scores of <-2, however it was only significant at θ=0.00. Therefore there was no distance of exclusion aside from the position of the marker itself. Marker D20S928 did not show significant LOD scores, and yielded an inconclusive LOD scores of -1.87 at θ=0.00. Figure 4.20 illustrates

the positions of the markers that obtained significant negative LOD scores and the corresponding regions of exclusion on chromosome 20.

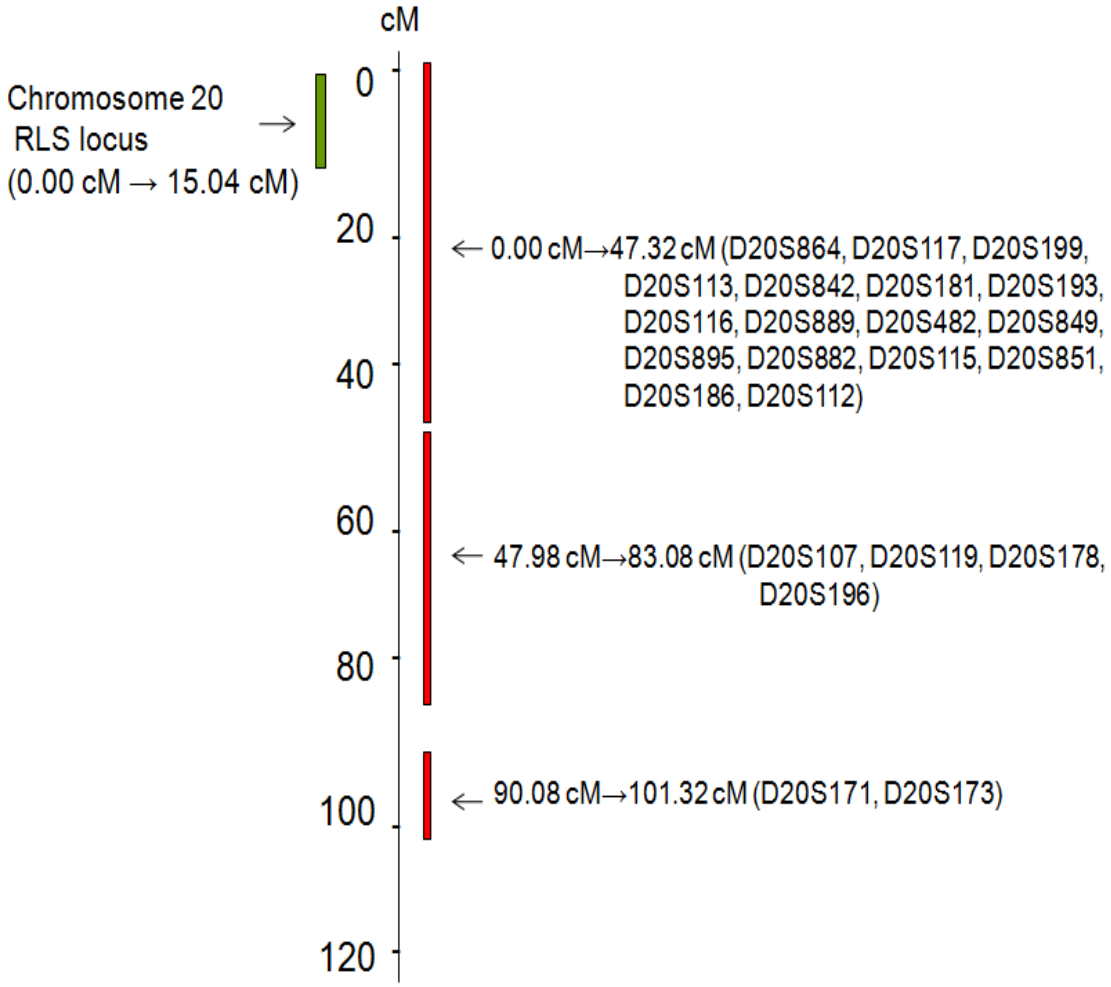


Figure 4.21: Illustration of areas of exclusion for Chromosome 20
Black vertical line with scales represents chromosome 20 (scales in cM). The red bars on the right illustrates the total areas excluded combining all markers from the two linkage set. The marker(s) which contributed to these exclusion values are also indicated.

The results of the genome-wide scan exclude a large amount of chromosome 20. Only two small gaps remain on the chromosome and these are positioned from 47.32 cM to 47.98 cM and from 83.08 cM to 101.32 cM. A total of 93.56 cM of chromosome 20 was excluded, which equals 92.4% of the entire chromosome. The highest LOD score seen for this chromosome was for D20S113 at 8.97 cM, which presented with a LOD score of 0.14 at θ=0.00. This LOD score was not high enough to require further investigation.

4.3.21 Chromosome 21

The Research Genetics Inc. mapping set contained only one marker for chromosome 21, which was a GATA tetranucleotide repeat. The Applied Biosystems mapping set contained five markers for chromosome 21. Table 4.24 lists the markers that were examined from both mapping sets, their positions (cM), maximum obtained LOD scores at theta equal to zero, the corresponding maximum excluded distances in cM, and the regions exclude. Markers highlighted in blue represent markers from the Research Genetics Inc. mapping set. Markers highlighted in pink represent markers from the Applied Biosystems mapping set.

Table 4.24: Markers used to examine chromosome 21.

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from (cM)**	**Area Excluded to (cM)**
D21S1914	19.39	-8.38	11.50	7.89	30.89
D21S263	27.40	-8.68	6.84	20.56	34.24
D21S1252	35.45	-8.68	9.93	25.52	45.38
D21S2055	40.49	-5.23	5.62	34.87	46.11
D21S266	45.87	-5.49	4.01	41.86	49.88

Five markers were examined for chromosome 21. All five markers from the two mapping sets obtained statistically significant LOD scores. Figure 4.21 illustrates the positions of the markers that obtained significant negative LOD scores and the corresponding regions of exclusion on chromosome 21.

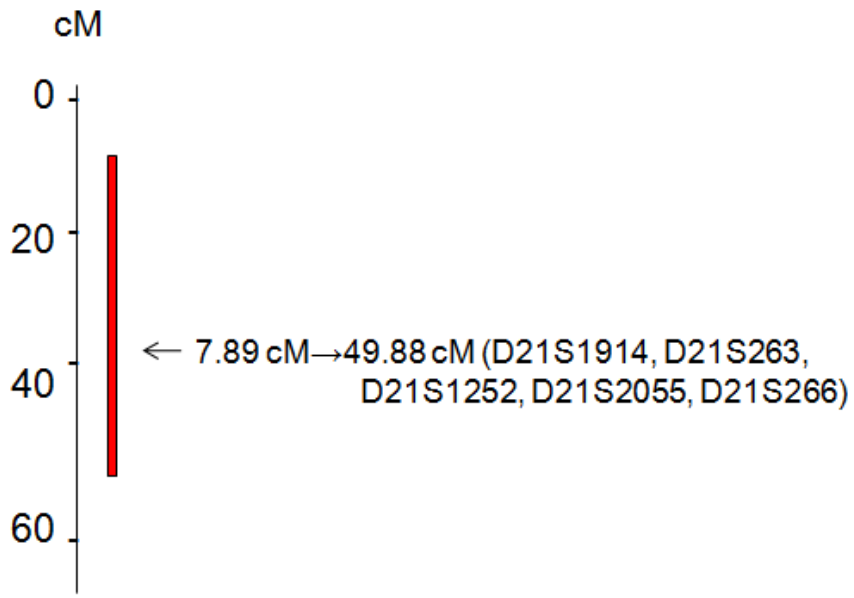


Figure 4.22: Illustration of areas of exclusion for Chromosome 21
Black vertical line with scales represents chromosome 21 (scales in cM). The red bars on the right illustrates the total areas excluded combining all markers from the two linkage set. The marker(s) which contributed to these exclusion values are also indicated.

The results of the genome-wide scan excluded a large section of chromosome 21, between 7.89 cM and 49.88 cM. A 7.89 cM gap remains at the telomeric end of the short arm of chromosome 21. A 7.90 cM gap remains at the opposite end of chromosome 21 also. A total of 41.99 cM of chromosome 21 was excluded, which equates to 72.7% of the entire chromosome.

4.3.22 Chromosome 22

The Research Genetics Inc. mapping set contained only one marker for chromosome 22, which was a GCT trinucleotide repeat. The Applied Biosystems mapping set contained seven markers for chromosome 22. Table 4.25 lists the markers that were examined from both mapping sets, their positions (cM), maximum obtained LOD scores at theta equal to zero, the corresponding maximum excluded distances in cM, and the regions excluded are listed here. Markers highlighted in blue represent markers from the Research Genetics Inc. mapping set. Markers highlighted in pink represent markers from the Applied Biosystems mapping set.

Table 4.25: Markers used to examine chromosome 22.

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from (cM)**	**Area Excluded to (cM)**
D22S420	4.06	-8.68	8.38	-4.32	12.44
D22S539	14.44	0.19	0.00	-	-
GCT10C10	18.10	-0.66	0.00	-	-
D22S315	21.47	-4.79	6.33	15.14	27.80
D22S280	31.30	-1.48	0.00	-	-
D22S283	38.62	-7.39	9.10	29.52	47.72
D22S423	46.42	-7.55	9.10	37.32	55.52
D22S274	51.54	-1.47	0.00	-	-

Four out of the eight markers from the two mapping sets obtained statistically significant LOD scores. Figure 4.22 illustrates the positions of the markers that obtained significant negative LOD scores and the corresponding regions of exclusion on chromosome 22.

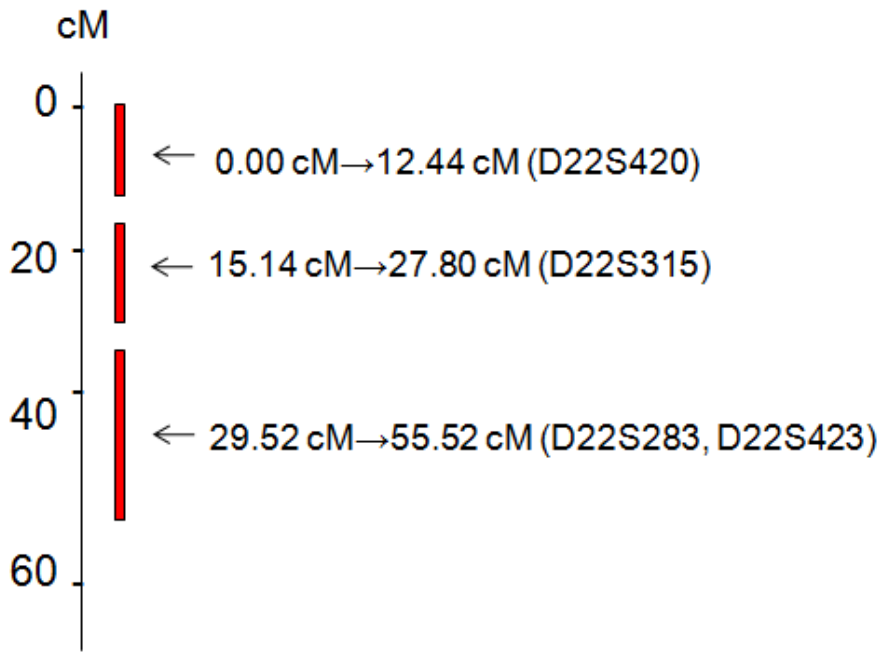


Figure 4.23: Illustration of areas of exclusion for Chromosome 22
Black vertical line with scales represents chromosome 22 (scales in cM). The red bars on the right illustrates the total areas excluded combining all markers from the two linkage set. The marker(s) which contributed to these exclusion values are also indicated.

These results exclude a large area of chromosome 22. Three gaps are present on the chromosome and are positioned between: 12.44 cM and 15.14 cM; 27.80 cM and 29.52 cM; and 55.52 cM and 62.32 cM. A total of 55.10 cM of chromosome 22 was excluded, which equates to 82% of the entire chromosome. The highest LOD score seen for this locus was for D22S539, which presented with a LOD score of 0.19 at θ=0.00. This LOD score was not high enough to require further investigation.

4.4 Areas of further Investigation

4.4.1 Chromosome 1

Marker D1S484 (169.69 cM) which was part of the Applied biosystems mapping set obtained a positive LOD score of 1.69 at θ=0.00. Although this LOD score is inconclusive, it was one of the highest LOD scores seen throughout the genome-wide scan; therefore it was necessary to investigate the region further. Marker D1S2878 (177.86 cM) which was also part of the Applied biosysystems mapping set is positioned 8.17 cM away from D1S484. Marker D1S2878 obtained a LOD score of -10.62 at θ=0.00, and excluded linkage from 168.35 cM to 187.37 cM. Therefore the region where D1S484 is positioned was excluded for linkage to RLS. Nevertheless to ensure that RLS is not linked to this region a haplotye analysis was carried out. The haplotype analysis was carried out using three other markers from the mapping sets. One marker was positioned distal to D1S484 and the other two were positioned further along chromosome 1. From looking at the haplotype analysis (figure 4.23), it becomes clear that there is no haplotype that is present in all the affected patients and not present in the unaffected individuals. So due to the fact that there is exclusion of the region and that haplotype analysis confirms exclusion of this region on chromosome 1, it can be safely assumed that the region surrounding marker D1S484 is not linked to RLS in this family.

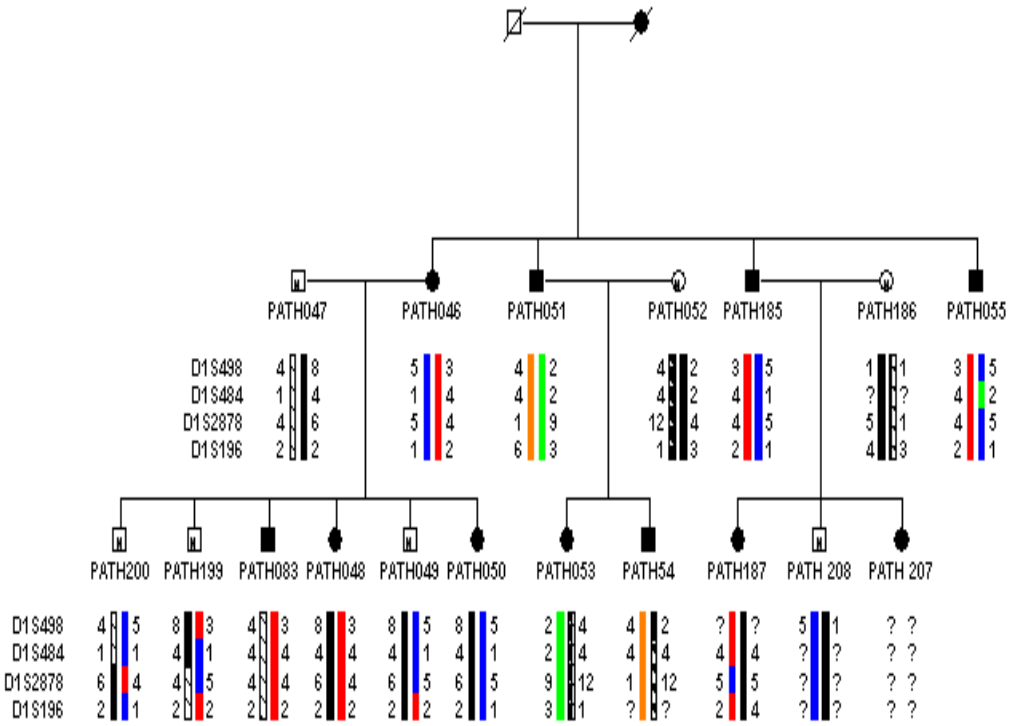


Figure 4.24: Haplotype analysis of RLS3002 pedigree for chromosome 1 region
A list of the examined markers is present on the left hand side. The alleles for each individual obtained from genotyping using these markers are listed below each individual. Each haplotype formed from the series of alleles is given a coloured bar so it can be recognised easily. All the coloured haplotypes are present in both affected (filled squares and circles) and unaffected (squares and circles containing the letter N) individuals. Hence there is no one shared haplotype between all the affected individuals that is absent in normal individuals. It is therefore possible to conclude that there is no linkage between RLS and the region surrounding marker D1S484 on chromosome 1.

4.4.2 Chromosome 10

Marker D10S548 (45.70 cM) which was part of the Applied biosystems mapping set showed a LOD score of 1.71 at θ=0.00. This LOD score was inconclusive however it was necessary to investigate it further as it was close to the value which would suggest linkage. Marker D10S548 is positioned at 45.70 cM on chromosome 10. This region was later excluded by marker D10S1423, which was also part of the Applied Biosystems mapping set. Marker D10S1423 (46.23 cM) is positioned only 0.53 cM away from marker D10S548, and showed a LOD score of -5.67 at θ=0.00, excluding from 39.39 cM to 53.07 cM. Therefore the position at which D10S548 is situated was excluded for linkage. To ensure that RLS is not linked to this region a haplotye analysis was carried out. Looking at the haplotype analysis (figure 4.24), it becomes clear that there is no haplotype that is present in all the affected patients and not present in the normal individuals. So due to the fact that there is exclusion of the region and that haplotype analysis confirms exclusion of this region on chromosome 10, it can be safely assumed that the region surrounding marker D10S548 is not linked to RLS in this family.

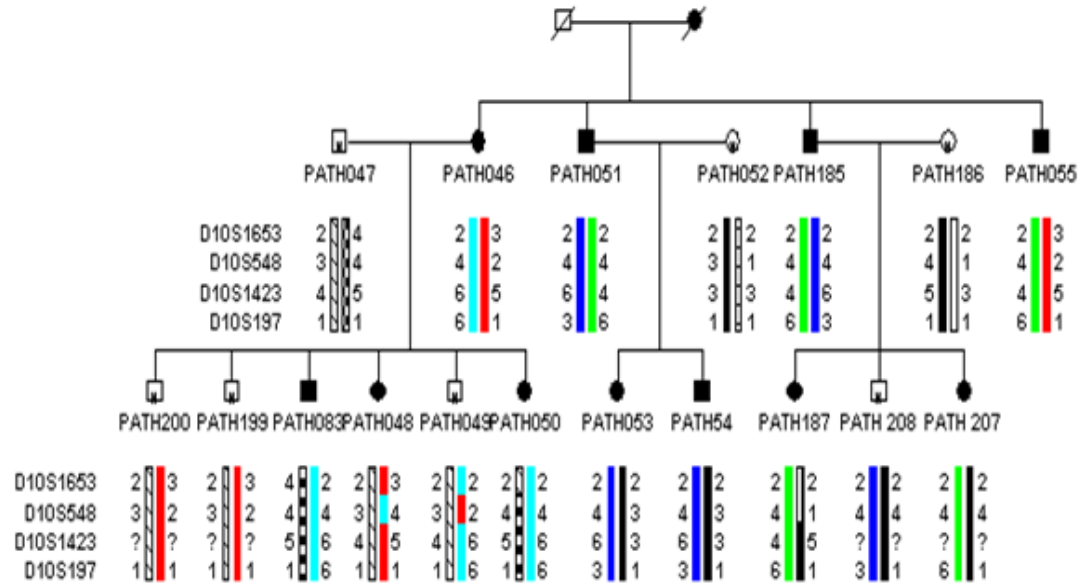


Figure 4.25: Haplotype analysis of RLS3002 pedigree for chromosome 10 region
A list of the examined markers is present on the left hand side. The alleles for each individual obtained from genotyping using these markers are listed below each individual. Each haplotype formed from the series of alleles is given a coloured bar so it can be recognised easily. All the coloured haplotypes are present in both affected (filled squares and circles) and unaffected (squares and circles containing the letter N) individuals. Hence there is no one shared haplotype between all the affected individuals that is absent in normal individuals. It is therefore possible to conclude that there is no linkage between RLS and the region surrounding marker D10S548 on chromosome 10.

4.4.3 Chromosome 17

Marker D17S944 (83.56 cM) which was part of the Applied Biosystems mapping set yielded a LOD score of 1.36 at θ=0.00. This area was not excluded by any other marker in the mapping sets. To ensure that RLS is not linked to this region a haplotye analysis was carried out. From looking at the haplotype analysis (figure 4.25), it becomes clear that there is no haplotype that is present in all the affected patients and not present in the normal individuals. Therefore, due to the fact that haplotype analysis confirms exclusion of this region on chromosome 17, it can be safely assumed that the region surrounding marker D17S944 is not linked to RLS in this family.

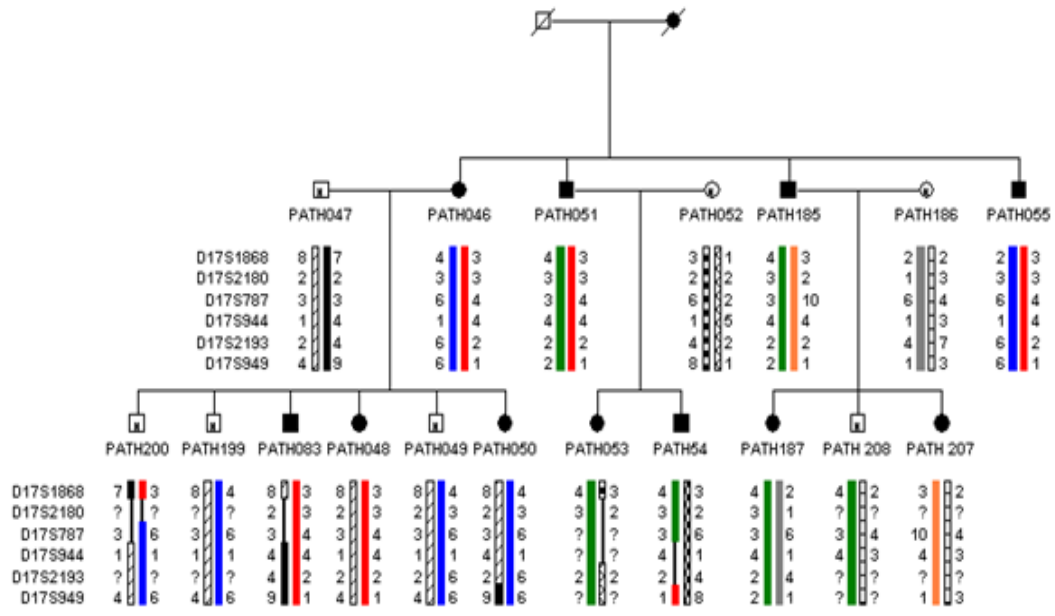


Figure 4.26: Haplotype analysis of RLS3002 pedigree for chromosome 17 region
A list of the examined markers is present on the left hand side. The alleles for each individual obtained from genotyping using these markers are listed below each individual. Each haplotype formed from the series of alleles is given a coloured bar so it can be recognised easily. All the coloured haplotypes are present in both affected (filled squares and circles) and unaffected (squares and circles containing the letter N) individuals. Therefore, there is no one shared haplotype between all the affected individuals that is absent in normal individuals.

4.4.4 Chromosome 18

Marker D18S1357 (88.62 cM) which was part of the Research Inc. mapping set yielded a LOD score of 0.68 at θ=0.00. This area was not excluded by any other marker in the mapping sets; therefore it was necessary to investigate this area further. Four additional markers in the area were chosen and ordered. Table 4.26 lists the markers that were ordered. Their positions (cM), size (bp), and the fluorescent tags with which they were labelled are listed here.

Table 4.26: Markers used to examine the area surrounding D18S1357.

Marker	**Position (cM)**	**Size (bp)**	**Fluorescent label**
D18S1134	88.62	204-232	6-FAM
D18S1148	90.60	132-154	6-FAM
D18S1147	90.60	207-219	HEX
D18S465	100.11	232-251	6-FAM

The four microsatellite markers surrounding marker D18S1357 were examined. Two of the four markers did not yield a statistically significant LOD score (i.e. <-2 or >3). Table 4.27 lists the markers that were examined, the LOD scores obtained at θ=0.00 and the corresponding excluded regions. Their positions (cM), maximum obtained LOD scores at theta equal to zero, the corresponding maximum excluded distances in cM, and the regions excluded are listed here.

Table 4.27: Markers used to examine the area surrounding D18S1357.

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from (cM)**	**Area Excluded to (cM)**
D18S1134	88.62	-2.04	0.10	88.52	88.72
D18S1148	90.60	-4.96	0.00	-	-
D18S1147	90.60	-1.49	0.00	-	-
D18S465	100.11	-1.76	0.00	-	-

The markers were not successful in excluding the area in question. The marker D18S1134 (88.62 cM) showed a significant LOD score of -2.04 at θ=0.00 and excluded 0.10 cM either side. The marker D18S1148 showed a significant score however it was only significant at θ=0.00. Hence there was no distance of exclusion except at the position of the marker. Therefore the region in question has not been excluded by the extra markers ordered in this area. However by looking at the haplotype analysis (figure 4.26), it becomes clear that there is no haplotype that is present in all the affected patients and not present in the normal individuals. So due to the fact that there is exclusion of D18S1148 at the exact position it is located and that haplotype analysis confirms exclusion of this region on chromosome 18, it can be safely assumed that the region surrounding marker D18S1357 is not linked to RLS in this family.

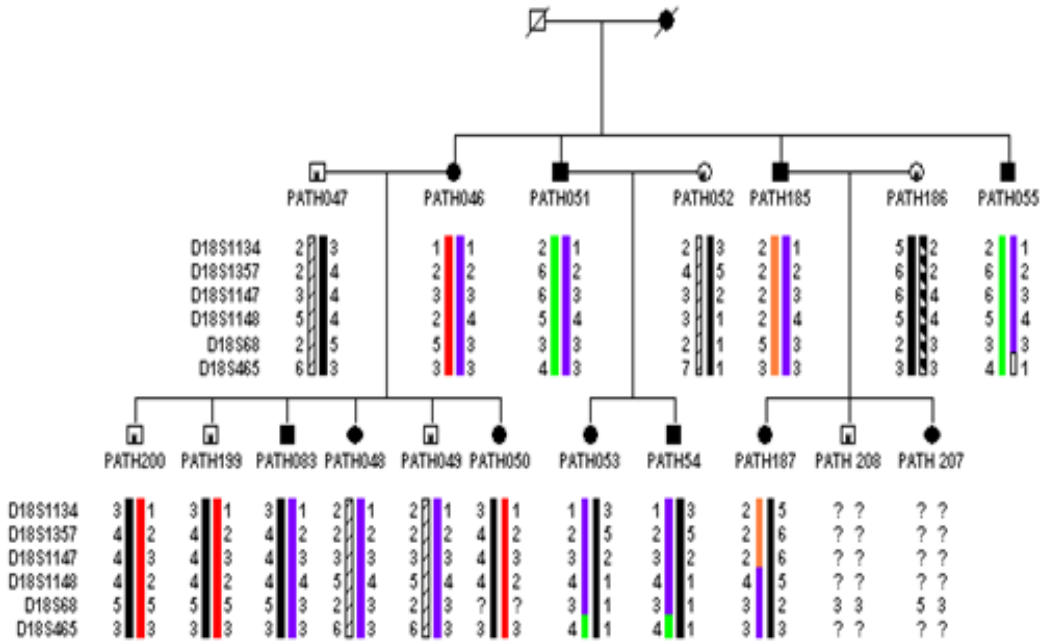


Figure 4.27: Haplotype analysis of RLS3002 pedigree for chromosome 18 region
A list of the examined markers is present on the left hand side. The alleles for each individual obtained from genotyping using these markers are listed below each individual. Each haplotype formed from the series of alleles is given a coloured bar so it can be recognised easily. All the coloured haplotypes are present in both affected (filled squares and circles) and unaffected (squares and circles containing the letter N) individuals. There is no one shared haplotype between all the affected individuals that is absent in normal individuals. Therefore, there is no linkage between RLS and the region surrounding marker D18S1357 on chromosome 18.

4.4.5 Chromosome 19

Marker D19S209 (10.97 cM) on chromosome 19, which was part of the Applied Biosystems mapping set yielded a LOD score of 2.01 at θ=0.67. This was the highest LOD score seen during the process of the genome-wide search and suggested linkage of RLS to the region. The nearest marker D19S216 (20.01 cM) was positioned almost 10.00 cM away and although it showed a significant LOD score of -2.62 at θ=0.00, it was only significant at this point. Hence, there was no distance of exclusion except at the position of the marker. The area surrounding D19S209 was not excluded by any other marker; therefore it was necessary to investigate this area further.

The highest LOD score for D19S209 was observed at θ=0.67. This indicates that the area of interest is approximately 6.00 cM away from the marker, which is positioned at 10.97 cM. Therefore our area of interest was at approximately 5.00 cM or at 17.00 cM. This was important information for ordering additional markers. Four additional markers in the area were chosen; the first positioned at 6.57 cM, the second positioned at 15.55 cM, a third positioned close to D19S209 at 9.84 cM and a final marker positioned at the telomere of chromosome 19. Table 4.28 lists the markers that were ordered. Their positions (cM), size (bp), and the fluorescent tags with which they were labelled are also listed.

Table 4.28: Markers used to examine the area surrounding marker D19S209.

Marker	**Position (cM)**	**Size (bp)**	**Fluorescent label**
D19S886	0.00	134-158	6-FAM
D19S878	6.57	208-230	6-FAM
D19S591	9.84	96-112	6-FAM
D19S894	15.55	144-168	HEX

The four markers surrounding marker D18S1357 were examined. Only one of the four markers yielded a statistically significant LOD score (i.e. <-2 or >3). Table 4.29 lists the markers that were examined Their positions (cM), maximum obtained LOD scores at theta equal to zero, the corresponding maximum excluded distances in cM, and the regions excluded.

Table 4.29: Markers used to examine the area surrounding D18S1357.

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded (cM)**	**Area Excluded from (cM)**	**Area Excluded to (cM)**
D19S886	0.00	1.87	0.00	-	-
D19S878	6.57	3.59	0.00	-	-
D19S591	9.84	1.81	0.00	-	-
D19S894	15.55	-5.44	0.60	14.95	16.15

These results provide evidence of linkage to chromosome 19. Marker D19S878 yielded a maximum LOD score of 3.59 at θ=0.00. A LOD score of >3 is required to be statistically significant. It was proposed that the locus would be positioned approximately 6.00 cM away from marker D19S209, which is located at 10.97 cM. Marker D19S878 is positioned 4.40 cM from D19S209 which is in the approximate location estimated. The marker D19S894 chosen to be 6.00 cM in the other direction away from D19S209, yielded a LOD score of -5.44 and excluded 0.60 cM either side of the position of the marker. The other two markers examined did not show statistically significant LOD scores, however both had LOD scores of approximately 1.80 at θ=0.00, which would have been questioned if found independently. Furthermore, by looking at the haplotype analysis (figure 4.27), it becomes obvious that there is one haplotype (red bar) that is present in all the affected individuals and not present in the unaffected individuals. Therefore following further investigation, it can be concluded that this region on chromosome 19 is linked to RLS in our Irish RLS3002 pedigree.

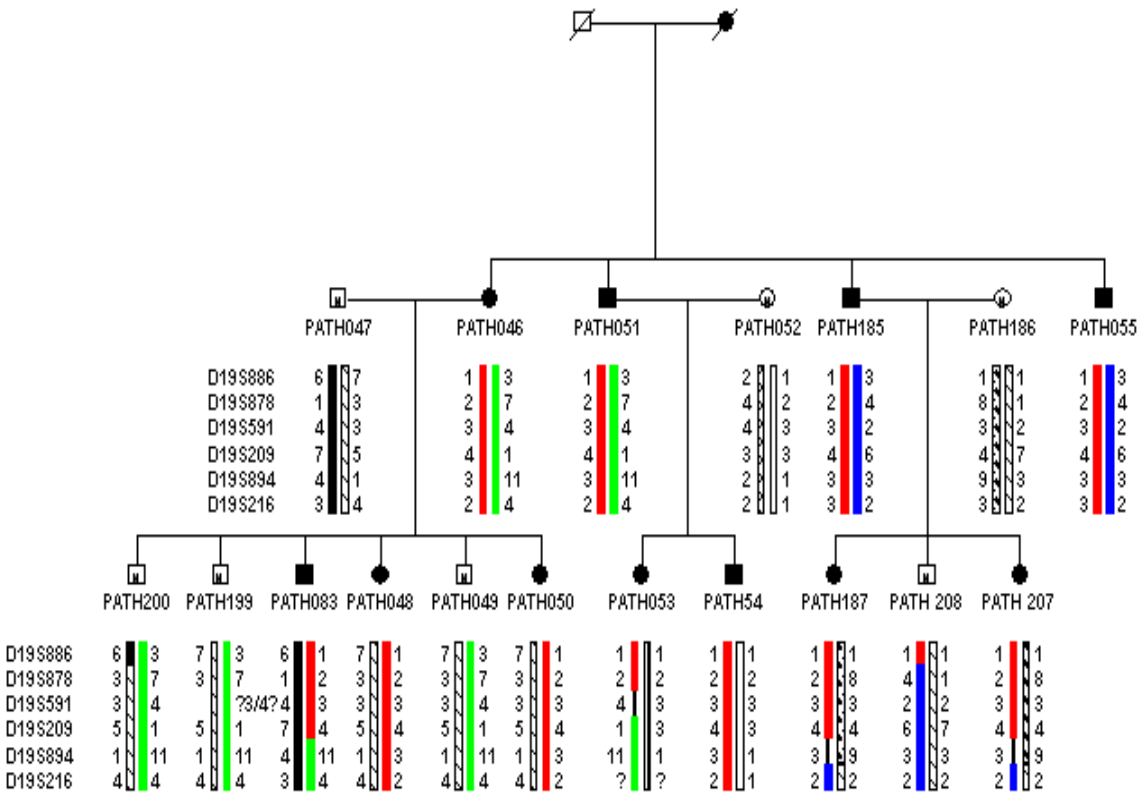


Figure 4.28: Haplotype analysis of RLS3002 pedigree for chromosome 19 region
A list of the examined markers is present on the left hand side. The alleles for each individual obtained from genotyping using these markers are listed below each individual. Each haplotype formed from the series of alleles is given a coloured bar so it can be recognised easily. The red bar (haplotype) is in all affected individuals (filled squares and circles) and a green or blue bar (haplotype) is present in the unaffected (squares and circles containing the letter N) individuals. Hence there is a shared haplotype between all the affected individuals that is absent in normal individuals. It is therefore possible to conclude that there is linkage between RLS and the region on chromosome 19.

4.5 Discussion

Following exclusion of pedigree RLS3002 to all known RLS loci and associated regions it was necessary to conduct a genome-wide search. The aim of this approach was to find out the rough location of the causative gene relative to a microsatellite marker, which has it position already known. The first mapping set used was the Research Genetics Inc. mapping set. This mapping set consisted of microsatellite markers spaced approximately every 20 cM along the entire genome. The majority of the markers in this set were tetranucleotide. Following completion of this mapping set, no positive LOD score of >3.00 was observed. Therefore it was necessary to continue the genome-wide search.

For the second mapping set, it was decided that a mapping set with greater coverage was necessary. Therefore the Applied Biosystems mapping set version 2.5-MD10 was used. This mapping set has approximately 400 microsatellite markers spaced at 10 cM intervals along the genome. This mapping set also had an average heterozygosity of 0.79.

Subsequently four areas of interest were found and required further investigation. The first region of interest was observed on chromosome 1 where marker D1S484 showed a LOD score of 1.69 at θ=0.67. This region was excluded for linkage by marker D1S2878, and a haplotype analysis confirmed that RLS3002 was not linked to this region.

The second area of interest was on chromosome 10, where a LOD score of 1.71 at θ=0.00 was observed for marker D10S548. This region had also been excluded by

marker D10S1423, however a haplotype analysis confirmed pedigree RLS3002 was not linked to this region.

The third area which was investigated further was a region on chromosome 18. Marker D18S1357 showed a LOD score of 0.68 at θ=0.00, and although this value was inconclusive, a large area surrounding the marker was not excluded for linkage and therefore required further investigation. Four additional markers surrounding D18S1357 were ordered and investigated. The markers were mostly inconclusive, however following haplotype analysis it was clear that pedigree RLS3002 was not linked to this.

A positive LOD score of 2.01 at θ=0.67 obtained for marker D19S209 suggested linkage of RLS to the region on chromosome 19. This was the fourth area which required investigation. Four adjacent markers were ordered and examined. Linkage analysis of marker D19S878 yielded a LOD score of 3.59 at θ=0.00, thereby providing evidence of linkage to this region. SLINK simulation analysis indicates that the maximum LOD score achievable with this pedigree is 3.41 at θ=0.01. To confirm that this region on chromosome 19 is linked to RLS in pedigree RLS3002, a haplotype analysis was performed. For the haplotype analysis the four newly ordered markers and two markers from the Applied Biosystems mapping set were used. These markers were located between 0.00 cM and 20.01 cM on chromosome 19. Haplotype analysis of the genotype data revealed a disease haplotype that was present in all 11 affected members. Therefore the haplotype analysis confirmed that this region was linked to RLS in our Irish pedigree.

Chapter 5

Fine-Mapping Chromosome 19

RLS locus

5.1 Introduction

Linkage to RLS in our Irish pedigree RLS3002 was confirmed to chromosome 19p. Marker D19S878 yielded a LOD score of 3.59 at θ=0.00, thereby providing evidence of linkage to this region. Haplotype analysis of the region also confirmed linkage. Initial detection of a genetic locus generally does not yield much precision on its location, thus leaving too vast an area to explore for candidate genes. It is therefore necessary to refine the location of the genetic determinants. Localisation or fine-mapping is the process of refining the location. Studies which have an increased number of meiosis between individuals, narrowing the expected preserved segment lengths and thus increasing the resolution, are most advantageous. Fine-mapping of a region can be achieved in a number of ways, the most common being; increasing marker density within the chromosomal area of interest or by increasing the number of individuals for which phenotype information can be obtained. Since all known members of the RLS3002 have been recruited and used for this study, it was necessary to identify microsatellite markers that were very tightly linked to the targeted area and thereby used to further refine the susceptibility region.

The region in question was a marker poor region; therefore, it was necessary to identify novel microsatellite markers in the region. This was essential for generation of a more detailed haplotype. In order to design more microsatellite markers it was necessary to examine DNA sequences in databases for clusters of short tandemly repeated nucleotide bases. The areas which were targeted were areas in which no known microsatellite markers were present. For this study repeats which were greater than 10 units long were considered for fine-mapping. Following

identification of short tandem repeats in the desired area, primers were designed flanking the area of interest (see section 2.7 for primer design).

5.2 Materials and Methods

5.2.1 Microsatellite marker selection

The area on chromosome 19 which was found to be linked to RLS in this Irish family required further examination in order to refine the region.

Known Microsatellite Markers

Following a search of genetic databases www.ncbi.nlm.nih.gov and www.genome.ucsc.org, only five other microsatellite markers in the region were identified. These microsatellite markers were D19S883, D19S565, D19S247, D19S120 and D19S424. The marker located nearest to the telomere was D19S883, which is located at 5.54 cM. There was no microsatellite marker in the databases between the telomere and 5.54 cM. In an attempt to refine this novel region it was necessary to design novel microsatellite markers.

Novel Microsatellite Markers

This region is a marker poor region, and it was therefore necessary to create additional microsatellite markers in the region. The regions which were marker poor and had no documented microsatellite markers were identified. The main region was located between the telomere and marker D19S883 (5.54 cM). Figure 5.1 shows this region (1 – 1,454,490 bp). The map shows genetic STS markers which have a red line beside their name, and radiation hybrid markers which have a black line beside their name. Only two genetic markers were observed in this map, and they were AFMA319WD9 (D19S886) and AFMA299YC1 (D19S883), which is highlighted in the figure. Microsatellite marker D19S886 had been previously used (chapter 4).

D19S886 is located at 998,664 bp on chromosome 19, therefore there was nearly a 1,000,000 bp region which was devoid of microsatellite markers. The aim was to attempt to design novel microsatellite markers in this region.

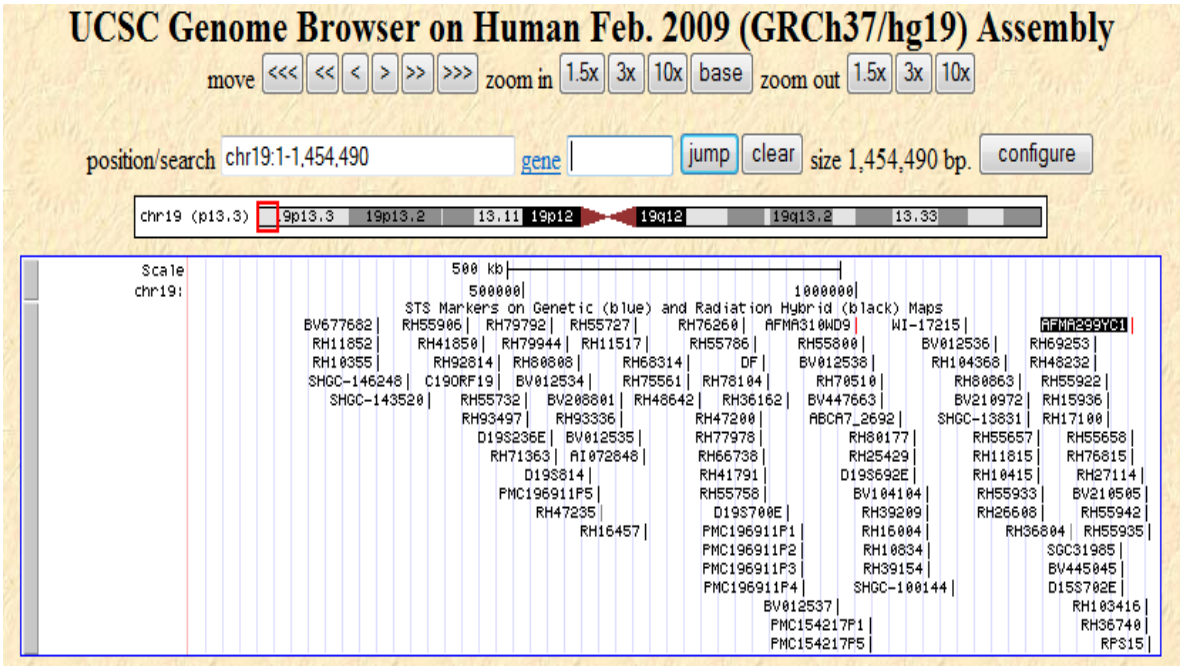


Figure 5.1:. Chromosome 19p13.3. microsatellite poor regions
This figure shows chromosome 19 ideogram with a red box indicating the location of the area shown. The area shown is 1-1,454,490 bp. Below the ideogram is a list of all STS genetic marker (red) and radiation hybrids (black). The highlighted marker is AFMA299YC1, also known as D19S883. There is a microsatellite poor region between the telomere and marker AFMA310WD9 (D19S886). UCSC Genome Browser on Human, Feb 2009 Assembly (http://genome.ucsc.edu).

Furthermore, the highest LOD score to date was 3.59 at θ=0.00 for Microsatellite marker D19S878 (section 4.4.2). A recombination event was observed in PATH053 between marker D19S878 and D19S209 (10.97 cM) (figure 4.27). Therefore it was necessary to investigate the region proximal to microsatellite marker D19S878 in the hope of reducing the locus further (Fig. 5.2).

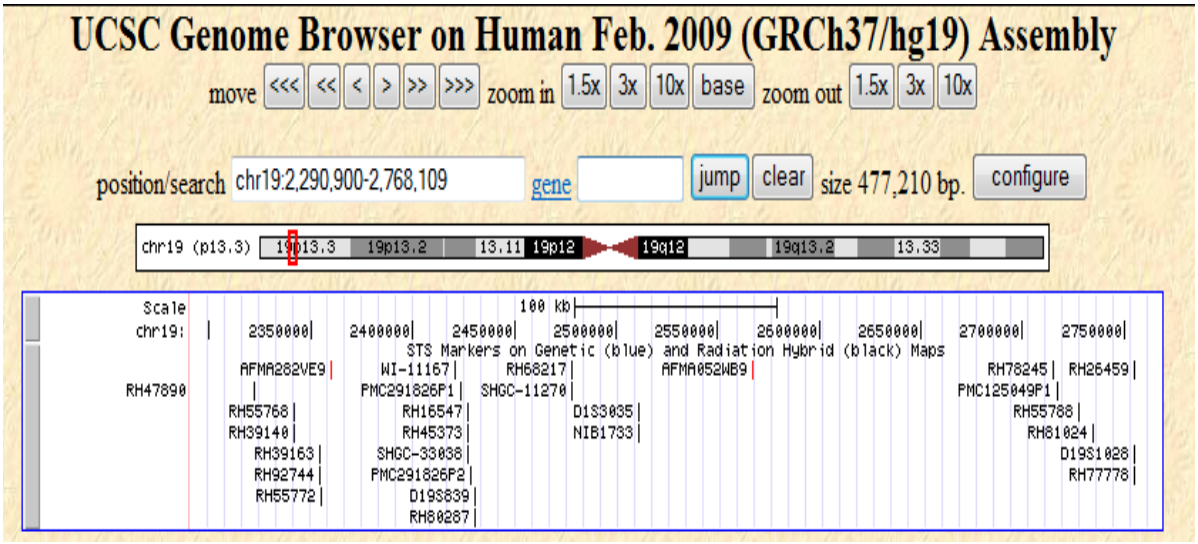


Figure 5.2: Region proximal to marker D19S878 on Chromosome 19p13.3.
This figure shows chromosome 19 ideogram with a red box indicating the location of the area shown. The area shown is 12,290,900-2,768,109 bp. Below the ideogram is a list of all STS genetic markers (red) and radiation hybrids (black). Two microsatellite markers are observed, AFMA282VE9 (D19S878) and AFMA052WB9 (D19S565). It is the region proximal to D19S878 which requires further investigation. UCSC Genome Browser on Human, Feb 2009 Assembly (http://genome.ucsc.edu).

The sequence of the microsatellite poor regions of interest were copied and pasted into *EditSeq* and the sequence was searched for multiple GA, GATA, CA, and CAG etc repeats. If a repeat was located the number of repeats were counted and if it consisted of more than ten repeats, it was accepted. One could not be certain it these regions were polymorphic but it was assumed that if there were at least 10 repeat units there was some evidence that the region might be polymorphic. Primers were designed for these region as per primer design (section 2.8).

5.2.2 Calculation of Genetic position

A method was required to calculate the genetic position of microsatellite markers which were not found in the database and were designed by relying on the position of known microsatellite repeats in the region. Linear interpolation based on physical positions was used to estimate the genetic position (Levchenko, Montplaisir et al. 2008). Figure 5.3 represents the genetic map and the physical map where the new microsatellite marker (B) is located. Microsatellite markers A and C are known microsatellite markers for which their genetic and physical position is known. The physical location of marker B is known, however the genetic position requires calculation.

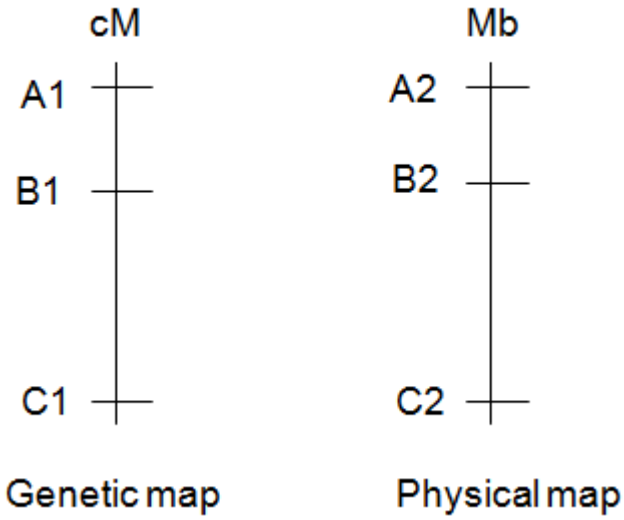


Figure 5.3: Genetic and physical maps required when determining the genetic distance of marker B
The black vertical lines represent a chromosome, with relative positions at markers A, B and C. A1, B1 and C1 represent the genetic positions of markers A, B and C indicated. A2, B2 and C2 represent the physical positions of markers A, B and C respectively. Marker B represents the microsatellite marker with an unknown genetic location. Using the known locations of the other markers and the known physical location of marker B, a formula is used to find the genetic position of marker B.

To find the genetic location of marker B, the following formula was used:

$$\frac{B2 - A2}{C2 - A2} = \frac{x}{C1 - A1}, \text{ where } x = B1 - A1$$

The relative genetic distance 'x' is then added to the known genetic position of marker A1.

5.2.3 Genotyping

Genotyping of novel and known microsatellite markers to fine-map the chromosome 19 RLS locus was performed as per section 2.5.

5.2.4 Haplotype analysis

Haplotype analysis of the novel chromosome 19 locus was carried out according to section 2.7.

5.2.5 Multipoint Linkage Analysis

Multipoint linkage analysis is commonly used to evaluate linkage of a disease to multiple markers in a small region. It is an extension of two-point linkage analysis, where linkage of the disease trait is tested not to just a single microsatellite marker, but to a map of markers. The analysis usually involves three or more linked markers. In general multipoint linkage analysis is not used in the testing phase of a study, i.e. when using highly polymorphic markers to find linkage. Multipoint Linkage analysis is more beneficial when estimating the location of a linked disease gene.

In multipoint analysis, linkage analysis is carried out for a set of markers that are linked to each other against a disease. It is assumed that the marker order and the intermarker distances are known. The disease is then placed in each marker interval, as well as left and right of the leftmost and right most markers. Subsequently, the likelihood is computed for all possible positions of the trait locus in the considered chromosomal region. One can then estimate the position of the disease gene along a chromosomal region (Ziegler A 2010). Multipoint linkage analysis is more complex than two-point analysis. The order of the loci now matters. Furthermore the number of parameters, haplotypes, and genotypes increases drastically with the number of loci considered.

As before, the disease gene is an autosomal dominant disease with disease allele frequency of 0.01 and disease allele frequency of 0.95. For this study, LINKMAP was used for multipoint linkage analysis (ftp://linkage.rockefeller.edu/software/linkage). LINKMAP performs the calculations by allowing the user to fix the order and spacing of two or more of the markers. Likelihoods of the pedigree data are then calculated allowing the locus

whose position is unknown to be placed in all possible intervals. The software MAPFUN was utilized to convert between recombination fractions and morgans. A graph is used to interpret the results. From the graph it is possible to estimate the location of the disease gene.

Multipoint Linkage analysis requires a number of essential steps:

1. A batch file is prepared using LCP.
2. Recompile the LINKMAP program if necessary. The constants may need to be modified to suit the problem which is under consideration.
3. The analysis is performed.
4. The program does not produce the actual multipoint LOD scores, therefore it is necessary to calculate the multipoint LOD scores. The program LINKLODS will perform this calculation.
5. Convert the recombination fractions to map distance using MAPFUN.
6. Graph the results. The y axis is the multipoint lod score and the x axis is the map distance.
7. The graph is used to interpret the location of the disease gene.

There are two major advantages to multipoint LOD score analysis. The first is that it offers an opportunity to impute the genotype information at an original uninformative locus via haplotype information. Consequently, the linkage results are less sensitive to the uninformative or missing genotype at any given marker. Secondly, multipoint linkage analysis can be extremely useful to pintpoint a disease gene location in the fine-mapping of a Mendelian disorder (Haines J.L 1998). This can be accomplished by evaluating many positions $Z(x)$ on a fixed map. At a certain location (x), any true recombinant will contribute a strongly negative LOD score and decrease the possibility that his location contains a candidate locus. Thus this will help to further define the candidate region. On the other hand, the position where the

maximum multipoint LOD score is observed can pinpoint the location of the causative gene and can be used as a guide for selecting candidate genes.

5.3 Results

5.3.1 Novel Microsatellite Markers

The newly designed primers, their location (bp), the forward and reverse primer used, the fluorescent tag used to label the forward primer, and the expected size of the product are detailed in Table 5.1. Their physical positions (bp), primers used for amplification, the fluorescent tag, and the annealing temperature are listed here.

Table 5.1. List of newly designed microsatellite markers used to fine-map the chromosome 19 RLS locus.

Marker	**Location (bp)**	**Primers**	**Fluorescent Tag**	**Product Size (bp)**	**Annealing Temp °C**
CA19S322F	322,195	agacgctttcaaaactcaccact	HEX	326	*
CA19S322R		gggcacccacctcagacat			
AC19S189F	189,592	taagtgatccccatgcctctgtct	6-FAM	407	*
AC19S189R		aacatggtgaaaccccgtctctac			
CA19S872F	872,286	acagtgcaaatgcccatcaatcaa	6-FAM	411	67
CA19S872R		gtggcgccgtctcacctgaa			
GT19S949F	949,752	ctccctccgtccctccccagtc	6-FAM	271	68
GT19S949R		ctcccgacggtgtctccccagat			
ATA19S1001F	1,001,575	cctggctaacatggtgaaacagtg	6-FAM	409	67
ATA19S1001R		gacgtcgtggcccaggatgaag			
AC19S1117F	1,117,411	ccctccacatggattgaagacaca	6-FAM	414	67
AC19S1117R		cagggtggtgcggctttctgt			
CA19S2310F	2,310,554	cttcccgggcaacatggtgagac	6-FAM	270	67
CA19S2310R		agggcgttttctagggcactggtg			
ATA19S2359F	2,359,877	aaaaataaagttgccgggtgtggt	6-FAM	436	*
ATA19S2359R		ctcaggaggctaaggcaggagaat			
GT19S2518F	2,518,246	ttggccgtgatcattcctgtagtg	HEX	290	68
GT19S2518R		cctgggcaaaatggcgaaacc			

* represents markers which failed to amplify, therefore the annealing temperature remains unknown.

5.3.2 Calculation of Genetic positions

Using the formula described above, the genetic positions were calculated for the new microsatellite marker. Below is an example of how this formula was used to calculate the genetic distance of marker AC19S1117 located at 1,117,411bp on chromosome 19.

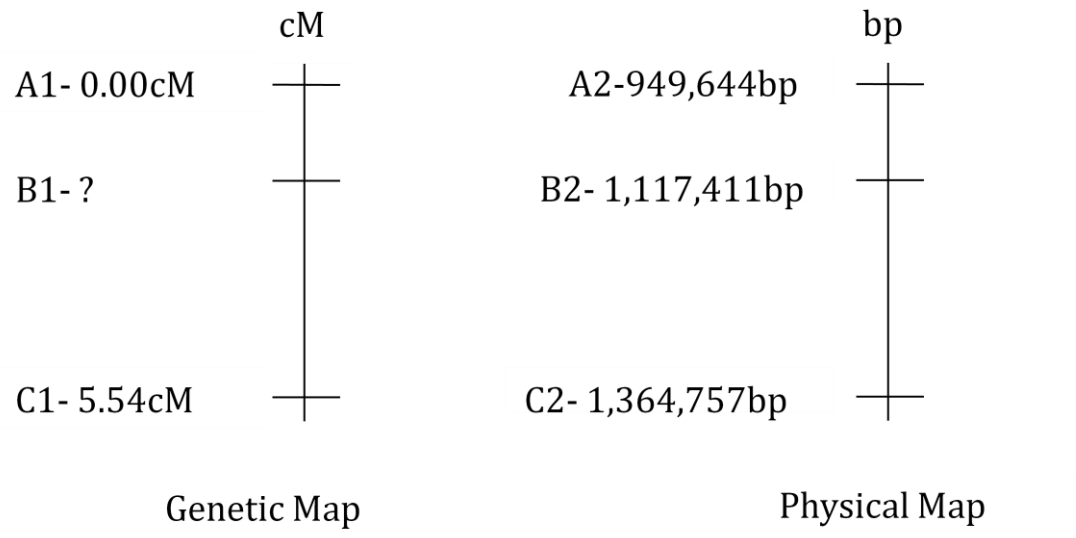


Figure 5.4: Calculating the genetic position of marker AC19S1117
The black horizontal lines represent chromosome 19. A1, B1 and B2 represent the genetic positions of markers D19S886, AC19S1117 and D19S883 respectively. A2, B2 and B3 represent the physical positions of markers D19S886, AC19S1117 and D19S883 respectively. Marker B represents the microsatellite marker AC19S1117 with an unknown genetic location. Using the known locations of the other markers and the known physical location of marker AC19S1117, a formula is used to find the genetic position of AC19S1117.

Using the formula the genetic position of marker AC19S1117 is calculated.

$$\frac{B2 - A2}{C2 - A2} = \frac{x}{C1 - A1}, \quad \text{where } x = B1 - A1$$

1,117,411 bp - 949,644 bp = X
1,364,757 bp- 949,644 bp 5.54 cM– 0.00 cM

167,767 bp= X
415,113 bp 5.54 cM

X = 2.23cM

The relative genetic distance 'X' is then added to the known genetic position of marker A1.

0.00 cM (A1) + 2.23 cM (X) = 2.23 cM

Therefore marker AC19S1117 is located at 2.23 cM on chromosome 19. The remaining new markers were calculated for genetic position. Table 5.2 lists the known markers that were used to calculate the genetic position (cM) of the novel microsatellite markers. As this was a marker poor region, there were no microsatellite markers located at the region distal to markers CA19S322, AC19S189 and CA19S872. Therefore a superficial marker 'X' was used which had a physical location at 0.00 bp and a genetic position at 0.00 cM. In table 5.2, Marker B represents the novel microsatellite marker for which the genetic location is unknown and the physical location (B2) is known. Markers A and B represent known marker for which the genetic location (A1 and B1) and the physical location (A2 and B2) is known. Marker 'X' represents the superficial marker used to calculate the genetic position of novel microsatellite markers CA19S322, AC19S189 and CA19S872.

Table 5.2: Known markers used for calculating the genetic position of novel microsatellite markers used for fine mapping the chromosome 19 locus.

Marker (B)	Known Marker A	Known Marker C	A1 (cM)	A2 (bp)	B2 (bp)	C1 (cM)	C2 (bp)
CA19S322	X	D19S886	0.00	0	322,195	0.00	949,644
AC19S189	X	D19S886	0.00	0	872,284	0.00	949,644
CA19S872	X	D19S886	0.00	0	872,390	0.00	949,644
GT19S949	D19S886	D19S883	0.00	949,644	949,752	5.54	1,364,757
ATA19S1001	D19S886	D19S883	0.00	949,644	1,001,575	5.54	1,364,757
AC19S1117	D19S886	D19S883	0.00	949,644	1,117,411	5.54	1,364,757
CA19S2310	D19S883	D19S878	5.54	1,364,757	2,310697	6.57	2,310,697
GT19S2359	D19S878	D19S565	6.57	2,310,697	2,359,877	6.57	2,518,075
GT19S2518	D19S565	D19S591	6.57	2,518,075	2,518,246	9.84	3,026,844

The genetic positions of the novel microsatellite markers were calculated using the figures in Table 5.2. The results of these calculations, along with the physical and genetic positions of known microsatellite markers are detailed in Table 5.3. This table lists their physical position (bp) and Genetic position (cM).

Table 5.3: Genetic positions of known and novel microsatellite markers examined for fine-mapping chromosome 19 RLS locus.

Marker	Physical position (bp)	Genetic position (cM)
CA19S322	322,195	0.00
AC19S189	872,284	0.00
CA19S872	872,390	0.00
GT19S949	949,644	0.00
ATA19S1001	1,001,575	0.69
AC19S1117	1,117,411	2.23
D19S883	1,413,918	5.54
CA19S2310	2,310,554	6.57
GT19S2359	2,359,877	6.57
GT19S2518	2,518,246	6.57
D19S565	2,567,233	6.57
D19S247	3,139,982	9.84
D19S120	3,189,343	10.97
D19S424	3,226,373	10.97

5.3.3 Genotyping results

Fourteen microsatellite markers were ordered for fine-mapping the chromosome 19 locus, nine of which were novel microsatellite markers. These microsatellites were newly designed. It was not known if these markers were polymorphic and therefore if they were going to be informative. Six of the nine novel microsatellite markers were amplified, all of which were polymorphic, and provided LOD scores. This was a fantastic result, as these were newly designed primers, and it was not known if the novel microsatellite markers would firstly amplify and secondly be polymorphic. Of the known microsatellite markers four out of five amplified and provided LOD scores. Four out of the fourteen known and novel markers ordered for fine-mapping the chromosome 19 locus were not informative: CA19S322, AC19S189, D19S883 and ATA19S2359. These microsatellite marker did not produce specific bands on agarose gel electrophoresis. Standard procedures were carried out to attempt to amplify these markers e.g. range of annealing temperatures, range of Mg^{2+} concentrations, and primers were re-suspended. Nonetheless, these markers failed to amplify.

As previously mentioned ten of the fourteen microsatellite markers were successfully amplified for the chromosome 19 RLS locus. Table 5.3 lists all the markers examined and outlines their genetic positions (cM), the LOD score obtained at θ=0.00, the corresponding maximum excluded distances in cM.

Table 5.4: Markers used to fine-map the chromosome 19 RLS locus.

Marker	**Position (cM)**	**Max LOD (θ=0)**	**Excluded cM**
CA19S872	0.00	2.47	0.00
GT19S949	0.00	2.12	0.00
ATA19S1001	0.69	2.16	0.00
AC19S1117	2.23	2.46	0.00
CA19S2310	6.57	3.46	0.00
D19S565	6.57	-0.78	0.00
GT19S2518	6.57	-0.89	0.00
D19S247	9.84	0.92	0.00
D19S120	10.97	-1.66	0.00
D19S424	10.97	-1.72	0.00

Of the ten microsatellite markers which worked, one of them obtained a statistically significant LOD score of >3, thereby confirming linkage. None of the microsatellite markers showed a negative LOD score of <-2, therefore there was no area of exclusion.

5.3.4 Haplotype Analysis

From these results it is not possible to determine the refined candidate region. In order to determine this region a haplotype for the chromosome 19 locus was reconstructed with the new microsatellite markers added. All 11 affected individuals carried a common haplotype which was identified by markers CA19S872, D19S886, GT19S949, AC19S1117, CA19S2310, and D19S878. Although individual PATH208 is unaffected, he does share part of the affected haplotype (figure 5.6).

A recombination event between markers D19S878 and D19S565 in individual PATH053 allowed for the defining of the RLS region. Both marker D19S878 and D19S565 are located at 6.57 cM, however D19S878 is located at 2.3 Mb and D19S565 is located at 2.5 Mb on chromosome 19. Therefore a recombination event took place somewhere within this 0.2 Mb region. Haplotype reconstruction allowed determination of the overall candidate region which is flanked by the telomere and microsatellite marker D19S565. This candidate region has a genetic distance of 6.57 cM and corresponds to a physical distance of 2.5 Mb on chromosome 19p13.3. Figure 5.5 represents the newly reconstructed haplotype for the chromosome 19 RLS locus.

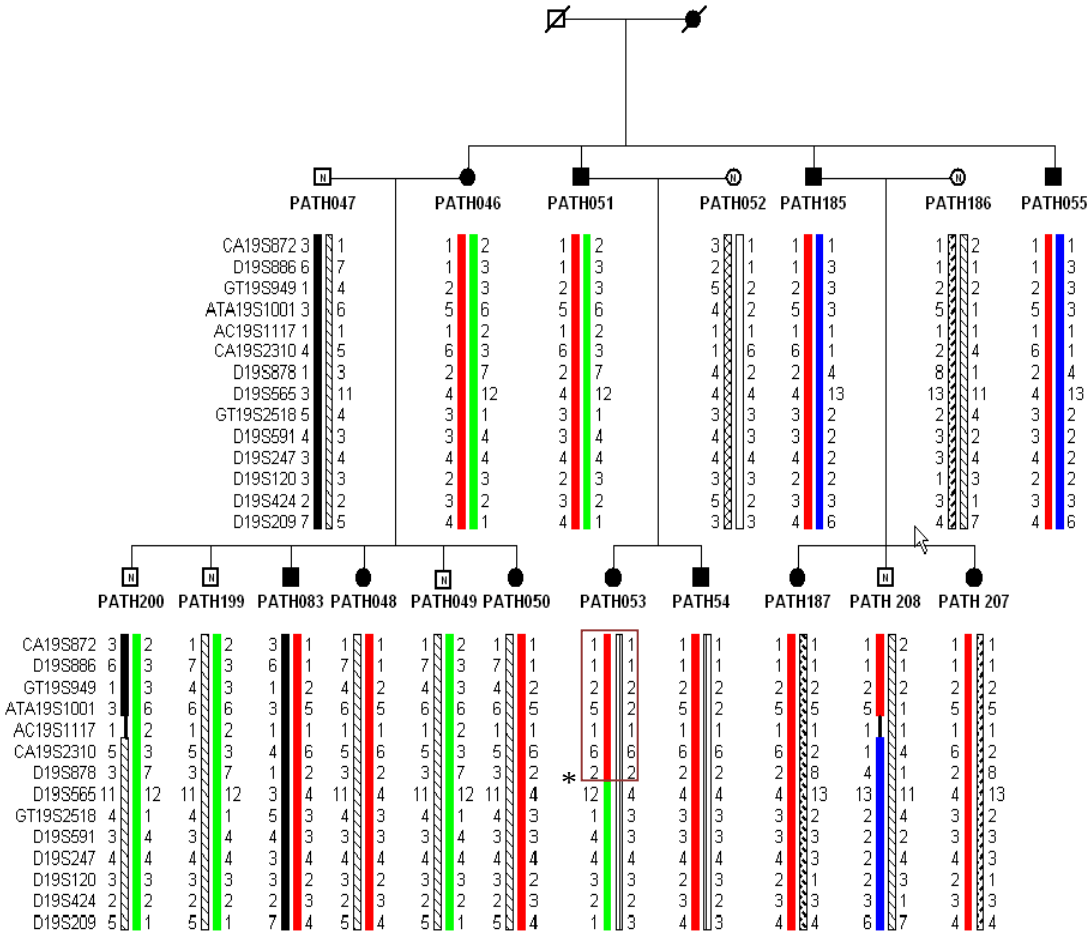


Figure 5.5: Haplotype analysis of RLS3002 pedigree for chromosome 19 region
A list of the examined markers is present on the left hand side. The alleles for each individual obtained from genotyping using these markers are listed below each individual. Each haplotype formed from the series of alleles is assigned a coloured bar so it can be recognised easily. The red bar (haplotype) is in all affected individuals (filled squares and circles), and it is assumed that this red bar is inherited from the affected grandmother in generation I. The unaffected (squares and circles containing the letter N) individuals do not inherit a red bar, and it is assumed they inherit their haplotype from the unaffected grandfather (generation I). Hence there is a shared haplotype between all the affected individuals that is absent in normal individuals. A recombination event was observed in individual PATH053 and the point of recombination is marked with an asterix (*). This recombination event defined the critical RLS gene location as distal to marker D19S565.

5.3.5 Multipoint Linkage analysis

Once linkage has been detected, multipoint linkage analysis can help localize the position of the gene more precisely (Elston and Olson 2002). Multipoint linkage analysis is the analysis of linkage data involving three or more linked loci. Multipoint linkage analysis was performed for this region for a number of different marker combinations. However the combination of markers which gave the highest LOD score was: D19S886 (0.00 cM), D19S878 (6.57 cM) and D19S591 (9.84 cM). A maximum multipoint LOD score of 3.77 was obtained at a position of 6.64 cM on chromosome 19, the nearest marker to this being D19S878 which is positioned at 6.57cM. This maximum multipoint LOD score is illustrated in figure 5.6 which also shows the surrounding markers. A number of markers are located at the same genetic position on chromosome 19, these markers are represented with a + sign.

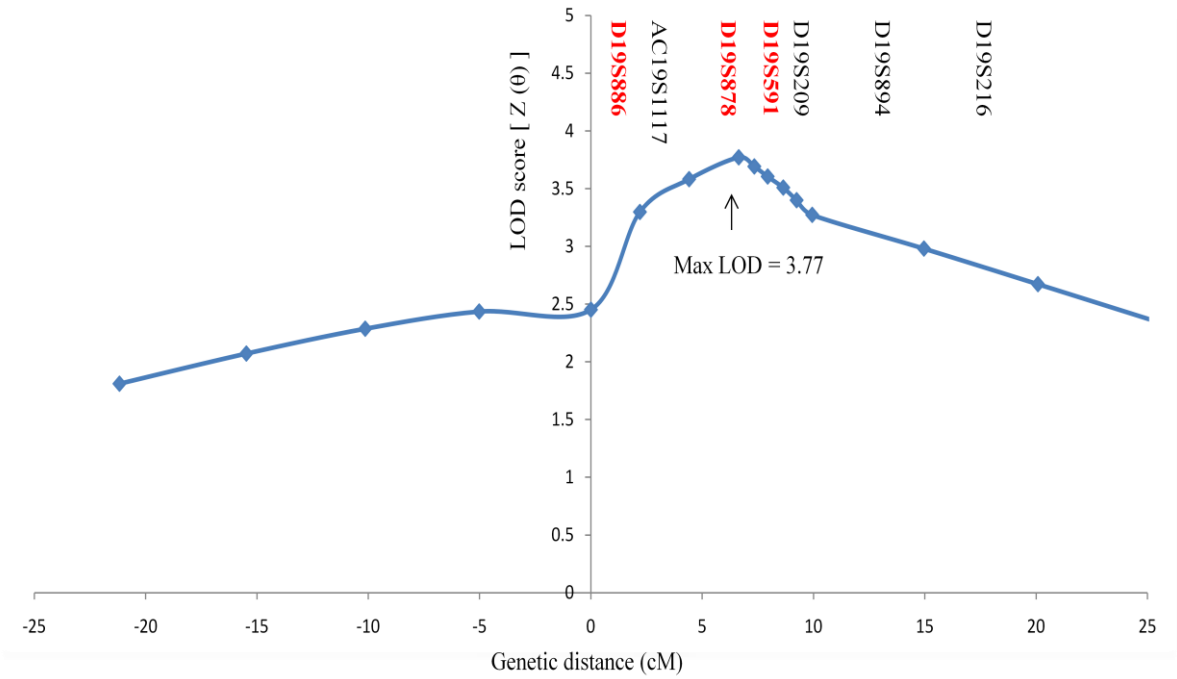


Figure 5.6: Graph of Multipoint parametric LOD scores for family RLS3002 on chromosome 19p

Genetic distance (cM) are plotted on the x axis and LOD score [Z (θ)] is plotted on the y axis. Multipoint Linkage analysis was performed with markers D19S886, D19S878 and D19S591, which are shown in red. Names of a number of microsatellite markers are not shown on the graph as a number of the markers share the same genetic position. The markers which generated the highest LOD scores are shown on this map.

Multipoint Linkage analysis can help localize the position of the gene more precisely. However, it can also support the findings of the genome-wide scan. The results of the multipoint linkage analysis in figure 5.6 support the findings that family RLS3002 is linked to chromosome 19 for RLS. A maximum multipoint LOD score of 3.77 was obtained at a position of 6.64 cM on chromosome 19.

From a multipoint LOD score graph it is usually possible to determine a support interval for the likely placement of the disease locus. Current Protocols of Human Genetics suggest that in order to do this , a horizontal line is to be drawn across the graph at a value equal to the highest LOD score minus 3 LOD units. All points on

the map that are above this horizontal line are included within the 3-LOD-unit support interval and cannot be entirely excluded. For two-point linkage analysis graph only a 1-LOD unit support is required, however the 3-LOD-unit support is needed for multipoint analysis to ensure a higher degree of confidence in locus placement for multipoint situation (Speer 2006). Unfortunately it is not possible to use this approach for our study. The maximum LOD score is equal to 3.77, which would indicate that a horizontal line be drawn at $Z(\theta=0.77)$. This line would not cross the LOD score curve at any point, therefore no support interval can be inferred.

Nevertheless, the graph can be used to interpret the location of the disease gene. The highest LOD score was obtained just proximal to microsatellite marker D19S878. The value of the maximum LOD score is equal to 3.77. Therefore the most likely location of the causative gene is in close proximity to microsatellite marker D19S878. This information should be considered when selecting candidate genes for screening.

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5.4 Discussion

Following confirmation of linkage to chromosome 19 it was necessary to refine the region. Refining the region will help to the smallest possible area will reduce the candidate region and will help locate the gene. Initial detection of a genetic locus generally does not yield much precision on its location, thus leaving too vast a region for choosing candidate genes. Fine-mapping refines the genetic region, thereby reducing the number of genes within the candidate region. Fine mapping is therefore an essential step before beginning candidate gene selection in order to reduce the number of potential candidate genes**.**

To fine map the chromosome 19 candidate region it was necessary to locate other microsatellite markers in the region. Only four other microsatellite markers were available in this region, (www.ncbi.nih.nlm, and www.ensembl.org). These markers were: D19S565, D19S247, D19S120 and D19S424. This region is a microsatellite poor region; therefore it was necessary to find additional microsatellite repeats in the region and to design primers to amplify these regions. An additional 10 microsatellite markers were ordered. Linkage analysis results of these markers obtained a maximum LOD score of 3.46 for marker CA19S2310 which is located at 6.57 cM.

Haplotype reconstruction revealed that all 11 affected individuals carried a common haplotype. A recombination event between markers D19S878 and D19S565 in individual PATH053 allowed for the defining of the 19p13.3 RLS region flanked by the telomere and microsatellite marker D19S565. Therefore an overall candidate region of 6.57 cM corresponding to 2.5 Mega bases (Mb) on chromosome 19p13.3 has been identified.

Individual PATH208, is unaffected, however shares part of the affected haplotype. Two theories can explain this individual's phenotype. In the first scenario, individual PATH208 is a carrier of only a portion of the overall candidate region, spanning 1.1 Mb on the physical map. The gene responsible for RLS in this family may be positioned elsewhere on the chromosome 19p13.3 locus. The second theory is that PATH208 is a non-symptomatic member. He may carry the affected haplotype but symptoms and genetic traits are not being expressed (incomplete penetrance). It has been previously noted that incomplete penetrance is seen in one third of cases with autosomal dominant RLS (Hamet and Tremblay 2006).

A maximum multipoint LOD score of 3.77 was obtained at a position of 6.64 cM on chromosome 19, the nearest marker being D19S878 which is positioned at 6.57 cM. This confirmed linkage to the chromosome 19p13.3 region and also localises the position of the gene more precisely. It is probably not a coincidence that the highest two-point LOD score was also observed at this microsatellite marker, D19S878 (LOD=3.59 at θ=0.00). Therefore the multipoint analysis shows that the most likely map location for the RLS gene is close to D19S878. Multipoint LOD scores are potentially more informative than two point LOD scores as they have the ability to pinpoint a disease gene location in the fine-mapping of a Mendelian disorder. Nonetheless, as seen in this study multipoint LOD scores will not produce a higher absolute value at a specific marker if the marker itself is already fully informative for linkage. In this study a maximum LOD score of 3.77 (θ=0.00) was observed, which was only slightly higher than that seen for the two-point LOD score (3.59 at θ=0.00).

In conclusion a novel locus for RLS has been identified on chromosome 19p13.3. The next step is to search the region for candidate genes. It can be anticipated that

the detection of the underlying gene defect at 19p13.3 will add to our current understanding of the underlying pathophysiology of RLS.

Chapter 6

Sequencing

6.1 Introduction

The novel RLS locus which was identified spanned a region of 6.57 cM corresponding to 2.5 Mb on chromosome 19p13.3. The next obvious step was to search the region for candidate genes in the hope of finding the underlying gene defect.

The first stage of this part of the study was to select candidate genes from within thes novel locus. Due to the fact that no causative gene had yet been identified in any of the previously described loci, it was not possible to choose candidate genes based on known genes. The candidate genes therefore were selected on the rationale that the pathophysiology of RLS may be closely linked to the dopaminergic system and/or iron metabolism. As previously mentioned (section 1.6), the pharmacological data that strongly lend support to a dopaminergic abnormality and iron depletion is regarded as the most relevant additional factor for the clinical manifestations of the syndrome. The chromosome 19p13.3 locus is flanked by the telomere and microsatellite marker D19S565. The region is 6.57 cM (genetic length) corresponding to 2.5 Mb (physical length). There are approximately 100 genes in this region and were identified using the human genome public database websites http://genome.ucsc.edu/ and www.ensembl.org. Many of the genes had known functions, for example *MADCAM1,* which is a cell adhesion leukocyte receptor expressed by mucosal venules; and *PPAP2C*, which is a member of the phosphatidic acid phosphatise family and is found mainly in the brain and placenta. Other genes were expressed in tissues unlikely to be related to RLS, for example *C19orf22* which is expressed in whole blood, B cells and T cells. The genes within the chromosome

19p13.3 locus were narrowed down to 8 possible candidate genes, and from this two of the more promising genes within the candidate interval were chosen for screening.

6.1.1. *KLF16*

The first gene selected for screening was *KLF16* (Krueppel-like factor 16) otherwise known as Transcription factor *BTEB4* (Basic transcription element-binding protein 4) and *DRRF* (dopamine receptor regulating factor). Transcriptional regulation is governed by the coordinated action of regulatory factors that bind to specific DNA elements. *KLF16* (*DRRF*) binds to GC and GT boxes in the D1 and D2 dopamine receptor promoters and effectively displaces Sp1 and Sp3 from these sequences. Subsequently, *DRRF* can modulate the activity of these dopamine receptor promoters. In a study published in 2001, it was reported that mice treated with drugs that increase extracellular striatal dopamine levels (cocaine), block dopamine receptors (haloperidol), or destroy dopamine terminals (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) show significant alterations in *DRRF* mRNA (Hwang, D'Souza et al. 2001). *KLF16*, therefore, is an excellent candidate gene for RLS as it is important for modulating dopaminergic transmission in the brain.

6.1.2 *GAMT*

The second gene selected for screening was *GAMT* (guanidinoacetate N-methyltransferase. It is also known as *PIG2* and *TP53I2*. *GAMT* supplies instructions for making the enzyme guanidinoacetate methyltransferase. It is active in the pancreas, liver and kidneys. This enzyme participates in the two-step synthesis of the compound creatine from the amino acids glycine, arginine and methionine. *GAMT* gene mutations impair the ability of the guanidinoacetate methyltransferase enzyme to participate in creatine synthesis (http://ghr.nlm.nih.gov/). The effects of guanidinoacetate methyltransferase deficiency are most severe in organs and tissues that require large amounts of energy, such as the brain and muscles. Increased serum concentration of muscle creatine phosphokinase is observed in the majority of affected individuals with choreoacanthocytosis (ChAc) (Hiersemenzel, Johannes et al. 1996). Choreoacanthocytosis is characterised by a progressive movement disorder. Although the movement disorder is mostly limb chorea, some individuals present with a parkinsonian syndrome (http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=gene&part=chac).

6.1.3 DNA sequencing

The principles of DNA sequencing is based on the dideoxy sequencing method developed by Fred Sanger. The DNA is present in a single stranded form and acts as a template for making a new complementary DNA strand in vitro using a suitable DNA polymerase. Cycle sequencing is similar to PCR as it includes denaturation, annealing and DNA synthesis and it uses a heat stable DNA polymerase. In addition, however, it uses dideoxynucleotide triphosphates (ddNTPs), and only a single oligonucleotide primer is required. The ddNTPs are closely related to the normal dNTPs; they differ only in that they lack a hydroxyl group at the 3' carbon position as well as the 2' carbon. Due to the fact that ddNTPs lack a 3' hydroxyl group, any ddNTP that is incorporated into a growing DNA chain cannot participate in phosphodiester bonding at it 3' carbon atom. This thereby causes abrupt termination of chain synthesis. By setting the concentration of the ddNTP to be much lower than that of the dNTPs, there will be competition between a specific ddNTP molecule and an surplus of analogous dNTP molecules for incorporation into the growing DNA chain. Therefore if a dNTP is incorporated, chain extension will continue; however if a ddNTP is (occasionally) included it causes chain termination. Thus, each reaction is only a partial reaction, as chain termination will take place randomly at one of the possible choices for a specific type of base in any one DNA strand. The sequence cycle will therefore result in the synthesis of differently sized labelled DNA fragments. Figure 6.1 demonstrates the dideoxy DNA sequencing with the random incorporation of ddNTP causing a chain termination (Strachan and Read 2003).

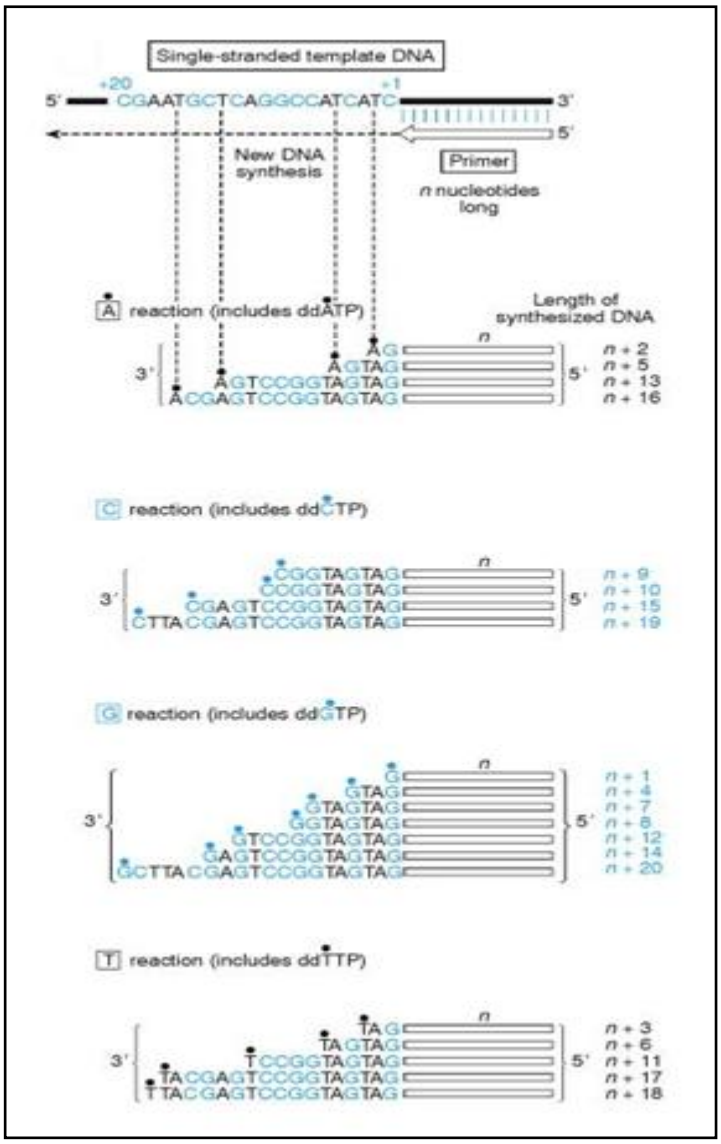


Figure 6.1: A schematic diagram explaining Sanger's dideoxy DNA sequencing

The sequencing primer binds to a region 3′ of the desired DNA sequence and primes synthesis of a complementary DNA strand in the indicated direction. Four parallel base-specific reactions are carried out, each with all four dNTPs and with one ddNTP. Competition for inclusion into the growing DNA chain between a ddNTP and its normal dNTP analog results in a population of fragments of different lengths. The fragments will have a common 5′ end (defined by the sequencing primer) but variable 3′ ends, depending on where a dideoxynucleotide (shown with a filled circle above) has been inserted. For example, in the A-specific reaction chain, extension occurs until a ddA nucleotide (shown as A with a filled black circle above) is incorporated. This will lead to a population of DNA fragments of lengths $n + 2$, $n + 5$, $n + 13$, $n + 16$ nucleotides, etc.

6.2 Materials and Methods

6.2.1 Primer design

KLF16

According to the UCSC Genome Browser (Feb. 2009), two versions (A and B) of *GAMT* exist (figure 6.2). Introns are depicted as lines with arrows indicating the direction of transcription. The arrows are pointing to the left, therefore transcription occurs on the negative strand. Version A of *KLF16* has two exons in total. The second exon, which is the one to the far right, is quite large, spanning approximately 2 kb. Version B of *KLF16* has three exons in total. Exon one is identical to that seen in version A. Exons two and three are located in the same location as the second exon in version A, the only difference being there is approximately 1.0 kb of an intronic region dividing the two exons. To ensure comprehensive screening of *KLF16*, both versions of the gene required screening. Exon 1 was identical in both versions and was designated x1. By screening the second exon of version A, exons two and three of version B was also covered.

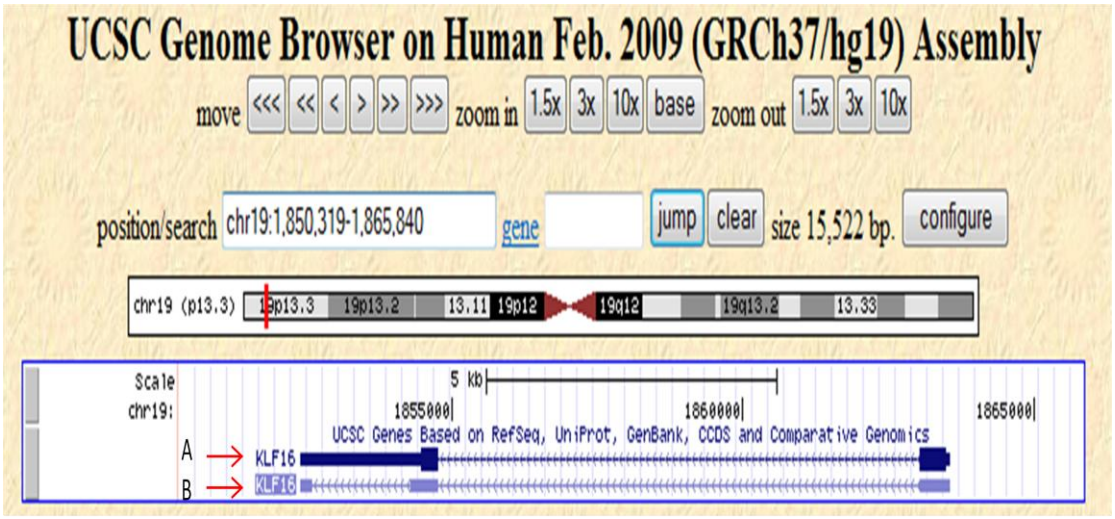


Figure 6.2: Chromosome 19 and *KLF16* gene

This figure shows the chromosome 19 ideogram, with a red vertical bar indicating the position of *KLF16* on the chromosome. **A** represents the UCSC version of the *KLF16* gene. The two exons are represented by the blue filled rectangles. The introns are represented by the arrowed lines in between the exons, with the arrows indicating the direction of transcription. The arrows are pointing to the left, therefore transcription occurs on the negative strand. **B** is a representation of the *KLF16* gene based on another database. This version only has three exons. UCSC Genome Browser, February2009 Assembly (http://genome.ucsc.edu).

GAMT (Primer Design)

According to the UCSC Genome Browser (Feb. 2009), two versions of *GAMT* exist. These two versions can be observed in figure 6.3 below. Introns are depicted as lines with arrows indicating the direction of transcription. In this case intron orientation is negative, therefore arrows point to the left. Version **A** of *GAMT* has six exons (black rectangles). Version **B** of *GAMT* has only five exons, however. Exons 1 to 4 (represented by the blue rectangles) are identical to exons 1-4 in version **A**. Nonetheless, exon 5 is somewhat larger than that of version **A**, and exon 6 does not exist. Hence, to ensure comprehensive screening of *GAMT*, both versions of the gene required screening. Exons 1 to 4 were identical and were therefore designated x1, x2, x3, x4. By screening the fifth exon of version **B**, exon 5 of version **A** was also covered. The fifth exon of version **B** was designated x5. Exon 6 of version **A** was designated x6. According to ensembl (www.ensembl.org) only one version of *GAMT* exists, and this is identical to version A (http://genome.ucsc.edu).

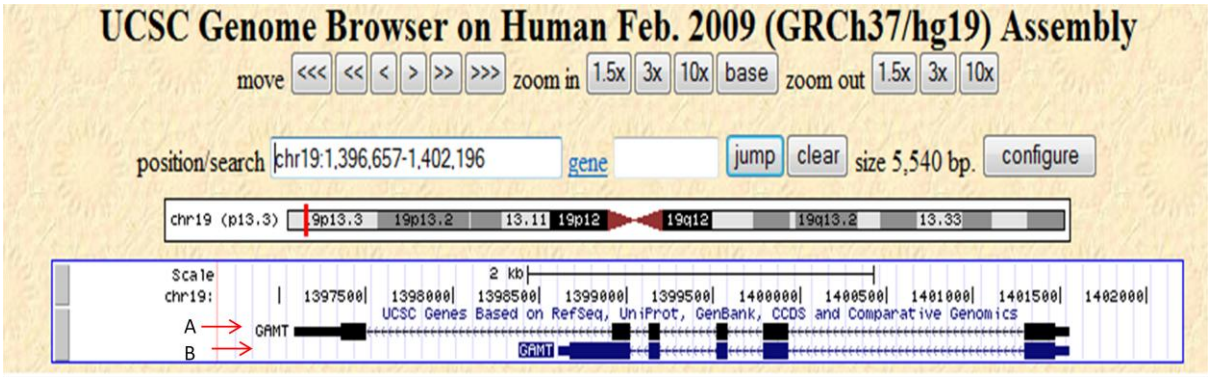


Figure 6.3: Chromosome 19 and GAMT gene
This figure shows the chromosome 19 ideogram, with a red vertical bar indicating the position of *GAMT* on the chromosome. **A** represents the UCSC version of the *GAMT* gene. The six exons are represented by the black filled rectangles. The introns are represented by the arrowed lines in between the exons, with the arrows indicating the direction of transcription. The arrows are pointing to the left, therefore transcription occurs on the negative strand. **B** is a representation of the *GAMT* gene based on another database. This version only has five exons. UCSC Genome Browser, February2009 Assembly (http://genome.ucsc.edu).

6.2.2 Pedigree RLS3002

When carrying out the genome-wide scan it was necessary to genotype all members of the RLS3002 family. However, when sequencing a candidate gene, it is not necessary to screen every member of the family. For the purpose of screening the two candidate genes *KLF16* and *GAMT*, DNA from one unaffected and two affected members of the pedigree were screened. The two affected members screened were first cousins, PATH048 and PATH054. One unaffected member of the family was screened, PATH047. This individual is the father of individual PATH048, which would mean that any variant observed in the daughter (PATH048) and not in the father would have been inherited from the affected mother or less likely, it would be a novel mutation. PATH047 is married into pedigree RLS3002, which also means that any variation he has is unlikely to be disease causing. The three members chosen for screening the candidate gene can be observed in figure 6.4.

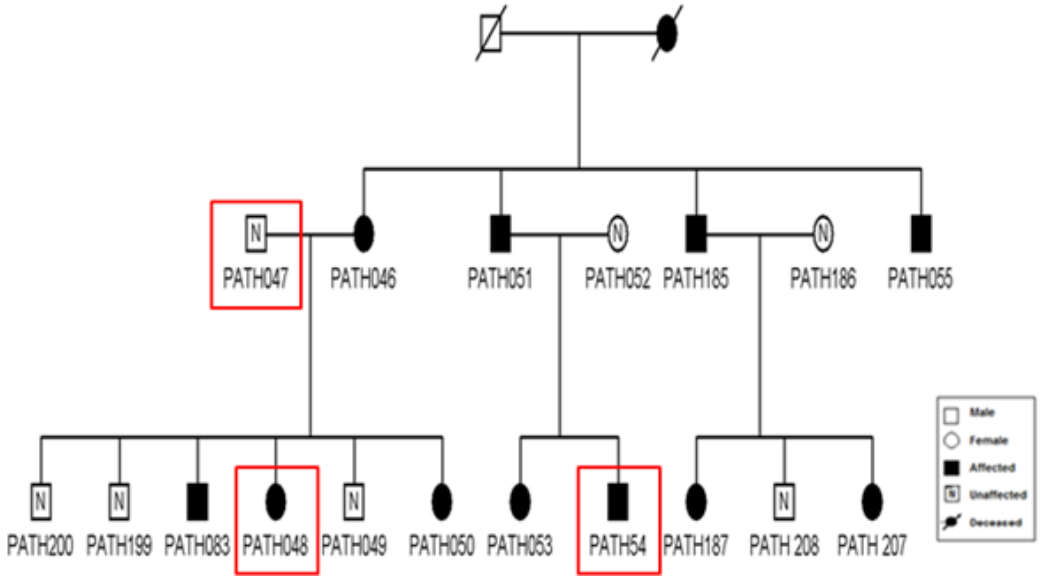


Figure 6.4: Members of RLS3002 chosen for candidate gene screening
Only three members of RLS3002 were used for screening the candidate genes. Two affected members, PATH048 and PATH054, who are first cousins, were screened. PATH047, father of PATH048 is an unaffected member and was also screened. The three individuals used for this part of the study are indicated by the red boxes.

6.2.3 PCR

PCR was carried out in 50 µl reactions. A total of 5 µl of genomic DNA was used as a template for amplification reactions. Table 6.1 provides details of the reagents used. The table contains the name of the reagent, the stock concentration, the concentration used, and the amount of each reagent in a 50 µl reaction volume.

Table 6.1 Reagents used for PCR in 50µl reaction volumes.

Reagent	**[Stock]**	**[Final]**	**1 sample (µl)**
10x buffer	10x	1x	5.00
$MgCl_2$	25 mM	1.5 mM	3.00
dNTP	10 mM	0.2 mM	1.00
Forward Primer	100 ng/µl	100 ng	1.00
Reverse Primer	100 ng/µl	100 ng	1.00
Taq polymerase	5 U/µl	0.05 U	0.15
H_2O			33.85
DNA		50-100 ng	5.00
Total Volume			50.0

Once the samples were prepared, each 50 µl reaction was placed in a 0.2 ml capped PCR tube and placed on the GStorm Thermal Cycler PCR machine. A temperature gradient was initially used to discover the optimal annealing temperature for each individual marker (as outlined earlier in section 3.2). The success of PCR was verified by agarose gel electrophoresis (as outlined in section 2.4).

6.2.4 DNA sequencing

Automated DNA sequencing requires 1) fluorescence labelling of DNA and 2) a detection system. Dideoxynucleotides to which fluorophores (chemical groups capable of fluorescing) are attached are used. This results in each nucleotide base being attached to a specific fluorophore. Since a different label is used for each nucleotide, the cycle sequencing will produce differently sized DNA fragments of the desired sequence with each base in the sequence tagged with a different fluorescent label. During electrophoresis, using the ABI3130 Genetic Analyser, the fluorescence signal is detected and recorded as the DNA passes through a fixed point in the capillary. The output is in the form of intensity profiles for each of the differently coloured fluorophores and the information is simultaneously stored electronically (Strachan and Read 2003).

Following amplification of the desired region by PCR (section 6.2.2 above), the sequencing protocol used for this study comprises of the following steps:

a. PCR purification;
b. Sequencing reaction;
c. Post-sequencing purification;
d. Detection of sequences using the ABI3130 genetic analyser.

a. PCR purification

To achieve good quality sequence data using the Sanger dideoxy sequencing method and fluorescently labelled nucleotides, it is necessary to remove the excess primer and dNTPs from the initial PCR reaction performed (section 6.2.2). JETQUICK Spin Columns were used according to the manufacturer's guidelines as described in section 2.8.

b. Sequencing reaction

The purified PCR products were then sequenced. As previously mentioned this technique is based on Fred Sanger's dideoxy sequencing method (section 6.1.3). This will result in differently sized DNA fragments of the desired sequence with each base in the sequence tagged with a different fluorescent label. The reaction was carried out according to the protocol detailed in section 2.9.

c. Post-Sequencing Purification

Purification of sequencing products was carried out using the AmpliPurify^{TM} ExTerminator Kit. This optimised purification procedure removes unincorporated dyes, dNTPs and excess salt. It also removes the sequencing enzyme. This was performed according to the protocol outlined in section 2.10.

d. Detection of sequences using the ABI3130 genetic analyser

The instrument is used to perform the electrophoresis step and to monitor the different fluorophores (colours) as they pass by and are detected by the laser. The samples are run on the ABI3130 according to guidelines outlined in section 2.11.1.

6.2.5 Sequence Analysis

The sequences acquired from the ABI3130 were analysed using SeqMan programme from DNAstar software. SEQMAN provides a number of useful tools for simplifying the process of assembling data from a sequencing project. It allows the user to perform alignments and discover variants, along with many other analyses on de novo or template assemblies. SeqMan allows the user to assemble anything from two sequences to tens of thousands of sequences into contigs (figure 6.5).

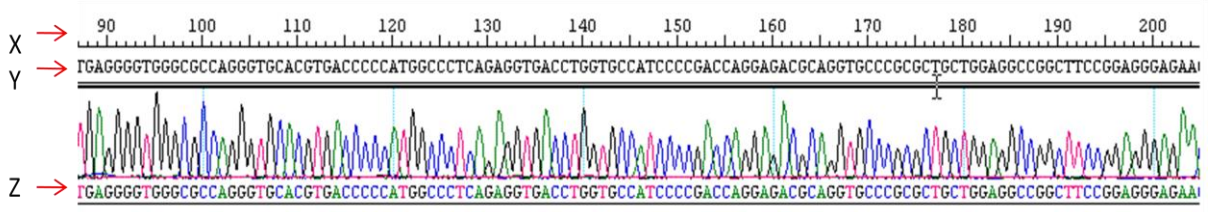


Figure 6.5: DNA sequence data from the Seqman programme
A series of peaks are seen each of which corresponds to a specific base. Blue = C (Cytosine), black = G (Guanine), green = A (Adenine) and red = T (Thymine). X points to the position of the sequence (bp), Y points to the consensus sequence and Z points to the sequence of the sample. The example illustrated here shows a section of sequence from *GAMT* exon 6a, sample PATH054.

Sequences were compared with the expected amplicon and with other samples. Any variation present was recorded and both affected and unaffected individuals were compared for the presence or absence of variations. For a variation to be significant within this family, it must be present in samples from both of the affected individuals (PATH048 and PATH054) and not present in the unaffected individual (PATH047). If a variation was detected in both affected individuals, various websites (http://genome.ucsc.edu, http://www.ensembl.org and http://www.ncbi.nlm.nih.gov/) should be searched to determine whether these variations are known single nucleotide polymorphisms (SNPs).

6.3. Results

6.3.1 Primer Design

KLF16

As described in section 6.2.1, two versions of *KLF16* exist according to UCSC Genome Browser, February2009 Assembly (http://genome.ucsc.edu). However, by screening version A of *KLF16*, version B is included within this. Therefore, two exons in total required screening.

The first exon (*KLF16*x1) was 524 bp long. Initially, only one set of primers was designed to amplify this exon. However, after numerous attempts to amplify this fragment, another set of primers were designed. One of the main limitation within a sequencing reaction is sequence length, therefore this exon was divided into smaller overlapping fragments. Exon 1 was divided into four overlapping fragments, x1a, x1b, x1c, and x1d. Only a forward primer was ordered for x1c, as the reverse of x1d was to be used to amplify this inner region of exon 1.

The second exon, *KLF16*x2, was a large exon, encompassing 2,362 bp. Similar to exon 1 this exon was also divided into smaller overlapping fragments. Exon 2 was divided into five overlapping fragments, which were designated: x2a, x2b, x2c, x2d and x2e (illustrated in figure 6.6).

As work progressed, a further three sets of primers were ordered. This was because gaps remained between sequences after analysis of the sequences using the original primers and so a complete sequence of exon 2 was not obtained. One gap was located between x2b and x2c. In total, two more pairs of primers (*KLF16*x2gap1 and *KLF16*x2bgap) were required to obtain sequences to close this gap.

A second gap of sequence was present between x2d and x2e. It was necessary to design and order an additional set of primers (*KLF16*x2gap2) to cover this gap.

The primer sequences, annealing temperatures, and expected sizes of all the *KLF16* fragments are shown in table 6.2. A number of the fragments failed to amplify, therefore an asterix (*) represents unknown annealing temperature in Table 6.2. Figure 6.6 shows *KLF16* and its the positions of the fragments used to sequence the gene.

Table 6.2: Features of primer fragments used to screen *KLF16*.

Fragment	**Forward Primer Sequence**	**Reverse Primer Sequence**	**Annealing Temp (°C)**	**Size (bp)**
KLF16x1	CCCCACGCGCCACGCAGTTT	GGGCGGGGCGGGCTGTG	*	761
KLF16x1a	AGAGGCCCAGCAGGAGCAGGAAC	AGCACGTCGGCGGCGAAGTAATC	*	1865
KLF16x1b	TCGCCGCCGACGTGCTCAT	TCAATCCCGCCCGCAGACCTC	*	691
KLF16x1c	GCGCGGCTCGGAGAGTGGTG		*	*
KLF16x1d	CCGTGGCGTGCGTGGATTACTT	CTGGCGGGGGAGGGGTCTC	*	509
KLF16x2a	GGGGGCGGGTAAGGGTTGTATTCT	GGGGGCAGGGGTGTCTTCAGG	67	600
KLF16x2b	GCCCTCGTGCAGCCCACCCTGAC	CGCTGCCCCACTCCTGGCTCTACC	70	630
KLF16x2c	CCACCGAAAGCCATGGGAGTAGTT	CACAGCCAGCCCTCTGCTTCCAGT	70	657
KLF16x2d	CCGCCGGGGTTGCAGGCTCTT	TCCCCCACCCACCCCCACAGACTA	69	766
KLF16x2e	GGGGGCTTTGGATGGCACTG	CCCTCCGCGGGGCTTAGG	69	750
KLF16x2gap1	GGGTGACGGGGGCTTGTGTCTGTA	CATCCTAACGCTGCCCCACTCCTG	70	447
KLF16x2gap2	GTTGGCTGCAGGAGGTTTTTG	GAGGGGCCCAGTTATAGAGTTCTG	63	413
KLF16x2bgap	GGGTGGAGTGGACTTCTGG	TCAACCCCCGACTCCTCT	66	105

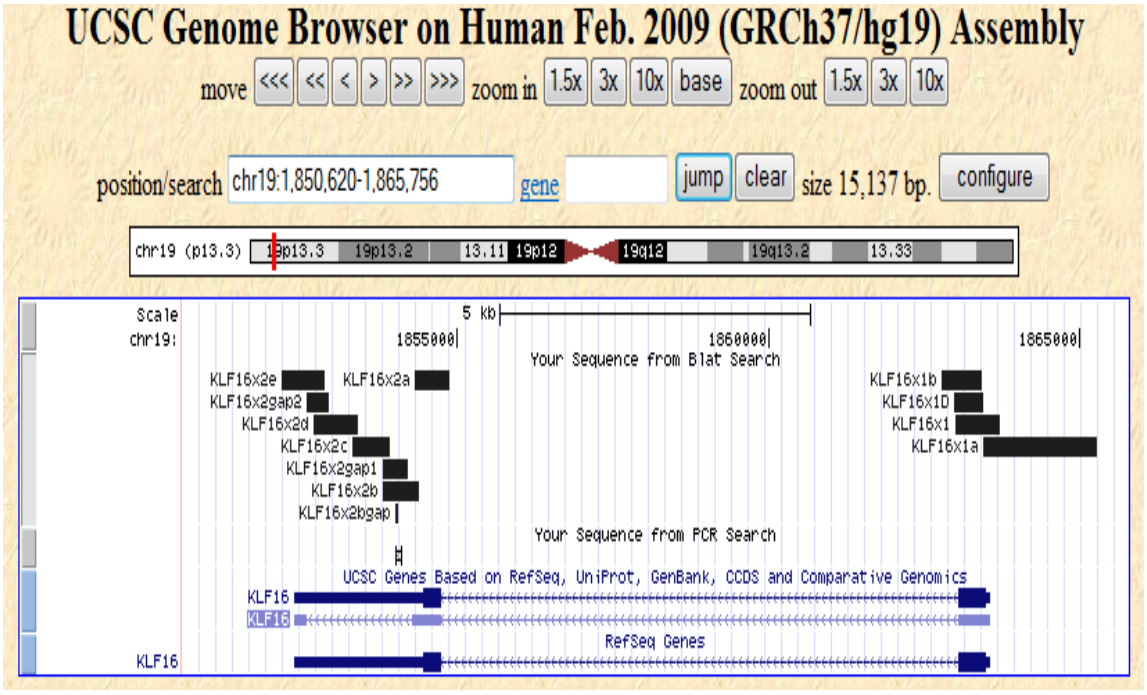


Figure 6.6: *KLF16* gene, its exons and amplicons (primer fragments)

The horizontal line with arrows at the base of the picture represents the *KLF16* gene to be sequenced. The two blue rectangles at either end represent exon 1 and exon 2. The fragments to be sequenced using the primers which were previously described (Table 6.2) are represented by the black rectangles. The name of each fragment is indicated. Both exons are covered by the fragments and from the diagram it is clear that many of the exons overlap. This is to ensure that all nucleotides are examined.

GAMT

As previously mentioned (section 6.2.1), two versions of the *GAMT* gene exist. To ensure comprehensive screening of *GAMT*, it was necessary to screen both versions of the gene. *GAMT* had six exons which required screening and they were designated x1, x2, x3, x4, x5 and x6. Initially five sets of primers were designed using the PrimerSelect program, and were ordered from Biomers (http://biomers.net). Exons 1 and 6 had one set of primers each (*GAMT*x1 and *GAMT*x6). Three sets of primers were designed to cover exons 2 to 3 (x2-3) and exons 4 to 5 (x4-5a and x4-5b). As work progressed, a further two sets of primers were required to obtain sequences not detected by the original primer products. The first additional set which was designed included primers for fragments to cover x1 (*GAMT*x1a and *GAMT*x1b), and primers for fragments to cover x6 (*GAMT*x6a and *GAMT*x6b). It was later necessary to design and order an additional set of primers (*GAMT*x1.2) to cover a large gap which remained in x1. The primer sequences, annealing temperatures, and expected sizes of the *GAMT* fragments are shown in Table 6.3.

Table 6.3: Features of primer fragments used to screen *GAMT*. (*) represents unknown annealing temperatures.

Fragment	**Forward Primer Sequence**	**Forward Primer Sequence**	**Annealing Temp (°C)**	**Size (bp)**
GAMTx1	CCGACCCCACTCCCGCCACCTCTC	GCCCCCACCTGCAGCCCCACTTC	*	509
GAMTx1a	GTAAGCGGCAAGGCGGGGATTC	CAGCGCGTGCATATAGGGGGTCTC	*	465
GAMTx1b	CCCAGCGCGACCCCCATCTT	GGACCCGGTTCCACCCAGTTTCA	63	477
GAMTx1.2	AGCGCCTGGAAGATCCTCAGAT	CAGCCCCGGCCTCAGTTT	53	631
GAMTx2-3	CGCCCCCTCCTCCAGGCAGTCT	CGCCCCCTCCTCCAGGCAGTCT	63	701
GAMTx4-5a	GGGTGTGGGCCTGGCTGTGG	CACCCCCATCCAAGGTCACTTCCT	70	555
GAMTx4-5b	GGAGGCCCTCGGGGAAGCG	TACCCAGGCTGGAGTGCAGTGGTT	70	702
GAMTx6	CCCGCACTGGGGAGCTGGAGATTC	CTCCAGGTTTCTGAGCGGCCCCAG	*	615
GAMTx6a	CACTGGGGAGCTGGAGATTCC	AGCGCCGGCGTTTACTTCAC	69	574
GAMTx6b	CCGCTACTACGCCTTCCC	GGGGCCGCTCAGAAACC	69	422

Figure 6.7 illustrates the positions of the fragments used to sequence the *GAMT* gene.

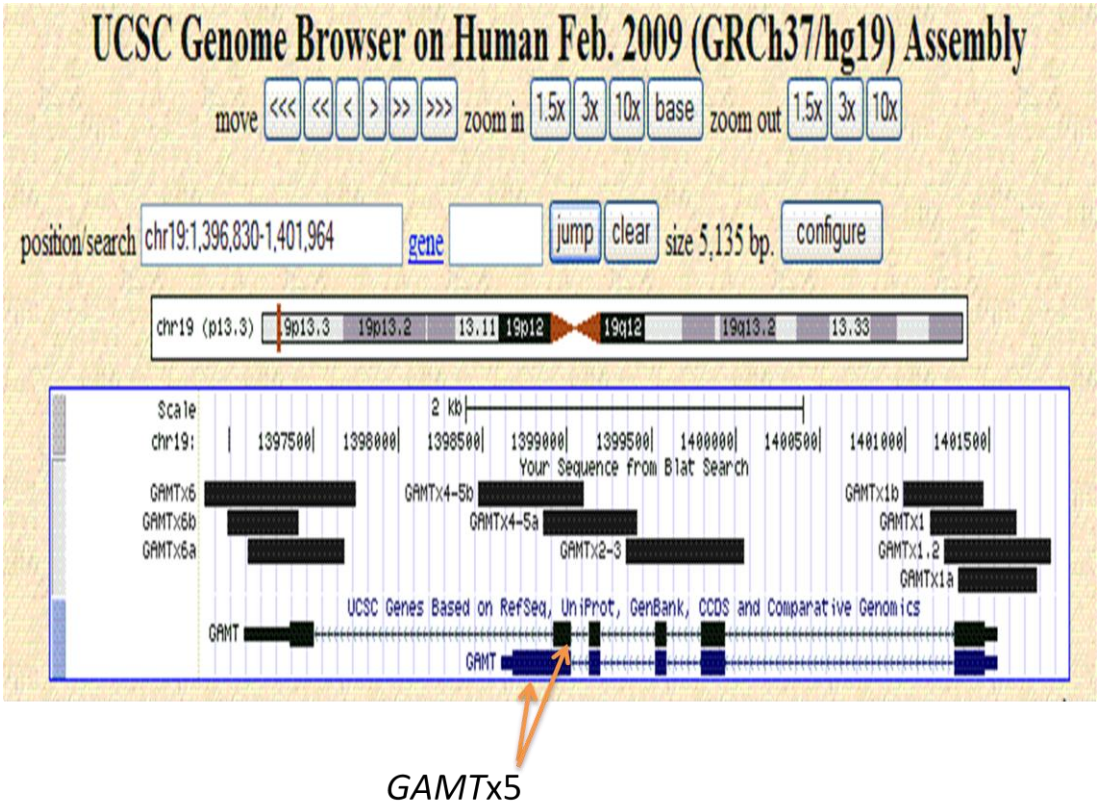


Figure 6.7. *GAMT* gene, its exons and amplicons (primer fragments)
The two horizontal lines with arrows at the base of the picture represents the two versions of *GAMT* to be sequenced. The black and blue rectangles located at intervals along these lines represent exons 1 to 6. From the figure, it can be seen that two versions of exon 5 exist. The larger exons was screened. The fragments to be sequenced using the primers which were previously described (Table 6.3) are represented by the black rectangles. The name of each fragment is indicated to the left hand side. All six exons are covered by the fragments and from the diagram it is clear that many of the exons overlap. This is to ensure that all nucleotides are examined.

6.3.2 PCR

As mentioned for the purpose of screening KLF16 and GAMT for mutations, three individuals from RLS3002 were selected, two affected individuals (PATH048 and PATH054) and one unaffected individual (PATH047). DNA was amplified for all fragment following PCR optimisation.

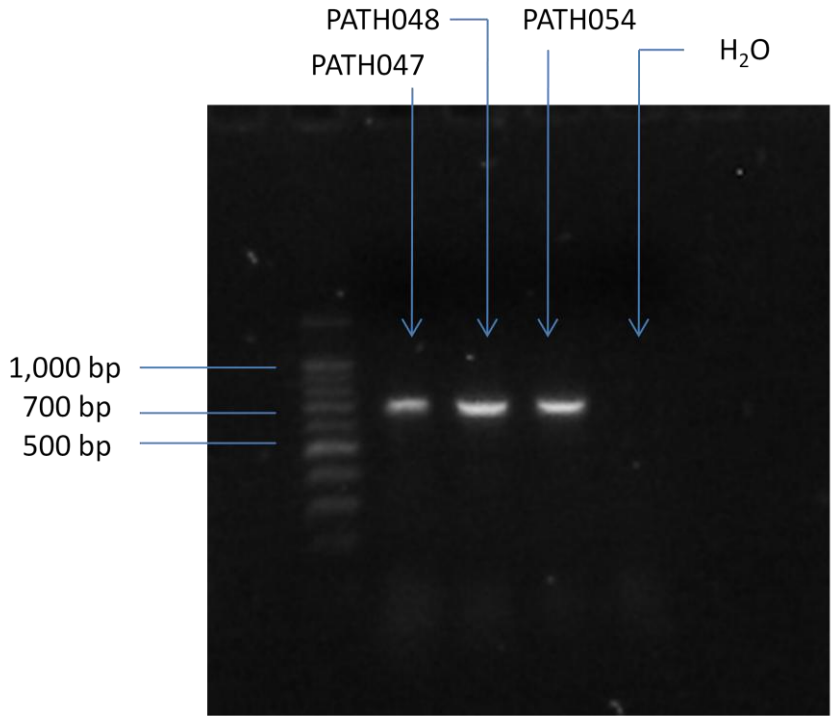


Figure 6.8: Agarose gel electrophoresis of PCR products of *GAMT* x2-3
Samples used are PATH047, PATH048, PATH054 and H_2O (negative control). The 100 bp ladder is to the far left of the gel and the 500 bp, 700 bp and 1,000 bp bands are indicated. The ladder allows assessment of size of the products and also the quality of gel staining. The three samples (PATH047, PATH048 and PATH054) have one single band each, which is located at approximately 700 bp. This is consistent with the expected PCR product size of 701 bp.

KLF16

All fragments for KLF16x2 (KLF16x2a, KLF16x2b, KLF16x2c, KLF16x2d, KLF16x2e) were amplified successfully. An additional three sets of primers (KLF16x2gap1, KLF16x2gap2, KLF16x2bgap) were ordered to cover gaps in the sequence. Fragment KLF16x1 failed to amplify. A number of approaches were applied to attempt to amplify the fragment, e.g. altering annealing temperature, increasing/decreasing $MgCl_2$, however none of these approaches were successful. Upon examining the content of the region, the reason that the fragment was failing to amplify became clear. The region has a very high GC content (81%), and amplification of these regions is usually very difficult. Problems usually observed with GC-rich DNA are constraint of template amplification by stable secondary structures that stall or reduce the DNA polymerase progress, and the presence of secondary annealing sites giving rise to nonspecific amplified bands. Four additional primer sets were designed and ordered for exon 1 (KLf16x1a, KLf16x1b, KLf16x1c, KLf16x1d). When designing these primers, it was ensured that the primers themselves did not have a high GC content. Unfortunately, these four primer pairs failed to amplify also. Therefore, exon 1 of *KLF16* was not sequenced.

GAMT

Following determination of PCR conditions, GAMTx1b, GAMTx2-3, GAMTx4-5a and GAMTx4-5 were amplified successfully. GAMTx1a and GAMTx6 did not amplify successfully. Upon examining the content of GAMTx1, it was discoved that this region has a high GC content (79%) and was possibly the reason GAMTx1a failed to amplify. After several attempts to amplify this region e.g increasing annealing temperature, adjusting $MgCl_2$ concentration, and using betaine, the fragment failed to amplify. An additional set of primers was designed and ordered for this region (GAMTx1.2). These primers were successful in amplifying the remaining region on exon 1. GAMTx6 also failed to amplify after several attempts. Two new sets of primers were ordered for this region (GAMTx6a and GAMTx6b). These primers allowed successful amplification of exon 6.

6.3.3 DNA sequencing and Sequencing analysis

The next step was DNA sequencing and sequence analysis of all three individuals to check for variation in the exons of *KLF16* and *GAMT*. Sequences were compared with the expected amplicon and with one another. Any variation present was recorded and examined for presence/absence on the affected and unaffected individuals. For a variation to be significant with respect to RLS within this family, it must be present in the affected individuals (PATH048 and PATH054), and not in the unaffected one (PATH047).

KLF16

Three variants were detected in KLF16x2. Table 6.4 lists these variations, the chromosome position, the variant type, and the SNP title.

Table 6.4: Variants found in gene *KLF16*.

Exon	**Chromosome 19 Position (bp) [UCSC Genome Browser Feb. 2009]**	**Variant type**	**Comment**	**SNP title**
KLF16x2b	1,805,159	C→G	Present in an unaffected individual (PATH107) and affected family members.	rs12972720
KLF16x2b	1,805,260	GG Insertion	Present in unaffected (PATH047) and affected family members (PATH048 and PATH054)	New variant
KLF16x2d	1,853,667	G→C (Het)	Present only in unaffected (PATH047)	rs3746043

The first SNP detected was rs12972720 in exon 2b of *KLF16*. The SNP is a known C to G change in the coding sequence if *KLF16* exon 2. The unaffected member PATH047 was homozygous C/C; his affected son PATH048 was heterozygous (C/G) at that position; and the affected individual PATH054 was homozygous G/G. Therefore both affected individuals carry at least one G, while the unaffected sample has no G. An additional unaffected control PATH107 (which has been used for other RLS studies in the Dept. of Pathology) was sequenced; this was to ensure that the SNP was not significant with respect to RLS within this family. PATH107 was also homozygous G/G.

Although the two affected individuals (PATH048 and PATH054) do not have the same genotype at this position, both do carry at least one G while the unaffected sample has no G (PATH047). Furthermore, the G in PATH048 must have been inherited from her affected parent as the unaffected parent is CC at this position. Another unaffected sample (PATH107) was screened and was determined to be GG at this position. This individual does not have RLS; therefore, the genotype is not the causative variant. Furthermore, the frequency data on the SNP website www.ncbi.nlm.gov/projects/SNP/ published data for four populations for this SNP rs12972720. The European data (pilot.1.CEU) found that 54.2% of the population have a C at this position, while 45.8% of the population have a G.

One can conclude that this SNP is not a disease causing variant for two reasons. Due to the fact that the SNP was also seen in PATH107, an unaffected control, and that it is a known variant common in the European population, one can conclude that this is not disease causing variant.

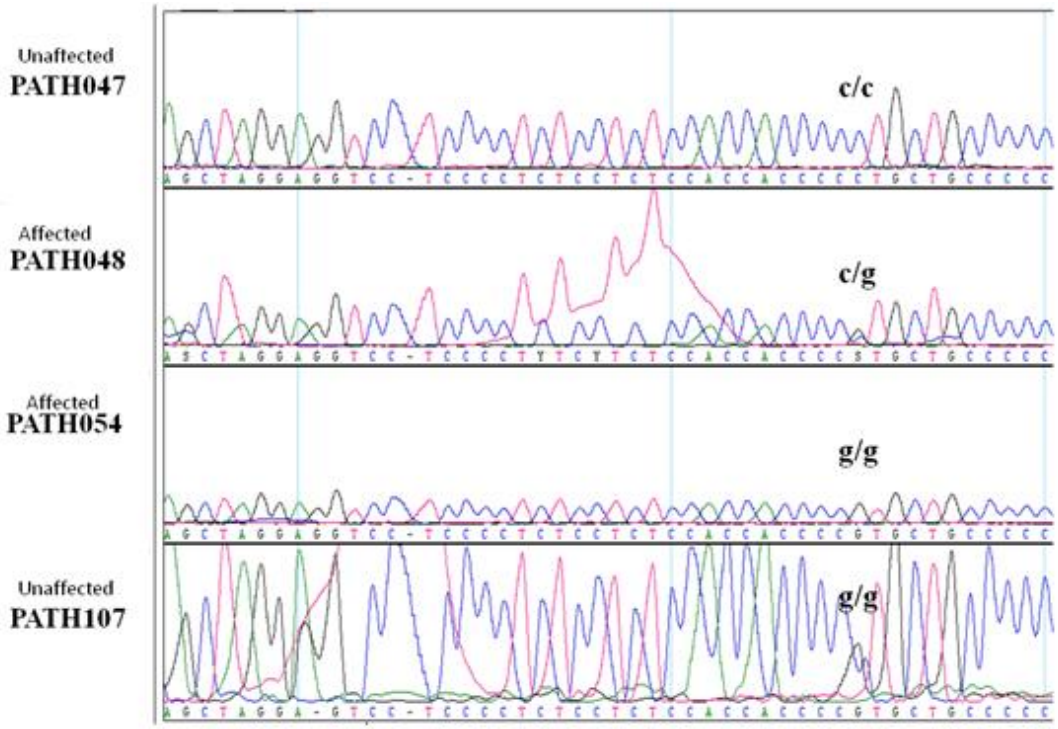


Figure 6.9: A segment of DNA sequence data demonstrating C→G heterozygous change

Exon 2b of *GAMT*. The four individuals screened for the variant are identified in the figure. The unaffected individual has a single blue peak at this position, and is therefore homozygous (C/C). Instead for PATH048 two overlapping peaks (black and blue) are present representing a C→G heterozygous change. Furthermore, a C→G homozygous change is present in PATH054 and PATH107, represented by a single black peak. This change is present in both an affected individual and an unaffected control. Blue = C (Cytosine), black = G (Guanine), green = A (Adenine) and red = T (Thymine).

The second variant detected in KLF16x2 was a single nucleotide insertion seen in both the affected and unaffected family members. PATH054 is homozygous for a single insertion of a G. The insertion was also observed in PATH048, however this sample is heterozygous, carrying one copy of the fragment with the insertion and one copy of the fragment without the insertion. This results in non-identical sequences downstream of the insertion point and this is observed in sequence data when there are two peaks at each nucleotide position. The position is 507bp from the beginning of exon 2 in an untranslated region (UTR). The consensus sequence has 5 Guanines (ggggg); however, PATH054 has 6 Guanines (gggggg) in both copies of sequence.

PATH048 has one copy of each. A number of other affected and unaffected family members were sequenced for this region to determine if they also had this insertion (Table 6.5). Both affected and unaffected family members have a heterozygous insertion and a homozygous G insertion. Hence, it is non-disease segregating.

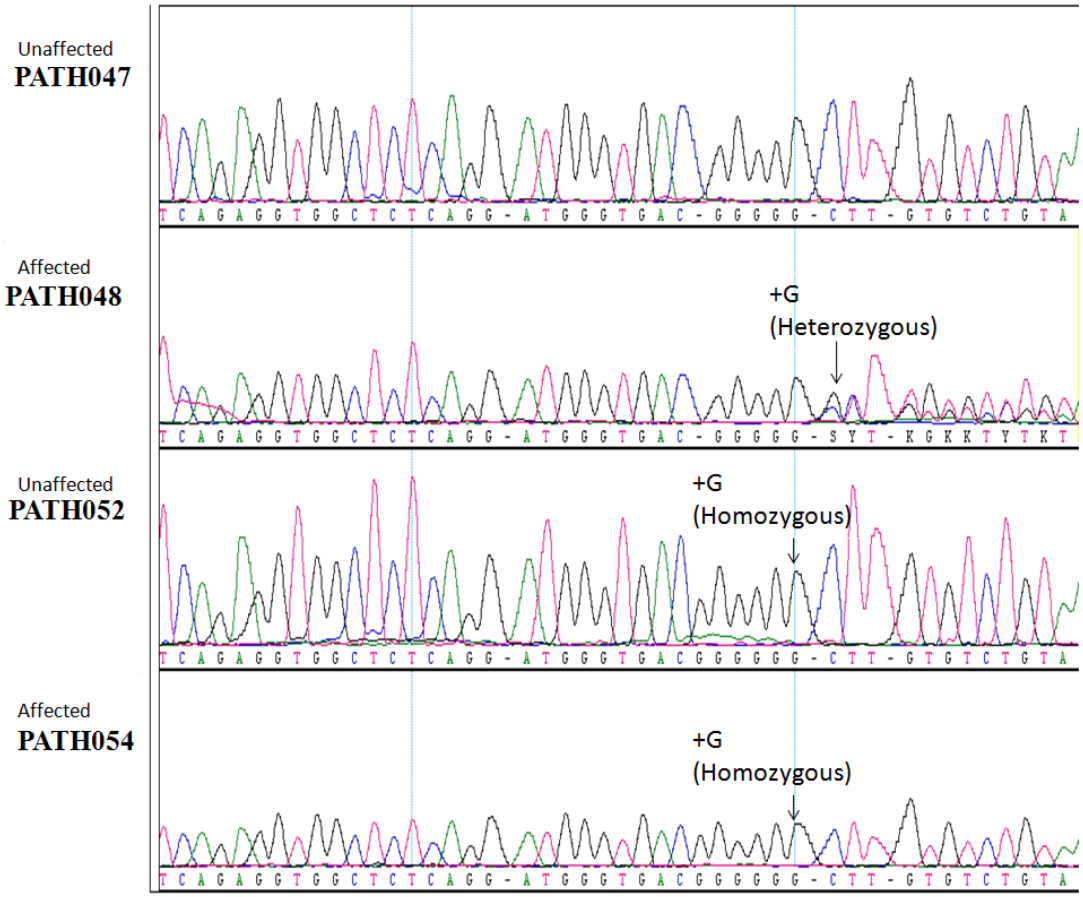


Figure 6.10: A segment of DNA sequence data demonstrating an insertion
The arrows point to positions on PATH047, PATH052 and PATH054 where according to the database sequence there should be a single blue peak representing the base C. However, PATH048 has a G (heterozygous) insertion at this position, represented by the blue and black peak. PATH052 and PATH054 both have a G (homozygous) insertion at this position represented by the single black peak. This inserion is present in both affected and unaffected members of Pedigree RLS3002. Blue = C (Cytosine), black = G (Guanine), green = A (Adenine) and red = T (Thymine).

Table 6.5: Results of family members tested for the G/G insertion in KLF16 x2.

Individual	Status	Insertion
PATH046	Affected	G/G (Hom)
PATH048	Affected	G (Het)
PATH053	Affected	G/G (Hom)
PATH054	Affected	G/G (Hom)
PATH047	Unaffected	Not present
PATH052	Unaffected	G/G (Hom)
PATH199	Unaffected	G (Het)
PATH200	Unaffected	G (Het)

The third variant detected was a G (Guanine) to C (Cytosine) heterozygous shift, in the coding sequence of *KLF16* exon 2. This variant was detected in the control/unaffected family member, PATH047 (figure 6.9). The variant was not present in either of the two affected family members. A number of other family members were sequenced for this SNP, two affected members (PATH053, PATH055) and two unaffected members (PATH052, PATH186). This variant was not detected in any of the other family members. Therefore this is not the disease causing mutation. It was only detected in one 'married in' family member.

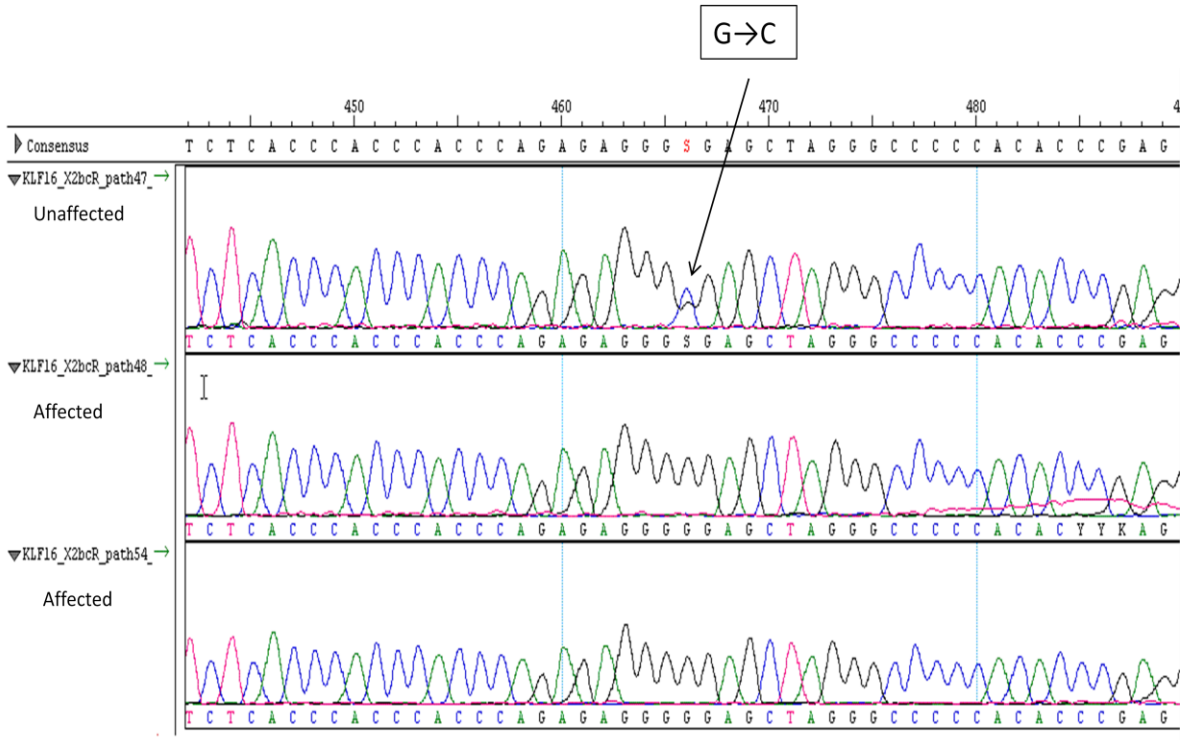


Figure 6.11: A segment of DNA sequence data demonstrating G→C heterozygous change.

Exon 2bc of *GAMT* . The arrow points to a position (PATH047) where according to the amplicon should have a single black peak representing the base G, but instead two overlapping peaks (black and blue) are present representing a G→C heterozygous change. This is only present in PATH047 (unaffected parent) and not present in the affected individuals. Blue = C (Cytosine), black = G (Guanine), green = A (Adenine) and red = T (Thymine).

KLF16x1 failed to amplify therefore it could not be sequenced.

GAMT

GAMT was successfully sequenced and analysed. No variants were detected within any of the amplicons. The UCSC genome browser database (www.genome.ucsc.edu) was used to investigate if there were any known SNPs within the regions amplified for GAMT. Figure 6.10 illustrates the SNPs located within the amplified regions of *GAMT*.

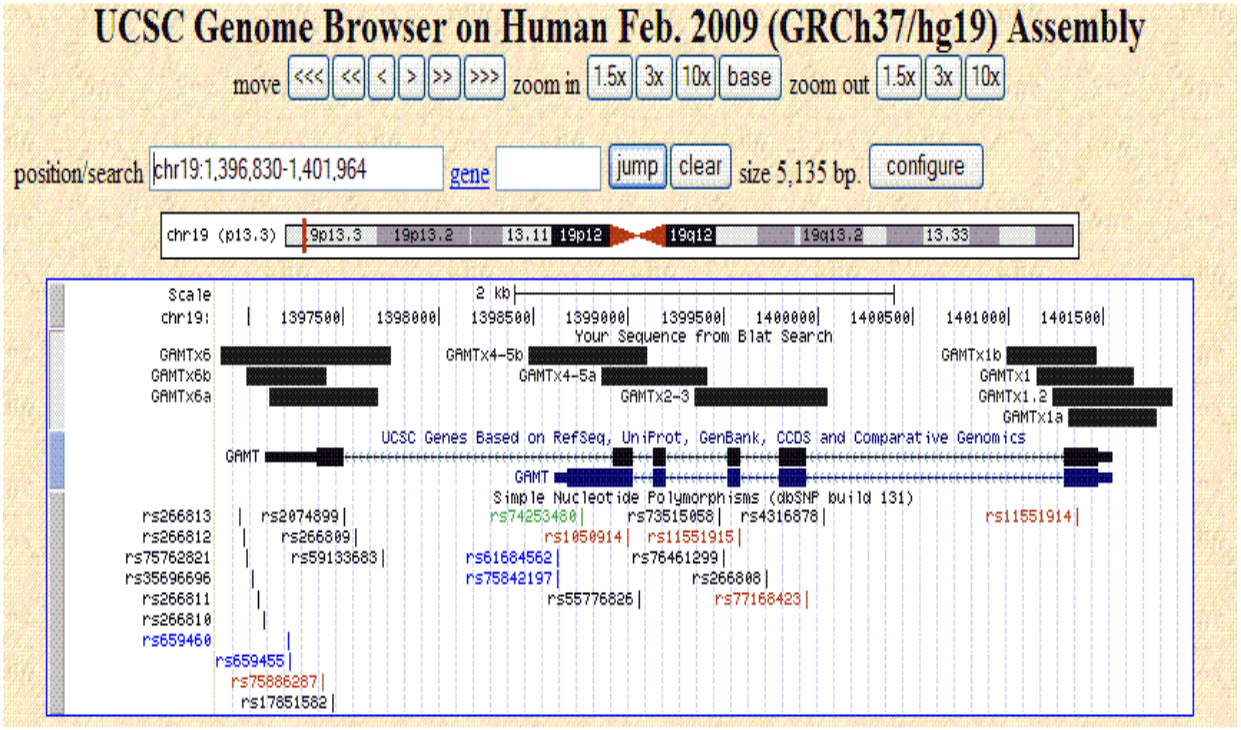


Figure 6.12: SNPs within the GAMT amplicons
The amplicons are represented by the black rectangles. Each amplicon has its own unique name e.g. GAMTx6a, which indicates the exon the fragment is incorporating. The SNPs are shown below the two versions of *GAMT*. There are 25 SNPs within these amplified regions. The SNPs are represented by colours. Black represents Unknown, Untranslated, Locus and Intron SNPs. Green represents Coding/Synonymous SNPs. Red represents Splice sites and Coding/Non-synonymous SNPs. UCSC Genome Browser, February2009 Assembly (http://genome.ucsc.edu).

None of the above known SNPs or no new variant was detected within the three investigated members of pedigree RLS3002 for the gene *GAMT*.

6.4 Discussion

This part of the study examined two candidate genes within the novel RLS locus on chromosome 19p13.3. The first candidate gene which was studied was *KLF16*. Although this candidate region was not very near to the area which gave the highest multipoint LOD score, it was an excellent candidate gene. *KLF16* is important for modulating dopaminergic transmission in the brain. The pathophysiology of RLS is thought to be closely linked to the dopaminergic system and iron metabolism.

Exon 2 of *KLF16* was large and therefore had to be divided into five overlapping fragments of sizes suitable for sequencing. Amplification and sequencing of this exon was successful and three variants were detected. The first SNP detected was rs12972720. This is a synonymous SNP present in the untranslated region of exon 2. The C→G change was observed in both unaffected individuals PATH048 and PATH054. However a second unaffected control for RLS was sequenced and was found to also have this variation. This SNP was a known variant, and according to the population data both the C and G are common in the European population. Therefore this disease most likely has no role in the pathogenesis of RLS.

The second variant was a G insertion in the untranslated region of exon 2. A number of additional family members were examined for this variation and it was found that both affected and unaffected family members carry either a heterozygous or homozygous G insertion. It did not have an effect on amino acid as it is in an untranslated region. This was an unknown SNP; therefore no frequency data was available. Due to the fact the variation was seen in both affected and unaffected family members, this variation was not disease segregating and most likely has no role in the pathogenesis of RLS.

The third variation that was observed in exon 2 of *KLF16* was a G to C change in the unaffected individual, PATH047. This is a known SNP (rs3746043), and is a synonymous SNP, therefore it does not have any effect on the resulting amino acid. A number of additional affected and unaffected family members were examined for this variation, and it was not detected in these samples. The frequency data on the SNP website www.ncbi.nlm.gov/projects/SNP/ published data for five studies for this SNP rs3746043. The European data (pilot.1.CEU) found that 26.4% of the population have a C at this position, while 73.6% of the population have a G. Subsequently, the variant rs3746043 detected in exon 2 of *KLF16* is common within the European population and is unlikely to have a role in the pathogenesis of RLS in this family.

Exon 1 of *KLF16* is not a very large exon, howver it has a high GC content (81%). This exon proved very difficult to amplify, and after several attemps including trying to amplify the fragment with betaine and reordering new primers it failed to amplify. Therefore, *KLF16* cannot be fully excluded as to having a role in the aetiology of RLS in this pedigree.

The second candidate gene which was investigated was *GAMT*. *GAMT* is required for producing the enzyme guanidinoacetate methyltransferase. A deficiency in this enzyme mainly affects organs and tissues that require large amounts of energy, such as the brain and muscles. RLS is a neurological disorder which is characterised by cramps in the lower limbs and often accompanied with muscle spasms.

Exon 1 of *GAMT* was difficult to amplify as it has a high GC content. Additional primers were ordered for this region, and were designed avoiding the GC rich areas.

All of the *GAMT* fragments were amplified successfully for the three family members PATH047, PATH048 and PATH 054.

No SNP or new variant was detected within any of the amplicons analysed for the members of pedigree RLS3002. Firstly there were not many SNPs within the amplicons. Figure 6.12 confirms that there were a total of 25 SNPs within these amplified regions. One of these SNPs, rs73525059, was deleted from the database on May 13, 2010. Therefore there are a total of 24 SNPs within the amplicons of *GAMT*. Furthermore, when the database of these SNPs were investigated further, it was became clear that many of these SNPs are not common in people of European descent. For example, SNP rs266811 had frequency data for two populations. The first was for pilot.1.YRI (Nigerian population) which found an allele frequency of 0.24 for C and an allele frequency of 0.76 for G. The CEPH data (European population), also for SNP rs266811, found an allele frequency of 1.00 for G. Another SNP, rs1050914, found an allele frequency of 1.00 for G. In addition to this, a number of SNPs had only been detected in non-European populations. For example, rs76461299 and rs73515058, had only been detected in Chinese/Japanese and Nigerian population respectively. Therefore it is not unusual that any of the known variants were not detected in the members of pedigree RLS3002. The SNPs within the amplified region were seen mostly within non-European populations, and are not common within the European population.

All six exons of *GAMT* were analysed including splice sites. No mutation was found within the exons of *GAMT* when comparing the two affected members (PATH047 and PATH054) to the unaffected member (PATH047). Sequencing of the candidate

gene *GAMT* did not identify mutations, however variants in either the promoter region or the intronic regulatory elements cannot be excluded.

Chapter 7

Discussion

7.1 Introduction

Restless Legs Syndrome is one of the most common disorders seen in sleep clinics. It is a sensorimotor disorder, characterised by the desire to move the lower limbs due to unpleasant sensations that are worse at rest and in the evenings and are relieved, at least temporarily, by movement. It can be either primary (genetic/idiopathic) or secondary (caused by other conditions). RLS causes tremendous distress and affects the quality of life of those affected. Generally speaking, RLS is not well understood in terms of pathophysiology and symptomatology. Ironically, RLS can be described as one of the most common conditions one has never heard of.

In order to find the causative gene for primary RLS, several genome wide studies have been completed and 10 loci have been identified on chromosomes 5q, 12q, 14q, 9p, 2q, 20p, 4q, 17p, 16p and 19p. Several association studies have also been performed and to date five genomic regions associated with RLS have been identified on chromosomes 6p, 2p, 15q, 9p and 12q.

The UCC Pathology lab has identified and collected Irish families with RLS. In one large autosomal dominant (AD) family, a novel RLS locus has been determined. The research lab also identified and recruited a second Irish AD RLS family (RLS3002). Previous research on this family had partially excluded four of the known loci (Abdulrahim 2008). The aim of this study was to examine all known loci and associated regions for linkage to this family, and if linkage was not found to perform a genome-wide search to map and identify the causative gene in this RLS family

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7.2 Discussion

RLS is a difficult to define condition that is possibly more prevalent than previously thought. Public and professional awareness of RLS has certainly increased in the last number of years, but questions, even skepticism about the very existence of the condition, persist (Varga, Ako-Agugua et al. 2009). According to Professor Richard P. Allen, Secretary of the World Association of Sleep Medicine and Committee Member of World Sleep Day 2009 he has " conducted medical research in the field of RLS for over 30 years, but still encounter many misconceptions about the condition."

In 2005 Requip (ropinirole) became the first drug approved by the FDA for treatment of RLS. This was followed, in 2006, by Mirapex (pramipexole). In 2006 an article published in the Public Library of Science Medicine Journal, discussed the issue of "disease mongering" in relation to RLS. This is the effort by pharmaceutical companies (or others with similar financial interests) to enlarge the market for a treatment by convincing people that they are sick and need medical intervention. They state that "the restless legs campaign has developed into a multimillion dollar international effort to push restless legs syndrome into the consciousness of doctors and consumers alike" (Woloshin and Schwartz 2006).

Although there are a number of negative claims regarding RLS, scientific evidence proves this is a "real" syndrome. The oldest reference of 'Restless Legs syndrome' syndrome on PubMed (www.ncbi.nlm.nih.gov/pubmed), dates back to 1960 (Ekbom 1960). Ekbom also published and presented eight cases in a paper published in 1945, titled 'Restless Legs.' It was claimed that Mirapex and Requip were in fact failed sleep medications, however, both of these medications were also approved by the

FDA as treatments for Parkinson ’s disease. The drugs are dopamine agonists, which means they act in the brain mimicking the neurotransmitter dopamine, which is lacking in PD patients. The pathophysiology of RLS is thought to be closely linked to dopamine dysfunction; L-dopa (a precursor to dopamine) relieves the symptoms of RLS. Furthermore akathisia, which is present in PD patients, is similar in character to RLS. It was therefore understandable why dopamine agonists, such as Mirapex and Requip, were considered for the treatment of RLS. It was not surprising that such drugs helped relieve symptoms of RLS.

RLS is a known disorder known for over four decades, it fits well within our knowledge of movement disorders and the role of dopamine agonists in treating them.

The UCC Pathology lab recruited pedigree RLS3002 in 2004/2005. There was a strong family history seen in this family, the grandmother (generation I), all of her four children (generation II) and seven of her grandchildren (generation III) being affected with RLS. Suggestive family history is therefore consistent with an autosomal mode of inheritance. All 18 family members answered a questionnaire and all affected individuals undertook a neurological examination. Age of onset in most affected individuals in this family ranged from 3 years of age to the teens. Although most RLS patients have lower extremity symptoms, arm restlessness occurs in up to 49% of patients with RLS (Freedom and Merchut 2003). In pedigree RLS3002, most of the individuals experienced symptoms confined to the lower limbs bar one who reported upper limb and trunk symptoms as well. Of the 11 affected individuals, five were affected mildly and the remaining six individuals

were moderately affected according to the IRLSSG rating scale for RLS severity (section 1.3.2).

Iron deficiency and chronic renal failure can play a part in the pathophysiology of RLS and basic biochemical tests such as serum iron and renal function can be carried out on affected individuals to rule out secondary RLS. Such tests were not performed on affected individuals of pedigree RLS3002 as the familial pattern, the early age of onset and the absence of any clinical history of other disease indicates primary/familial RLS in this family.

Mode of inheritance refers to the way in which genes are passed down from generation to generation (e.g. autosomal dominant, autosomal recessive, X-linked, multifactorial and mitochondrial inheritance). By analysing mode of inheritance, researchers can estimate the relative risk for and penetrance of a disease. Determining the mode of inheritance was crucial for this study. In order to perform linkage analysis and to compute a LOD score it is necessary to specify the mode of genetic transmission of the family being investigated. RLS has been identified as a Mendelian autosomal dominant disease with variable expressivity and high penetrance ((Winkelmann, Muller-Myhsok et al. 2002; Bonati, Ferini-Strambi et al. 2003; Levchenko, Montplaisir et al. 2004; Pichler, Marroni et al. 2006). An autosomal dominant mode of inheritance was displayed in pedigree RLS3002 as was reported in the other loci. This was evident by the fact that all the affected individuals have one parent who is affected. The chromosome 12q locus mapped by Desautels et al in a French-Canadian (FC) family was the only exception to an autosomal dominant mode of inheritance in an RLS pedigree. This family displayed autosomal recessive inheritance (Desautels, Turecki et al. 2001). However, Desautels

and associates explained the autosomal recessive mode of inheritance in their family as pseudodominant mode of inheritance. This means that the real transmission of the gene is autosomal recessive but given a high frequency of the disease allele(s) there is a higher proportion of affected individuals, thus giving the appearance of a dominant pattern of inheritance. Desautels et al supported their pseudodominant mode of transmission, by stating that 1) the occurrence of RLS in the FC population was one of the highest in the world; 2) only FC families were linked to that locus at that time; and 3) that a founder effect has been well described in the FC population. The paper also maintained that RLS may be similar to Parkinson disease, where the presence of intrafamilial allelic heterogeneity is highly possible. After the original report of recessive juvenile Parkinsonism, a number of other studies later identified further mutations in the same gene and have also identified a mixed mode of inheritance of different mutations within and between families (Desautels, Turecki et al. 2005).

Linkage analysis of pedigree data is a powerful tool for mapping genomic regions that are likely to contain genes influencing human diseases. It is assumed that the phenotype arises from a single major gene effect with almost full (95%) penetrance i.e. it is expressed regardless of other genetic or environmental factors. It has been successful in mapping hundreds of Mendelian diseases including Huntington's disease and cystic fibrosis. However for many common diseases with high prevalence, their penetrance are often incomplete. Also, phenocopies may exist. It is therefore difficult to specify the genetic model parameters in these circumstances. Misspecification of the model parameters as a result of incomplete penetrances and/or phenocopies may lead to a reduction in power to detect linkage. The reason there may be a reduction in linkage power is, incomplete penetrances have the ability

to reduce evidence for linkage. Furthermore, phenocopies can give false information about recombination patterns. For linkage calculations in this study, RLS was modelled as an autosomal dominant trait with a 95% penetrance as used in other RLS linkage studies (Chen et al, 2004). The reported value varies from 86% to full penetrance in most families; Trenkwalder *et al*., 1996; Lazzarini *et al*., 1999).

For many years, genetic mapping was limited by the lack of available markers. In 1980, it was suggested that restriction-fragment length polymorphisms (RFLPs) be used as simple Mendelian co-dominant genetic markers. Although RFLPs generally did not lie within a gene of interest, they had the advantage of requiring no prior knowledge about the sequence of DNA in which they lie, or of its function. Since the 1980's the field of molecular biology has grown rapidly, and there are currently a number of dense genetic maps available. Microsatellite markers are now more commonly used, and were therefore employed for this study. While this was efficient and powerful for the gene mapping part of this study, once linkage was estabished fine-mapping of our RLS locus was hampered by the lack of available markers in the area. Novel microsatellite markers were identified and primers were designed for these areas. It was not known if these novel microsatellites would be polymorphic, and subsequently, informative. Despite the great advances in genomic technology, the availability of microsatellite markers for fine-mapping the chromosome 19p13.3 region was limited.

Apart from a number of microsatellite markers failing to amplify i.e. those used for fine-mapping (section 5.3.3), no problems with genotyping was encountered throughout the study. I found it was best to attain the alleles without reference to the pedigree, and then to consider the results in the context of the family tree. This

avoided the subconscious bias of results. If there was doubt over a particular individual or a number of individuals in the family, these samples were re-analysed together with parent samples if available. This made it much easier to determine whether or not they all share the same allele. When genotyping, it is important to bear in mind that an inconsistency can arise through mutation of the microsatellite from one generation to the next. This is the process of how microsatellite polymorphisms occur over time. This, however, was not observed during this study.

When this study was initiated only four loci were identified. It was postulated that the newly recruited Irish family might share the same disease locus as the other Irish family in which the chromosome 5 locus was identified rather than being linked to the other loci identified in families of other origins. Hence, the remaining gap on chromosome 5 was examined first. A total area of 38.31 cM was excluded extending from 44.92 cM (flanked by the marker D5S418) and 83.23 cM (flanked by the marker D5S2003). The results exclude a large area which included the locus. All other nine loci identified by linkage analysis studies were examined for linkage to RLS3002. Linkage to all nine loci was successfully examined and excluded in this RLS family.

In 2007 the first RLS association studies were published. To date four RLS regions have been identified by association studies. This is a high-throughput approach which scans the entire genome or a specific region in an unbiased manner, using statistical methods to determine associations between chromosomal loci and a given phenotype. It requires a large number of cases and a large number of controls which is often difficult to collect, therefore would not have been a suitable method to use for our family. However, it was important to investigate if pedigree RLS3002 was

linked to any of these novel RLS regions. Following linkage analysis, our analysis shows that RLS3002 is not linked to any of these known risk-associated genes.

The RLS3002 pedigree must therefore carry a mutation in a novel genetic locus. Two microsatellite marker sets were used during the genome wide scan of family RLS3002. The first marker set used was the Research Genetics Inc. mapping set. When this panel of markers was completed and no linkage had been found, there were many questions raised as to whether another microsatellite marker set should be used or if another route should be taken. Two possible options to locate the RLS locus were considered. The first option was another microsatellite marker set with a higher density of markers. The second options considered was using microarray-based single nucleotide polymorphism (SNP) data. The second option was becoming more widely used as markers for linkage analysis in the identification of loci for disease-associated genes. Both options were considered. Although microarray based SNP analyses have markedly reduced genotyping time and cost compared with microsatellite-based analyses, applying these enormous data to linkage analysis programs is a time-consuming step. The Microsatellite marker set which was considered was the Applied Biosystems Linkage mapping set version 2.5, which has a resolution of 10 cM. The first mapping, Research Genetics Inc. has a resolution of approximately 20 cM.

Furthermore, since the beginning of the initial genome-wide scan an additional seven members of the RLS3002 family had been examined and had blood samples taken which significantly increase the power of the study. The maximum possible positive LOD score with initial eleven individuals in the study was calculated to be 1.86. In order to detect linkage reliably, the family size must be large enough to give a

statistically significant answer. This means that in practice there must be at least ten potentially informative meiosis (those in which potential cross-overs could be recognised) within a family in order to give a LOD score $\geq$ 3.00. With the addition of the seven other members, power analysis with the SLINK software was performed again and it was found that the maximum possible LOD score for this family was now 3.41. This was sufficient to prove linkage as a LOD score of > 3.00 is required to prove linkage. It was therefore decided that the Applied Biosystems Linkage mapping set version 2.5 would be used to continue the investigation of RLS in pedigree RLS3002.

Several epidemiological investigations have shown a female preponderance of RLS(Berger, Luedemann et al. 2004). This could indeed be a result of reporting bias. As pedigree RLS3002 is quite small it is difficult to say if there is a female prevalence. There are six affected females (seven if the grandmother is included), and there are five affected males.

There are considerable geographical variations in the prevalence of RLS. The cause of these variations are not well understood. The majority of these prevalence studies have also been performed on Caucasian populations. Prevalence estimates in East Asians include 0.1% in a Singapore survey (Tan, Seah et al. 2001) and 12% in a Korean survey (Kim, Choi et al. 2005). This study on an Irish Caucasian family is consistent with these findings.

The racial differences in RLS have yet to be understood. The prevalence of RLS in some ethnic groups may have been underestimated due to their under-representation in RLS speciality clinics. In a study by Lee et al. (2006) the prevalence of RLS in African-Americans and Caucasians were compared in a biracial community sample.

The prevalence of RLS in the overall population was 4.1%. The rates were similar for African–Americans (4.7%) and Caucasians (3.8%). After adjustment for age, gender, medical comorbidities, and socioeconomic status, no difference in the prevalence of RLS was found between African–Americans and Caucasians. It is quite possible that under-representation difficulties are present in prevalence studies in countries such as India where access to speciality clinics may also be a problem. Barriers affecting access to care settings for RLS patients worldwide should be investigated in the future. Further investigations into the geographical and racial differences in RLS need to be carried out also.

Two candidate genes within the novel RLS locus 19p13.3 were screened. Exon 1 of *KLF16* failed to sequence; however the results show no disease causing mutations in the exons and exon-intron junction regions of *KLF16* exon 2 and *GAMT*. There is a possibility that the mutation is one which is not detectable using current sequencing techniques, for example, large deletions.

In order to exclude mutations in the remaining introns of *KLF16* and *GAMT* further work is needed. There may be possible mutations within the introns of these genes that cause a cryptic splice site. These mutations would not have been detected by the approaches used in this study.

Further research is needed to screen other candidate genes within the disease gene interval. Genes which play a role in iron and dopamine pathways will be identified and prioritised for mutation screening. Failing to find a disease causing mutation in these genes, all other genes within the locus need to be screened as many do not have a known function as yet. Furthermore new mechanisms which have not yet been described may be involved in the pathophysiology. Reducing the size of the RLS

locus would reduce the number of genes to be examined. A number of approaches could be used to potentially decrease the size of the locus. Since all family members of RLS3002 have been recruited this approach is not an option. One approach to narrow the linkage region is to add more markers to the region to find additional recombinants. Although all known microsatellite markers in the region were examined, there is a possibility new markers may be identified in the future. Additional clinically defined RLS families could also be studied to determine whether any might be linked to the RLS 19p13.3 locus. This might allow for reduction of the locus

7.3 Conclusion and Future work

The results obtained in the Irish pedigree RLS3002 with autosomal dominant RLS showed evidence of a new RLS locus located on chromosome 19p13.3. To date linkage studies in RLS families have revealed 11 loci but no causally related sequence variant has yet been identified using this approach. Replication of RLS linkage studies has met with limited success. The first RLS locus, reported on chromosome 12q in a study of a single French-Canadian family, was confirmed in an additional 5 of 18 families (Desautels, Turecki et al. 2005) , and extended to a study of 100 families in Iceland (Hicks AA 2005). RLS2, the second susceptibility locus identified on chromosome 14q was initially seen in two large families from South Tyrol (Bonati, Ferini-Strambi et al. 2003) and subsequently identified in a single French-Canadian family (Levchenko, Montplaisir et al. 2004). This suggests that RLS is a genetically highly-heterogeneous disorder thus presenting a challenge to researchers hoping to unravel its pathophysiology. Further confirmation of existing RLS loci in different ethnic groups would be important as these might help prioritise and promote efforts to identify potential causative genes.

Symptoms of RLS are quite common. The prevalence of RLS severe enough to cause impairment in quality of life (QOL) and warrant medical treatment is probably around 2-3%. This makes clinically significant RLS 2 to 6 times as common as epilepsy in developed countries (estimated prevalence of epilepsy is 0.5-1.0%) (Natarajan). RLS can cause significant impairment of sleep and about 94% of patients report sleep disturbance due to RLS. As a result patients have daytime fatigue, reduced motivation, emotional stress, anxiety and difficulty accomplishing their jobs and participating in social activities. Studies have shown that RLS patients

are more prone to headaches than healthy subjects. Headaches are similar to migraine in character, though with an element of tension-type headache (d'Onofrio, Bussone et al. 2008). In addition, RLS may be linked to cardiovascular disease (Winkelman, Shahar et al. 2008) Currently, RLS is diagnosed solely on the clinical history, there are four essential criteria and all of them must be established to make a definite diagnosis. In addition there are three supportive criteria that are not necessary for a diagnosis of RLS but are admittedly helpful in the differential diagnosis. No other method has yet been discovered to diagnose RLS as effectively as the clinical criteria. The treatment of RLS is mainly symptomatic. Many therapeutic measures have been considered, some less effective than others. At present dopaminergic receptor agonists are considered the first choice in treating moderate to severe cases of RLS. However they can often be problematic, causing side effects such as augmentation; where in some patients the symptoms begin in late afternoon or in early evening as a result of treatment. Patients should also realize that pharmacological treatment is life-long in most cases. Ultimately if the causative gene(s) for RLS is identified, it would be a stepping stone to unravelling the etiology, and the development of therapeutic drugs to treat, prevent, and hopefully to cure this chronic distressing condition.(Garcia-Borreguero, Allen et al. 2007).

Bibliography

Abdulrahim, M. M. A. (2008). Gene Mapping & Candidate Gene Analysis in Autosomal Dominant Restless Legs Syndrome. Dept. of Pathology, U.C.C.

Adler, C. H., K. A. Gwinn, et al. (1998). "Olfactory function in restless legs syndrome." Mov Disord **13**(3): 563-5.

Aisen, P., C. Enns, et al. (2001). "Chemistry and biology of eukaryotic iron metabolism." Int J Biochem Cell Biol **33**(10): 940-59.

Allen, R. (2004). "Dopamine and iron in the pathophysiology of restless legs syndrome (RLS)." Sleep Med **5**(4): 385-91.

Allen, R. P., P. B. Barker, et al. (2001). "MRI measurement of brain iron in patients with restless legs syndrome." Neurology **56**(2): 263-265.

Allen, R. P. and C. J. Earley (1996). "Augmentation of the restless legs syndrome with carbidopa/levodopa." Sleep **19**(3): 205-13.

Allen, R. P. and C. J. Earley (2000). "Defining the phenotype of the restless legs syndrome (RLS) using age-of-symptom-onset." **1**(1): 11-19.

Allen, R. P. and C. J. Earley (2001). "Restless legs syndrome: a review of clinical and pathophysiologic features." J Clin Neurophysiol **18**(2): 128-47.

Allen, R. P. and C. J. Earley (2007). "The role of iron in restless legs syndrome." Mov Disord **22 Suppl 18**: S440-8.

Allen, R. P., D. Picchietti, et al. (2003). "Restless legs syndrome: diagnostic criteria, special considerations, and epidemiology. A report from the restless legs syndrome diagnosis and epidemiology workshop at the National Institutes of Health." Sleep Med **4**(2): 101-19.

Allen, R. P., A. S. Walters, et al. (2005). "Restless legs syndrome prevalence and impact: REST general population study." Arch Intern Med **165**(11): 1286-92.

Appiah-Kubi, L. S., S. Pal, et al. (2002). "Restless legs syndrome (RLS), Parkinson's disease, and sustained dopaminergic therapy for RLS." Sleep Med **3 Suppl**: S51-5.

Aukerman, M. M., D. Aukerman, et al. (2006). "Exercise and restless legs syndrome: a randomized controlled trial." J Am Board Fam Med **19**(5): 487-93.

Bagetta, G., G. De Sarro, et al. (1988). "Ventral tegmental area: site through which dopamine D2-receptor agonists evoke behavioural and electrocortical sleep in rats." Br J Pharmacol **95**(3): 860-6.

Barriere, G., J. R. Cazalets, et al. (2005). "The restless legs syndrome." Prog Neurobiol **77**(3): 139-65.

Bell, J. and J. B. Haldane (1986). "The linkage between the genes for colour-blindness and haemophilia in man. By Julia Bell and J.B.S. Haldane, 1937." Ann Hum Genet **50**(Pt 1): 3-34.

Bentley, A. J., K. D. Rosman, et al. (2006). "Gender differences in the presentation of subjects with restless legs syndrome." Sleep Med **7**(1): 37-41.

Benz, R. L., M. R. Pressman, et al. (1999). "A preliminary study of the effects of correction of anemia with recombinant human erythropoietin therapy on sleep, sleep disorders, and daytime sleepiness in hemodialysis patients (The SLEEPO study)." Am J Kidney Dis **34**(6): 1089-95.

Berger, K., J. Luedemann, et al. (2004). "Sex and the risk of restless legs syndrome in the general population." Arch Intern Med **164**(2): 196-202.

Bjorvatn, B., L. Leissner, et al. (2005). "Prevalence, severity and risk factors of restless legs syndrome in the general adult population in two Scandinavian countries." Sleep Med **6**(4): 307-12.

Bliwise, D. L., A. Freeman, et al. (2005). "Randomized, double-blind, placebo-controlled, short-term trial of ropinirole in restless legs syndrome." Sleep Med **6**(2): 141-7.

Boehm, G., T. C. Wetter, et al. (2008). "Periodic leg movements in RLS patients as compared to controls: Are there differences beyond the PLM index?" Sleep Med.

Bonati, M. T., L. Ferini-Strambi, et al. (2003). "Autosomal dominant restless legs syndrome maps on chromosome 14q." Brain **126**(Pt 6): 1485-92.

Brodeur, C., J. Montplaisir, et al. (1988). "Treatment of restless legs syndrome and periodic movements during sleep with L-dopa: a double-blind, controlled study." Neurology **38**(12): 1845-8.

Broman, K. W., J. C. Murray, et al. (1998). "Comprehensive human genetic maps: individual and sex-specific variation in recombination." Am J Hum Genet **63**(3): 861-9.

Brown, L. K., D. L. Dedrick, et al. (2005). "Antidepressant medication use and restless legs syndrome in patients presenting with insomnia." Sleep Med **6**(5): 443-50.

Bucher, S. F., K. C. Seelos, et al. (1997). "Cerebral generators involved in the pathogenesis of the restless legs syndrome." Ann Neurol **41**(5): 639-45.

Bucher, S. F., C. Trenkwalder, et al. (1996). "Reflex studies and MRI in the restless legs syndrome." Acta Neurol Scand **94**(2): 145-50.

Chahine, L. M. and Z. N. Chemali (2006). "Restless legs syndrome: a review." CNS Spectr **11**(7): 511-20.

Chaudhuri, K. R. (2003). "Restless legs syndrome." N Engl J Med **349**(8): 815.

Chaudhuri, K. R., L. S. Appiah-Kubi, et al. (2001). "Restless legs syndrome." J Neurol Neurosurg Psychiatry **71**(2): 143-6.

Chen, P. K., J. L. Fuh, et al. (2010). "Association between restless legs syndrome and migraine." J Neurol Neurosurg Psychiatry **81**(5): 524-8.

Chen, S., W. G. Ondo, et al. (2004). "Genomewide linkage scan identifies a novel susceptibility locus for restless legs syndrome on chromosome 9p." Am J Hum Genet **74**(5): 876-85.

Clark, M. M. (2001). "Restless legs syndrome." J Am Board Fam Pract **14**(5): 368-74.

Connor, J. R., P. J. Boyer, et al. (2003). "Neuropathological examination suggests impaired brain iron acquisition in restless legs syndrome." Neurology **61**(3): 304-9.

Connor, J. R., X. S. Wang, et al. (2004). "Decreased transferrin receptor expression by neuromelanin cells in restless legs syndrome." Neurology **62**(9): 1563-7.

d'Onofrio, F., G. Bussone, et al. (2008). "Restless legs syndrome and primary headaches: a clinical study." Neurol Sci **29 Suppl 1**: S169-72.

D.B. Reye, A. A. H., K. Kristjansson, T. Sigmundsson, A. Sigurdssson and H. Hakonarson *et al* (2005). "Population-based confirmation of the 12q RLS locus in Iceland." Sleep **(Abstract supplement)**(28): p. 0824.

Dausset, J., H. Cann, et al. (1990). "Centre d'etude du polymorphisme humain (CEPH): collaborative genetic mapping of the human genome." Genomics **6**(3): 575-7.

Davis, B. J., A. Rajput, et al. (2000). "A randomized, double-blind placebo-controlled trial of iron in restless legs syndrome." Eur Neurol **43**(2): 70-5.

Desai, A. V., L. F. Cherkas, et al. (2004). "Genetic influences in self-reported symptoms of obstructive sleep apnoea and restless legs: a twin study." Twin Res **7**(6): 589-95.

Desautels, A., G. Turecki, et al. (2002). "Evidence for a genetic association between monoamine oxidase A and restless legs syndrome." Neurology **59**(2): 215-219.

Desautels, A., G. Turecki, et al. (2001). "Identification of a major susceptibility locus for restless legs syndrome on chromosome 12q." Am J Hum Genet **69**(6): 1266-1270.

Desautels, A., G. Turecki, et al. (2005). "Restless legs syndrome: confirmation of linkage to chromosome 12q, genetic heterogeneity, and evidence of complexity." Arch Neurol **62**(4): 591-6.

Duggal, H. S. and D. N. Mendhekar (2007). "Clozapine-associated restless legs syndrome." J Clin Psychopharmacol **27**(1): 89-90.

Dzaja, A., R. Wehrle, et al. (2009). "Elevated estradiol plasma levels in women with restless legs during pregnancy." Sleep **32**(2): 169-74.

Earley, C. J. (2003). "Clinical practice. Restless legs syndrome." N Engl J Med **348**(21): 2103-9.

Earley, C. J. and R. P. Allen (1996). "Pergolide and carbidopa/levodopa treatment of the restless legs syndrome and periodic leg movements in sleep in a consecutive series of patients." Sleep **19**(10): 801-10.

Earley, C. J., R. P. Allen, et al. (2000). "Insight into the pathophysiology of restless legs syndrome." J Neurosci Res **62**(5): 623-8.

Earley, C. J., P. B. Barker, et al. (2006). "MRI-determined regional brain iron concentrations in early- and late-onset restless legs syndrome." Sleep Medicine. In Press, Corrected Proof.

Earley, C. J., J. R. Connor, et al. (2000). "Abnormalities in CSF concentrations of ferritin and transferrin in restless legs syndrome." Neurology **54**(8): 1698-700.

Earley, C. J., D. Heckler, et al. (2004). "The treatment of restless legs syndrome with intravenous iron dextran." Sleep Med **5**(3): 231-5.

Earley, C. J., K. Hyland, et al. (2006). "Circadian changes in CSF dopaminergic measures in restless legs syndrome." Sleep Med **7**(3): 263-8.

Ekbom, K. and J. Ulfberg (2009). "Restless legs syndrome." J Intern Med **266**(5): 419-31.

Ekbom, K. A. (1945). "Restless legs: a clinical study." Acta Med Scand. **158 (suppl)**: 1-123.

Ekbom, K. A. (1955). "[Restless legs as an early symptom of cancer.]." Sven Lakartidn **52**(30): 1875-83.

Ekbom, K. A. (1960). "Restless legs syndrome." Neurology **10**: 868-873.

Elston, R. C. and J. Olson (2002). Biostatistical genetics and genetic epidemiology

Wiley.

Erikson, K. M., B. C. Jones, et al. (2001). "Iron deficiency decreases dopamine D1 and D2 receptors in rat brain." Pharmacol Biochem Behav **69**(3-4): 409-18.

Evans, A. H. and J. R. Stegeman (2009). "Punding in patients on dopamine agonists for restless leg syndrome." Mov Disord **24**(1): 140-1.

Ferini-Strambi, L., M. T. Bonati, et al. (2004). "Genetics in restless legs syndrome." Sleep Med **5**(3): 301-4.

Freedom, T. and M. P. Merchut (2003). "Arm restlessness as the initial symptom in restless legs syndrome." Arch Neurol **60**(7): 1013-5.

Garcia-Borreguero, D., R. P. Allen, et al. (2007). "Diagnostic standards for dopaminergic augmentation of restless legs syndrome: report from a world association of sleep medicine - international restless legs syndrome study group consensus conference at the max planck institute." Sleep Med **8**(5): 520-30.

Garcia-Borreguero, D., O. Larrosa, et al. (2002). "Treatment of restless legs syndrome with gabapentin: a double-blind, cross-over study." Neurology **59**(10): 1573-9.

Garcia-Borreguero, D., P. Odin, et al. (2004). "Restless legs syndrome: an overview of the current understanding and management." Acta Neurol Scand **109**(5): 303-17.

Ghorayeb, I., B. Bioulac, et al. (2008). "Perceived severity of restless legs syndrome across the female life cycle." Sleep Med **9**(7): 799-802.

Goodman, J. D., C. Brodie, et al. (1988). "Restless leg syndrome in pregnancy." Bmj **297**(6656): 1101-2.

Guyatt, G. H., C. Patterson, et al. (1990). "Diagnosis of iron-deficiency anemia in the elderly." Am J Med **88**(3): 205-9.

Hadjigeorgiou, G. M., I. Stefanidis, et al. (2007). "Low RLS prevalence and awareness in central Greece: an epidemiological survey." Eur J Neurol **14**(11): 1275-80.

Haines J.L, a. P.-V. M. A. (1998). Approaches to gene mapping in complex human diseases, Wiley-liss.

Hamet, P. and J. Tremblay (2006). "Genetics of the sleep-wake cycle and its disorders." Metabolism **55**(10 Suppl 2): S7-12.

Happe, S., G. Klosch, et al. (2001). "Treatment of idiopathic restless legs syndrome (RLS) with gabapentin." Neurology **57**(9): 1717-9.

Happe, S., M. Vennemann, et al. (2008). "Treatment wish of individuals with known and unknown restless legs syndrome in the community." J Neurol **255**(9): 1365-71.

Hening, W. (2004). "The clinical neurophysiology of the restless legs syndrome and periodic limb movements. Part I: diagnosis, assessment, and characterization." Clin Neurophysiol **115**(9): 1965-74.

Hening, W., R. Allen, et al. (1999). "The treatment of restless legs syndrome and periodic limb movement disorder. An American Academy of Sleep Medicine Review." Sleep **22**(7): 970-99.

Hening, W., A. S. Walters, et al. (2004). "Impact, diagnosis and treatment of restless legs syndrome (RLS) in a primary care population: the REST (RLS epidemiology, symptoms, and treatment) primary care study." Sleep Med **5**(3): 237-46.

Hening, W. A., A. S. Walters, et al. (1999). "Circadian rhythm of motor restlessness and sensory symptoms in the idiopathic restless legs syndrome." Sleep **22**(7): 901-12.

Hicks AA, R. D., Kristjansson K (2005). "Population-based confirmation of the 12q RLS locus in Iceland." Mov Disord **20 (S10):S34**.

Hiersemenzel, L. P., S. Johannes, et al. (1996). "[Choreoacanthocytosis. A neurologic-hematologic syndrome]." Nervenarzt **67**(6): 490-5.

Hogl, B., D. Garcia-Borreguero, et al. (2009). "Progressive development of augmentation during long-term treatment with levodopa in restless legs syndrome: results of a prospective multi-center study." J Neurol **257**(2): 230-7.

Hogl, B., S. Kiechl, et al. (2005). "Restless legs syndrome: a community-based study of prevalence, severity, and risk factors." Neurology **64**(11): 1920-4.

Hornyak, M., C. Grossmann, et al. (2008). "Cognitive behavioural group therapy to improve patients' strategies for coping with restless legs syndrome: a proof-of-concept trial." J Neurol Neurosurg Psychiatry **79**(7): 823-5.

Hui, D. S., T. Y. Wong, et al. (2000). "Prevalence of sleep disturbances in chinese patients with end-stage renal failure on continuous ambulatory peritoneal dialysis." Am J Kidney Dis **36**(4): 783-8.

Hwang, C. K., U. M. D'Souza, et al. (2001). "Dopamine receptor regulating factor, DRRF: a zinc finger transcription factor." Proc Natl Acad Sci U S A **98**(13): 7558-63.

Inoue, Y., K. Kuroda, et al. (2010). "Long-term open-label study of pramipexole in patients with primary restless legs syndrome." J Neurol Sci.

Jeng, C. J., S. A. McCarroll, et al. (1998). "Thy-1 is a component common to multiple populations of synaptic vesicles." J Cell Biol **140**(3): 685-98.

Kageyama, T., M. Kabuto, et al. (2000). "Prevalences of periodic limb movement-like and restless legs-like symptoms among Japanese adults." Psychiatry Clin Neurosci **54**(3): 296-8.

Kang, S. G., H. J. Lee, et al. (2007). "Characteristics and clinical correlates of restless legs syndrome in schizophrenia." Prog Neuropsychopharmacol Biol Psychiatry **31**(5): 1078-83.

Keats, B., J. Ott, et al. (1989). "Report of the committee on linkage and gene order." Cytogenet Cell Genet **51**(1-4): 459-502.

Kemlink, D., G. Plazzi, et al. (2008). "Suggestive evidence for linkage for restless legs syndrome on chromosome 19p13." Neurogenetics **9**(2): 75-82.

Kemlink, D., O. Polo, et al. (2009). "Replication of restless legs syndrome loci in three European populations." J Med Genet **46**(5): 315-8.

Kim, J., C. Choi, et al. (2005). "Prevalence of restless legs syndrome and associated factors in the Korean adult population: the Korean Health and Genome Study." Psychiatry Clin Neurosci **59**(3): 350-3.

Kosambi, D. D. (1944). "The estimation of map disatnces from recombination values." Ann Eugen **12**: 172-175.

Kotagal, S. and M. H. Silber (2004). "Childhood-onset restless legs syndrome." Ann Neurol **56**(6): 803-7.

Kraus, T., A. Schuld, et al. (1999). "Periodic leg movements in sleep and restless legs syndrome probably caused by olanzapine." J Clin Psychopharmacol **19**(5): 478-9.

Krieger, J. and C. Schroeder (2001). "Iron, brain and restless legs syndrome." Sleep Med Rev **5**(4): 277-286.

Lathrop, G. M., J. M. Lalouel, et al. (1984). "Strategies for multilocus linkage analysis in humans." Proc Natl Acad Sci U S A **81**(11): 3443-6.

Lavigne, G. J. and J. Y. Montplaisir (1994). "Restless legs syndrome and sleep bruxism: prevalence and association among Canadians." Sleep **17**(8): 739-43.

Lee, B. Y. (2007). "in-vivo measurement of iron deficiency in rls with voxel-based MRI relaxometry." Neurology **68**(A356).

Levchenko, A., J. Y. Montplaisir, et al. (2008). "Autosomal-dominant locus for restless legs syndrome in French-Canadians on chromosome 16p12.1." Mov Disord.

Levchenko, A., J. Y. Montplaisir, et al. (2004). "The 14q restless legs syndrome locus in the French Canadian population." Ann Neurol **55**(6): 887-91.

Levchenko, A., S. Provost, et al. (2006). "A novel autosomal dominant restless legs syndrome locus maps to chromosome 20p13." Neurology **67**(5): 900-1.

Manconi, M., V. Govoni, et al. (2004). "Pregnancy as a risk factor for restless legs syndrome." Sleep Med **5**(3): 305-8.

Mata, I. F., C. L. Bodkin, et al. (2006). "Genetics of restless legs syndrome." Parkinsonism Relat Disord **12**(1): 1-7.

Michaud, M., A. Chabli, et al. (2000). "Arm restlessness in patients with restless legs syndrome." Mov Disord **15**(2): 289-93.

Michaud, M., J. P. Soucy, et al. (2002). "SPECT imaging of striatal pre- and postsynaptic dopaminergic status in restless legs syndrome with periodic leg movements in sleep." J Neurol **249**(2): 164-70.

Mizuno, S., T. Mihara, et al. (2005). "CSF iron, ferritin and transferrin levels in restless legs syndrome." J Sleep Res **14**(1): 43-7.

Mizuno, S., T. Miyaoka, et al. (2005). "Prevalence of restless legs syndrome in non-institutionalized Japanese elderly." Psychiatry and Clinical Neurosciences **59**(4): 461-465.

Montplaisir, J. (2004). "Abnormal motor behavior during sleep." Sleep Med **5 Suppl 1**: S31-4.

Montplaisir, J., S. Boucher, et al. (1997). "Clinical, polysomnographic, and genetic characteristics of restless legs syndrome: a study of 133 patients diagnosed with new standard criteria." Mov Disord **12**(1): 61-5.

Montplaisir, J., D. Lorrain, et al. (1991). "Restless legs syndrome and periodic leg movements in sleep: the primary role of dopaminergic mechanism." Eur Neurol **31**(1): 41-3.

Montplaisir, J., A. Nicolas, et al. (1999). "Restless legs syndrome improved by pramipexole: a double-blind randomized trial." Neurology **52**(5): 938-43.

Morris, R. (1985). "Thy-1 in developing nervous tissue." Dev Neurosci **7**(3): 133-60.

Natarajan, R. "Review of periodic limb movement and restless leg syndrome." J Postgrad Med **56**(2): 157-62.

National Heart, L., and Blood Institute Working Group on Restless Legs Syndrome (2000). "Restless legs syndrome: detection and management in primary care. ." Am Fam Physician **62**(1): 108-14.

Norlander, N. (1953). "Therapy in Restless legs." Acta Med Scand.(145): 453-457.

O'Keeffe, S. T., D. Egan, et al. (2007). "The frequency and impact of restless legs syndrome in primary care." Ir Med J **100**(7): 539-42.

O'Keeffe, S. T., K. Gavin, et al. (1994). "Iron status and restless legs syndrome in the elderly." Age Ageing **23**(3): 200-3.

Odin, P., M. Mrowka, et al. (2002). "Restless legs syndrome." Eur J Neurol **9 Suppl 3**: 59-67.

Ondo, W. G., K. D. Vuong, et al. (2002). "Exploring the relationship between Parkinson disease and restless legs syndrome." Arch Neurol **59**(3): 421-4.

Ondo, W. G., K. D. Vuong, et al. (2000). "Restless legs syndrome in monozygotic twins: clinical correlates." Neurology **55**(9): 1404-6.

Ott, J. (1985). Analysis of human genetic linkage, The John Hopkins University press.

Paulus, W. and C. Trenkwalder (2006). "Less is more: pathophysiology of dopaminergic-therapy-related augmentation in restless legs syndrome." The Lancet Neurology **5**(10): 878-886.

Pearson, V. E., R. P. Allen, et al. (2006). "Cognitive deficits associated with restless legs syndrome (RLS)." Sleep Med **7**(1): 25-30.

Pichler, I., F. Marroni, et al. (2006). "Linkage Analysis Identifies a Novel Locus for Restless Legs Syndrome on Chromosome 2q in a South Tyrolean Population Isolate." Am J Hum Genet **79**(4): 716-723.

Pichler, I., F. Marroni, et al. (2006). "Linkage analysis identifies a novel locus for restless legs syndrome on chromosome 2q in a South Tyrolean population isolate." Am J Hum Genet **79**(4): 716-23.

Rijsman, R. M. and A. W. de Weerd (1999). "Secondary periodic limb movement disorder and restless legs syndrome." Sleep Med Rev **3**(2): 147-58.

Rothdach, A. J., C. Trenkwalder, et al. (2000). "Prevalence and risk factors of RLS in an elderly population: the MEMO study. Memory and Morbidity in Augsburg Elderly." Neurology **54**(5): 1064-8.

Rottach, K. G., B. M. Schaner, et al. (2008). "Restless legs syndrome as side effect of second generation antidepressants." J Psychiatr Res.

Ruottinen, H. M., M. Partinen, et al. (2000). "An FDOPA PET study in patients with periodic limb movement disorder and restless legs syndrome." Neurology **54**(2): 502-4.

Schmidauer, C., M. Sojer, et al. (2005). "Transcranial ultrasound shows nigral hypoechogenicity in restless legs syndrome." Ann Neurol **58**(4): 630-4.

Schormair, B., D. Kemlink, et al. (2008). "PTPRD (protein tyrosine phosphatase receptor type delta) is associated with restless legs syndrome." Nat Genet **40**(8): 946-8.

Sharifian, A., M. Firoozeh, et al. (2009). "Restless Legs Syndrome in shift workers: A cross sectional study on male assembly workers." J Circadian Rhythms **7**: 12.

Shinno, H., Y. Inami, et al. (2008). "Effect of Yi-Gan San on psychiatric symptoms and sleep structure at patients with behavioral and psychological symptoms of dementia." Prog Neuropsychopharmacol Biol Psychiatry **32**(3): 881-5.

Shinno, H., M. Yamanaka, et al. (2010). "Successful treatment of restless legs syndrome with the herbal prescription Yokukansan." Prog Neuropsychopharmacol Biol Psychiatry **34**(1): 252-3.

Silber, M. H., B. L. Ehrenberg, et al. (2004). "An algorithm for the management of restless legs syndrome." Mayo Clin Proc **79**(7): 916-22.

Speer, M. (2006). Current Protocols in Human Genetics, John Wiley & Sons, Inc.

Staedt, J., F. Wassmuth, et al. (1997). "Pergolide: treatment of choice in restless legs syndrome (RLS) and nocturnal myoclonus syndrome (NMS). A double-blind randomized crossover trial of pergolide versus L-Dopa." J Neural Transm **104**(4-5): 461-8.

Stefansson, H., D. B. Rye, et al. (2007). "A Genetic Risk Factor for Periodic Limb Movements in Sleep." N Engl J Med.

Stiasny, K., W. H. Oertel, et al. (2002). "Clinical symptomatology and treatment of restless legs syndrome and periodic limb movement disorder." Sleep Med Rev **6**(4): 253-65.

Stiasny, K., T. C. Wetter, et al. (2001). "Long-term effects of pergolide in the treatment of restless legs syndrome." Neurology **56**(10): 1399-402.

Strachan, T. and A. P. Read (2003). Human Molecular Genetics, Garland Publishing.

Sun, E. R., C. A. Chen, et al. (1998). "Iron and the restless legs syndrome." Sleep **21**(4): 371-7.

Suzuki, K., T. Ohida, et al. (2003). "The prevalence of restless legs syndrome among pregnant women in Japan and the relationship between restless legs syndrome and sleep problems." Sleep **26**(6): 673-7.

Takaki, J., T. Nishi, et al. (2003). "Clinical and psychological aspects of restless legs syndrome in uremic patients on hemodialysis." Am J Kidney Dis **41**(4): 833-9.

Tan, E. K., A. Seah, et al. (2001). "Restless legs syndrome in an Asian population: A study in Singapore." Mov Disord **16**(3): 577-9.

Tings, T., V. Schettler, et al. (2004). "Impact of regular LDL apheresis on the development of restless legs syndrome." Mov Disord **19**(9): 1072-5.

Tison, F., A. Crochard, et al. (2005). "Epidemiology of restless legs syndrome in French adults: a nationwide survey: the INSTANT Study." Neurology **65**(2): 239-46.

Trenkwalder, C., B. Hogl, et al. (2008). "Augmentation in restless legs syndrome is associated with low ferritin." Sleep Med **9**(5): 572-4.

Trenkwalder, C., B. Hogl, et al. (2009). "Recent advances in the diagnosis, genetics and treatment of restless legs syndrome." J Neurol **256**(4): 539-53.

Trenkwalder, C., H.-P. Hundemer, et al. (2004). "Efficacy of pergolide in treatment of restless legs syndrome: The PEARLS Study." Neurology **62**(8): 1391-1397.

Trenkwalder, C., W. Paulus, et al. (2005). "The restless legs syndrome." Lancet Neurol **4**(8): 465-75.

Trenkwalder, C., V. C. Seidel, et al. (1996). "Clinical symptoms and possible anticipation in a large kindred of familial restless legs syndrome." Mov Disord **11**(4): 389-94.

Trenkwalder, C., K. Stiasny, et al. (1995). "L-dopa therapy of uremic and idiopathic restless legs syndrome: a double-blind, crossover trial." Sleep **18**(8): 681-8.

Trenkwalder, C., A. S. Walters, et al. (1996). "Periodic limb movements and restless legs syndrome." Neurol Clin **14**(3): 629-50.

Trenkwalder, C., A. S. Walters, et al. (1999). "Positron emission tomographic studies in restless legs syndrome." Mov Disord **14**(1): 141-5.

Turjanski, N., A. J. Lees, et al. (1999). "Striatal dopaminergic function in restless legs syndrome: 18F-dopa and 11C-raclopride PET studies." Neurology **52**(5): 932-7.

Ulfberg, J., B. Bjorvatn, et al. (2007). "Comorbidity in restless legs syndrome among a sample of Swedish adults." Sleep Med **8**(7-8): 768-72.

Van Camp, G., A. Flamez, et al. (2004). "Treatment of Parkinson's disease with pergolide and relation to restrictive valvular heart disease." Lancet **363**(9416): 1179-83.

Van De Vijver, D. A., T. Walley, et al. (2004). "Epidemiology of restless legs syndrome as diagnosed in UK primary care." Sleep Med **5**(5): 435-40.

Varga, L. I., N. Ako-Agugua, et al. (2009). "Critical review of ropinirole and pramipexole - putative dopamine D(3)-receptor selective agonists - for the treatment of RLS." J Clin Pharm Ther **34**(5): 493-505.

Vilarino-Guell, C., M. J. Farrer, et al. (2008). "A genetic risk factor for periodic limb movements in sleep." N Engl J Med **358**(4): 425-7.

Walters, A. S. (1995). "Toward a better definition of the restless legs syndrome. The International Restless Legs Syndrome Study Group." Mov Disord **10**(5): 634-42.

Walters, A. S., K. Hickey, et al. (1996). "A questionnaire study of 138 patients with restless legs syndrome: the 'Night-Walkers' survey." Neurology **46**(1): 92-5.

Walters, A. S., C. LeBrocq, et al. (2003). "Validation of the International Restless Legs Syndrome Study Group rating scale for restless legs syndrome." Sleep Med **4**(2): 121-32.

Walters, A. S., D. Picchietti, et al. (1990). "Variable expressivity in familial restless legs syndrome." Arch Neurol **47**(11): 1219-20.

Wang, X., J. Wiesinger, et al. (2004). "Thy1 expression in the brain is affected by iron and is decreased in Restless Legs Syndrome." Journal of the Neurological Sciences **220**(1-2): 59-66.

Wetter, T. C., J. Brunner, et al. (2002). "Restless legs syndrome probably induced by risperidone treatment." Pharmacopsychiatry **35**(3): 109-11.

Wetter, T. C., K. Stiasny, et al. (1999). "A randomized controlled study of pergolide in patients with restless legs syndrome." Neurology **52**(5): 944-50.

Winkelman, J. W., G. M. Chertow, et al. (1996). "Restless legs syndrome in end-stage renal disease." Am J Kidney Dis **28**(3): 372-8.

Winkelman, J. W., E. Shahar, et al. (2008). "Association of restless legs syndrome and cardiovascular disease in the Sleep Heart Health Study." Neurology **70**(1): 35-42.

Winkelmann, J. and L. Ferini-Strambi (2006). "Genetics of restless legs syndrome." Sleep Med Rev **10**(3): 179-83.

Winkelmann, J., P. Lichtner, et al. (2006). "Evidence for further genetic locus heterogeneity and confirmation of RLS-1 in restless legs syndrome." Mov Disord **21**(1): 28-33.

Winkelmann, J., P. Lichtner, et al. (2008). "Variants in the neuronal nitric oxide synthase (nNOS, NOS1) gene are associated with restless legs syndrome." Mov Disord **23**(3): 350-8.

Winkelmann J, L. P., Kemlink D et al. (2006). "New loci for restless legs syndrome map to Chromosome 4q and 17p [abstract]." Mov Disord. **21**: 304.

Winkelmann, J., B. Muller-Myhsok, et al. (2002). "Complex segregation analysis of restless legs syndrome provides evidence for an autosomal dominant mode of inheritance in early age at onset families." Ann Neurol **52**(3): 297-302.

Winkelmann, J., J. Schadrack, et al. (2001). "Opioid and dopamine antagonist drug challenges in untreated restless legs syndrome patients." Sleep Med **2**(1): 57-61.

Winkelmann, J., B. Schormair, et al. (2007). "Genome-wide association study of restless legs syndrome identifies common variants in three genomic regions." Nat Genet.

Winkelmann, J., T. C. Wetter, et al. (2000). "Clinical characteristics and frequency of the hereditary restless legs syndrome in a population of 300 patients." Sleep **23**(5): 597-602.

Woloshin, S. and L. M. Schwartz (2006). "Giving legs to restless legs: a case study of how the media helps make people sick." PLoS Med **3**(4): e170.

Ye, Z. and J. R. Connor (2000). "Identification of iron responsive genes by screening cDNA libraries from suppression subtractive hybridization with antisense probes from three iron conditions." Nucleic Acids Res **28**(8): 1802-7.

Ziegler A , K. I. (2010). A Statistical Approach to Genetic Epidemiology:

Concepts and Applications Wiley-Vch.

Zucconi, M. and L. Ferini-Strambi (2004). "Epidemiology and clinical findings of restless legs syndrome." Sleep Med **5**(3): 293-9.

Appendices

Appendix I: Restless Legs Syndrome Rating Scale

The patient rated his/her symptoms for the ten questions listed below. Ratings range from: none (0); mild (1); moderate (2); severe (3); and very severe (4). Ratings for question 3 include: none (0); mild (1 day a week or less) (1); moderate (2 to 3 days per week) (2); severe (4 to 5 days per week) (3); and very severe (6 to 7 days per week) (4). Ratings for question 4 include: none (0); mild (less than 1 hour per 24 hour day) (1); moderate (1 to 3 hours per 24 hour day) (2); severe (3 to 8 hours per 24 day) (3); and very severe (8 hours per 24 hour day or more) (4).

	PATH046	**PATH048**	**PATH050**	**PATH083**	**PATH051**	**PATH053**	**PATH054**	**PATH055**	**PATH185**	**PATH187**	**PATH207**
1. Overall, how would you rate the RLS discomfort in your legs or arms?	1	2	2	2	3	2	2	1	1	1	2
2. Overall, how would you rate the need to move around because of your RLS symptoms?	0	3	2	2	4	3	4	3	4	2	3
3. Overall, how much relief of your RLS arm or leg discomfort do you get from moving around?	1	1	1	2	1	1	2	2	1-2 (1.5)	1	1
4. Overall, how severe is your sleep disturbances from your RLS symptoms?	0	2	3	0	0	1	0	0	1	1	0
5. How severe is your tiredness or sleepiness from your RLS symptoms?	0	1	1	0	0	0	0	0	0	0	0
6. Overall, how severe is your RLS as a whole?	1	2	1	1	2	2	1	1	3	1	2

	PATH046	PATH048	PATH050	PATH083	PATH051	PATH053	PATH054	PATH055	PATH185	PATH187	PATH207
7. How often do you get RLS symptoms?	1	2	1	1	1	4	1	2	1-2 (1.5)	1	1
8. When you have RLS symptoms, how severe are they on an average day?	0	1	1	2	1	4	1	1	1	1	1
9. Overall, how severe is the impact of your RLS symptoms on your ability to carry out your daily affairs, for example carrying out a satisfactory family, home, social, school, or work life?	0	0	0	0	1	0	0	0	1	0	0
10. How severe is your mood disturbance from your RLS symptoms – for example angry, depressed, sad, anxious or irritable?	0	0	1	0	2	0	0	0	1	0	0
Total score:	4	14	13	10	15	17	11	10	16	8	10

Scoring criteria are: None = 0 points; mild = 1 – 10 points; moderate = 11 – 20 points; severe = 21 – 30 points; and Very severe = 31-40 points.

Appendix II: Research Genetics Inc. mapping set

Chromosome	Microsatellite Marker	Fluorescent Label	Position (cM)	Repeat Type	Heterozygosity	Size Range (bp)
1	D1S1622	FAM	56.74	ATA	0.73	249-273
1	D1S3728	HEX	89.49	GATA	0.63	244-268
1	D1S1728	HEX	109.04	GATA	0.58	158-174
1	D1S3723	HEX	140.39	GATA	0.67	142-206
1	D1S1589	TET	192.05	ATA	0.69	201-214
1	GATA124F08	FAM	226.16	GATA	0.79	229-245
1	D1S3462	TET	247.23	ATA	0.75	256-271
1	D1S547	TET	267.51	GATA	0.87	282-308
2	D2S2952	TET	17.88	GATA	0.77	177-209
2	D2S1400	FAM	27.60	GGAA	0.50	111-140
2	D2S405	TET	47.97	GATA	0.68	240-256
2	D2S1356	TET	64.29	ATA	0.95	218-262
2	D2S2739	FAM	73.61	GATA	0.94	281-329
2	D2S1777	FAM	99.41	GATA	0.64	196-208
2	D2S1334	HEX	145.08	GATA	0.71	266-310
2	D2S1776	TET	173.00	GATA	0.88	288-312
2	D2S434	FAM	215.78	GATA	0.82	262-286
2	D2S2968	HEX	251.94	GATA	0.59	171-195
2	D2S125	HEX	260.63	AC	0.86	96-100
3	D3S1304	HEX	22.33	AC	0.86	253-269
3	D3S3038	HEX	44.81	GATA	0.81	187-219
3	D3S4542	FAM	89.91	GATA	0.68	236-260
3	D3S2406	HEX	102.64	GGAT	0.92	306-350
3	D3S3045	FAM	124.16	GATA	0.99	170-211
3	D3S1303	FAM	136.32	AC	0.93	196-220
3	D3S3053	TET	181.87	GATA	0.68	226-238

3	D3S1262	FAM	201.14	AC	0.78	112-126
3	D3S3023	TET	126.98	ATA	0.82	228-249
4	D4S403	TET	25.90	AC	0.61	217-231
4	D4S2632	FAM	50.53	GATA	0.82	124-164
4	D4S3248	FAM	72.52	GATA	0.65	233-257
4	D4S3243	TET	88.35	GATA	0.61	162-174
4	D4S1647	TET	104.94	GATA	0.79	132-156
4	D4S1629	HEX	157.99	GATA	0.68	137-157
4	D4S2417	TET	181.93	GATA	0.67	251-271
4	D4S408	HEX	195.06	AC	0.85	229-243
5	D5S817	TET	22.88	GATA	0.62	260-272
5	D5S1470	TET	45.34	GATA	0.85	163-207
5	D5S2500	FAM	69.23	GATA	0.75	149-181
5	D5S1725	FAM	97.82	GATA	0.86	188-212
5	D5S1505	FAM	129.83	GATA	0.74	243-275
5	D5S1480	TET	147.49	ATA	0.86	208-249
5	D5S211	HEX	182.89	AC	0.79	186-204
6	D6S2434	TET	25.08	ATA	0.57	224-236
6	D6S2439	FAM	42.27	GATA	0.76	218-258
6	D6S2410	HEX	73.13	GATA	0.56	238-254
6	D6S1040	TET	128.93	GATA	0.74	257-285
6	D6S2436	TET	154.64	GATA	0.63	121-149
7	D7S513	FAM	17.74	AC	0.84	173-201
7	D7S3051	HEX	29.28	GATA	0.68	146-182
7	D7S2846	TET	57.79	GATA	0.85	172-196
7	D7S3046	FAM	78.65	GATA	0.69	318-346
7	D7S821	FAM	109.12	GATA	0.82	238-270
7	D7S3061	FAM	128.41	GGAA	0.82	114-146
7	D7S1804	TET	136.95	GATA	0.86	250-290
7	D7S1823	HEX	173.71	GATA	0.76	207-235
8	D8S1130	HEX	22.41	GATA	0.78	132-156

8	D8S136	TET	43.96	AC	0.79	71-80
8	D8S1477	FAM	60.34	GGAA	0.89	139-179
8	D8S532	FAM	64.75	AC	0.86	239-255
8	D8S285	FAM	71.00	AC	0.81	108-124
8	D8S260	HEX	79.36	AC	0.81	187-213
8	D8S2324	HEX	94.08	GATA	0.74	196-216
8	GAAT1A4	TET	110.20	GAAT	0.67	140-160
8	D8S1179	TET	135.08	GATA	0.91	162-194
8	D8S256	FAM	148.12	AC	0.81	210-232
9	D9S921	HEX	21.88	GATA	0.87	175-232
9	D9S1121	FAM	44.28	GATA	0.71	184-212
9	D9S1118	TET	58.26	GATA	0.85	142-178
9	D9S1122	TET	75.88	GATA	0.79	186-217
9	D9S257	HEX	91.87	AC	0.93	259-285
9	D9S282	FAM	136.47	AC	0.74	233-242
9	D9S2157	TET	146.83	ATA	0.82	132-173
10	D10S189	HEX	19.00	AC	0.85	175-195
10	D10S1423	FAM	46.23	GATA	0.83	218-238
10	D10S1208	FAM	63.30	ATA	0.83	179-200
10	ATA5A04	FAM	88.41	GATA	0.63	184-200
10	D10S2327	HEX	100.92	GGAT	0.58	204-228
10	D10S1239	TET	125.41	GATA	0.78	160-184
10	D10S1230	TET	142.78	ATA	0.71	113-140
10	D10S212	TET	170.94	AC	0.65	189-201
11	D11S1981	FAM	21.47	GATA	0.84	134-178
11	D11S2371	FAM	76.13	GATA	0.64	188-220
11	D11S2000	HEX	100.62	GATA	0.92	199-235
11	D11S4090	FAM	105.74	CA	0.80	167-191
11	D11S1998	HEX	113.13	GATA	0.69	129-169
11	D11S912	TET	131.26	AC	0.93	101-123
12	D12S398	FAM	68.16	GGAT	0.71	120-144

12	D12S1300	TET	104.12	GATA	0.68	112-136
12	D12S2070	TET	125.30	ATA	0.89	89-107
12	D12S2078	TET	149.60	GATA	0.89	250-282
13	D13S1493	FAM	25.80	GGAA	0.92	215-260
13	D13S800	HEX	55.31	GATA	0.74	295-319
13	D13S793	TET	76.26	GATA	0.74	253-273
13	D13S779	TET	82.93	ATA	0.50	175-210
14	D14S608	HEX	28.01	GATA	0.78	188-224
14	D14S599	FAM	40.68	ATA	0.85	98-133
14	D14S978	HEX	53.19	CA	0.89	228-258
14	D14S989	FAM	55.29	CA	0.57	161-185
14	D14S587	HEX	55.82	GGAA	0.81	254-274
14	D14S592	TET	66.80	ATA	0.78	220-250
14	D14S588	TET	75.61	GGAA	0.68	110-145
14	D14S53	FAM	86.29	CA	0.66	144-161
14	D14S610	FAM	95.89	GATA	0.73	360-376
14	D14S1279	TET	95.89	GATA	0.68	107-143
14	D14S617	FAM	105.53	GGAA	0.76	141-173
15	D15S1507	TET	60.17	GATA	0.65	205-221
15	D15S211	HEX	75.85	AC	0.95	207-261
15	D15S816	FAM	100.59	GATA	0.56	130-150
16	D16S403	HEX	43.89	AC	0.86	120-160
16	D16S3253	TET	71.77	GATA	0.78	167-199
17	D17S974	FAM	22.24	GATA	0.82	201-217
17	D17S947	FAM	31.96	AC	0.82	250-280
17	D17S2196	FAM	44.62	GATA	0.87	139-163
17	D17S2180	HEX	66.85	ATC	0.64	116-128
17	D17S2193	TET	89.32	ATA	0.71	88-123
17	D17S784	TET	116.86	AC	0.78	226-238
18	D18S976	HEX	12.81	GATA	0.92	171-198
18	D18S542	FAM	41.24	GATA	0.75	178-205

18	D18S877	TET	54.40	GATA	0.75	110-150
18	D18S1357	HEX	88.62	ATA	0.82	126-147
18	ATA82B02	TET	106.81	ATA	0.86	172-196
19	D19S1034	FAM	20.75	GATA	0.71	216-232
19	D19S586	HEX	32.94	GATA	0.79	222-250
19	D19S178	FAM	68.08	AC	0.77	155-189
19	D19S589	HEX	87.66	GATA	0.64	161-181
20	D20S482	TET	12.12	GATA	0.75	151-167
20	D20S851	HEX	24.70	AV	0.73	128-150
21	D21S2055	HEX	40.49	GATA	0.85	119-199
22	GCT10C10	FAM	18.10	GCT	0.68	192-210

Appendix III: Applied Biosystems Linkage mapping set version 2.5

Panel	Chromosome	Microsatellite Marker	Fluorescent Label	Position (cM)	Het	Size Range (bp)
1	1	D1S2797	FAM	75.66	0.74	97-135
1	1	D1S249	FAM	220.65	0.87	160-190
1	1	D1S2800	FAM	252.12	0.77	205-221
1	1	D1S234	FAM	55.1	0.81	262-284
1	1	D1S450	FAM	20.61	0.81	315-341
1	1	D1S255	VIC	65.47	0.75	85-107
1	1	D1S2667	VIC	24.68	0.82	122-152
1	1	D1S2785	VIC	266.27	0.76	171-185
1	1	D1S2890	VIC	85.68	0.81	211-235
1	1	D1S484	VIC	169.68	0.64	272-286
1	1	D1S196	VIC	181.49	0.74	321-337
1	1	D1S213	NED	242.34	0.86	103-129
1	1	D1S2878	NED	177.86	0.84	148-176
1	1	D1S206	NED	134.2	0.82	205-223
1	1	D1S2836	NED	285.75	0.79	242-256
1	1	D1S2726	NED	144.38	0.75	280-294
1	1	D1S2842	NED	273.46	0.76	336-358
2	1	D1S199	FAM	45.33	0.83	94-120
2	1	D1S207	FAM	113.69	0.84	146-176
2	1	D1S2868	FAM	126.16	0.76	206-220
2	1	D1S413	FAM	212.44	0.76	249-265
2	1	D1S238	FAM	202.73	0.86	292-326
2	1	D1S252	VIC	150.27	0.81	86-112
2	1	D1S230	VIC	95.31	0.78	150-164
2	1	D1S468	VIC	4.22	0.76	191-211
2	1	D1S2841	VIC	106.45	0.78	230-250
2	1	D1S2697	VIC	37.05	0.70	286-302
2	1	D1S214	NED	14.04	0.78	117-147

2	1	D1S498	NED	155.89	0.82	187-209
2	1	D1S218	NED	191.52	0.83	265-291
2	1	D1S425	NED	231.11	0.81	332-358
3	2	D2S286	FAM	94.05	0.66	80-102
3	2	D2S165	FAM	47.43	0.85	141-175
3	2	D2S160	FAM	122.96	0.78	206-224
3	2	D2S2211	FAM	15.61	0.74	236-258
3	2	D2S367	FAM	54.96	0.86	306-340
3	2	D2S125	VIC	260.63	0.82	87-109
3	2	D2S206	VIC	240.79	0.8	125-163
3	2	D2S117	VIC	194.45	0.82	190-220
3	2	D2S142	VIC	161.26	0.76	235-255
3	2	D2S2333	NED	103.16	0.82	79-101
3	2	D2S126	NED	221.13	0.82	113-145
3	2	D2S325	NED	204.53	0.82	154-184
3	2	D2S364	NED	186.21	0.80	230-256
3	2	D2S337	NED	80.69	0.88	291-315
4	2	D2S112	FAM	141.62	0.71	73-89
4	2	D2S162	FAM	20.03	0.75	119-148
4	2	D2S2330	FAM	169.41	0.81	166-186
4	2	D2S2216	FAM	111.21	0.76	208-224
4	2	D2S347	FAM	131.51	0.80	267-299
4	2	D2S2259	FAM	64.29	0.79	321-341
4	2	D2S319	VIC	7.6	0.73	128-140
4	2	D2S168	VIC	27.06	0.82	156-180
4	2	D2S151	VIC	152.04	0.82	224-252
4	2	D2S2382	VIC	213.49	0.81	296-336
4	2	D2S2368	NED	85.48	0.83	93-117
4	2	D2S391	NED	70.31	0.79	143-159
4	2	D2S335	NED	175.91	0.79	183-205
4	2	D2S396	NED	232.9	0.83	232-250
4	2	D2S338	NED	250.54	0.81	264-288
4	2	D2S305	NED	38.87	0.72	314-336
5	4	D4S392	FAM	78.97	0.82	79-109

5	3	D3S1311	FAM	224.88	0.83	133-159
5	3	D3S1565	FAM	186.04	0.64	178-194
5	4	D4S406	FAM	117.06	0.87	242-268
5	4	D4S1575	FAM	132.05	0.65	287-305
5	3	D3S1271	VIC	117.76	0.73	84-104
5	3	D3S3681	VIC	109.22	0.83	121-161
5	4	D4S414	VIC	100.75	0.89	231-249
5	4	D4S405	VIC	56.95	0.86	281-309
5	3	D3S1614	NED	177.75	0.83	101-125
5	3	D4S1534	NED	95.09	0.77	147-169
5	3	D3S1263	NED	36.1	0.86	191-211
5	3	D3S1285	NED	91.18	0.73	233-251
5	3	D4S1597	NED	169.42	0.76	274-300
6	3	D3S1262	FAM	201.14	0.80	110-132
6	3	D3S1569	FAM	158.38	0.80	150-174
6	4	D4S1572	FAM	107.95	0.84	195-213
6	3	D3S1300	FAM	80.32	0.82	230-262
6	4	D4S413	FAM	157.99	0.85	282-334
6	4	D4S2935	VIC	13.96	0.62	85-105
6	4	D4S1592	VIC	69.53	0.72	113-141
6	4	D4S391	VIC	43.59	0.85	150-170
6	3	D3S1304	VIC	22.33	0.80	254-276
6	3	D3S1601	VIC	214.45	0.85	298-330
6	3	D3S1297	VIC	8.31	0.82	351-369
6	3	D3S1292	NED	146.6	0.85	111-145
6	4	D4S426	NED	206.98	0.76	160-180
6	4	D4S419	NED	33.42	0.77	225-245
6	4	D4S415	NED	181.36	0.80	264-300
7	4	D4S402	FAM	124.45	0.91	106-146
7	4	D4S403	FAM	25.9	0.77	170-186
7	4	D3S1580	FAM	207.73	0.84	215-235
7	4	D3S1279	FAM	169.6	0.85	268-286
7	4	D4S1539	FAM	176.19	0.68	316-326
7	4	D3S2338	VIC	42.1	0.86	89-109

7	4	D4S2964	VIC	88.35	0.76	119-143
7	4	D4S412	VIC	4.74	0.77	158-176
7	4	D4S424	VIC	144.56	0.83	194-212
7	4	D3S1278	VIC	129.73	0.87	232-260
7	4	D3S1266	VIC	52.6	0.73	289-305
7	4	D3S1267	NED	139.12	0.88	93-131
7	4	D3S1566	NED	97.75	0.84	155-177
7	4	D3S1289	NED	71.41	0.81	202-224
7	4	D4S1535	NED	195.06	0.77	248-262
7	4	D3S1277	NED	61.52	0.82	289-311
8	5	D5S407	FAM	64.67	0.86	82-110
8	6	D6S289	FAM	29.93	0.79	160-182
8	6	D6S1610	FAM	53.81	0.84	200-214
8	6	D6S1581	FAM	164.78	0.72	257-271
8	6	D6S422	FAM	35.66	0.77	298-320
8	5	D5S644	VIC	104.76	0.85	82-112
8	6	D6S281	VIC	190.14	0.68	131-151
8	6	D6S262	VIC	130	0.82	169-189
8	5	D5S424	VIC	81.95	0.76	212-234
8	5	D5S419	VIC	39.99	0.81	255-287
8	5	D5S433	NED	111.97	0.86	63-93
8	5	D5S422	NED	164.19	0.84	113-135
8	5	D5S406	NED	11.85	0.79	164-192
8	5	D5S400	NED	174.8	0.82	217-239
8	6	D6S309	NED	14.07	0.83	304-330
9	6	D6S264	FAM	179.07	0.70	108-130
9	6	D6S1574	FAM	9.18	0.84	146-172
9	6	D6S276	FAM	44.41	0.83	201-233
9	5	D5S408	FAM	195.49	0.73	249-285
9	6	D6S308	FAM	144.46	0.75	326-354
9	6	D6S287	VIC	121.97	0.85	105-139
9	6	D6S292	VIC	136.97	0.83	155-177
9	6	D6S434	VIC	109.19	0.86	202-246
9	5	D5S426	VIC	51.99	0.80	275-299

9	5	D5S1981	NED	1.72	0.73	115-125
9	6	D6S257	NED	79.92	0.87	167-195
9	6	D6S446	NED	189	0.62	217-229
9	5	D5S641	NED	92.38	0.77	299-339
10	5	D5S2027	FAM	119.5	0.78	180-202
10	5	D5S436	FAM	147.49	0.83	238-258
10	6	D6S460	FAM	89.83	0.81	279-303
10	5	D5S410	FAM	156.47	0.79	329-351
10	6	D6S462	VIC	99.01	0.68	104-121
10	5	D5S2115	VIC	138.64	0.76	142-170
10	5	D5S418	VIC	58.55	0.80	208-228
10	5	D5S428	VIC	95.4	0.76	241-259
10	5	D5S630	VIC	19.67	0.89	283-393
10	6	D6S470	NED	18.22	0.80	120-140
10	6	D6S441	NED	154.1	0.86	162-196
10	5	D5S471	NED	129.83	0.76	235-255
10	5	D5S416	NED	28.76	0.77	285-297
10	5	D5S647	NED	74.07	0.82	326-365
11	7	D7S484	FAM	53.5	0.74	97-115
11	8	D8S264	FAM	0.73	0.83	136-160
11	8	D8S260	FAM	79.36	0.81	191-217
11	7	D7S517	FAM	7.44	0.83	243-261
11	8	D8S1784	FAM	118.15	0.67	276-292
11	7	D7S2465	FAM	180.24	0.83	319-343
11	8	D8S549	VIC	31.73	0.63	73-83
11	7	D7S530	VIC	134.55	0.78	105-123
11	8	D8S258	VIC	41.55	0.70	142-156
11	7	D7S669	VIC	90.42	0.80	172-194
11	8	D8S272	VIC	154.02	0.81	211-261
11	7	D7S502	VIC	78.65	0.84	289-309
11	7	D7S630	VIC	98.44	0.73	327-355
11	7	D7S510	NED	59.93	0.77	79-95
11	7	D7S640	NED	137.83	0.85	110-150
11	7	D7S513	NED	17.74	0.83	168-198

11	8	D8S514	NED	130	0.77	212-232
11	7	D7S657	NED	104.86	0.81	244-270
11	7	D7S516	NED	41.69	0.76	306-326
11	8	D8S1771	NED	50.05	0.75	343-367
12	7	D7S507	FAM	28.74	0.89	81-109
12	7	D7S515	FAM	112.32	0.82	139-201
12	7	D7S486	FAM	124.08	0.81	221-235
12	7	D7S519	FAM	69.03	0.81	257-284
12	7	D7S661	FAM	155.1	0.75	305-337
12	7	D7S798	VIC	168.98	0.84	71.26-93
12	8	D8S505	VIC	60.87	0.79	110-124
12	8	D8S277	VIC	8.34	0.73	151-185
12	7	D7S493	VIC	34.69	0.88	203-235
12	8	D8S284	VIC	143.82	0.83	272-306
12	7	D7S684	VIC	147.22	0.81	341-363
12	8	D8S270	NED	103.69	0.79	101-117
12	7	D7S636	NED	162.33	0.90	136-172
12	8	D8S550	NED	21.33	0.87	187-217
12	7	D7S531	NED	5.28	0.77	276-294
12	8	D8S285	NED	71	0.78	314-330
13	11	D11S987	FAM	67.48	0.82	94-134
13	11	D11S937	FAM	79.98	0.88	144-180
13	11	D11S935	FAM	45.94	0.73	196-218
13	9	D9S1677	FAM	117.37	0.81	229-255
13	11	D11S1314	VIC	73.64	0.78	93-121
13	11	D11S902	VIC	21.47	0.80	148-170
13	11	D11S904	VIC	33.57	0.83	183-213
13	11	D10S547	VIC	29.15	0.74	236-257
13	11	D11S905	VIC	51.95	0.75	269-297
13	9	D9S285	NED	29.52	0.78	80-110
13	10	D10S249	NED	2.13	0.74	117-139
13	9	D9S171	NED	42.73	0.79	160-186
13	9	D9S273	NED	65.79	0.74	202-222
13	10	D10S192	NED	124.27	0.77	238-264

13	11	D11S4175	NED	91.47	0.89	288-340
14	9	D9S161	FAM	51.81	0.78	122-139
14	10	D10S197	FAM	52.1	0.75	166-180
14	10	D10S185	FAM	116.34	0.77	201-219
14	9	D9S175	FAM	70.33	0.85	255-289
14	11	D11S901	FAM	85.48	0.82	311-327
14	10	D10S1653	VIC	40.36	0.77	118-132
14	10	D10S212	VIC	170.94	0.71	189-207
14	10	D10S1686	VIC	105.04	0.86	243-281
14	9	D9S287	VIC	103.42	0.67	295-315
14	11	D11S4046	NED	2.79	0.86	101-125
14	9	D9S288	NED	9.83	0.84	132-154
14	10	D10S208	NED	60.64	0.79	173-193
14	9	D9S157	NED	32.24	0.84	225-249
14	9	D9S167	NED	83.41	0.87	304-338
15	9	D9S164	FAM	147.91	0.80	84-102
15	9	D9S286	FAM	18.06	0.88	136-170
15	9	D9S1690	FAM	106.63	0.78	226-240
15	11	D11S1320	FAM	141.91	0.68	259-277
15	11	D11S4151	FAM	127.33	0.79	331-345
15	11	D11S4191	VIC	60.09	0.87	89-119
15	11	D11S968	VIC	147.77	0.81	140-162
15	9	D9S1776	VIC	123.33	0.84	172-210
15	11	D11S1338	VIC	12.92	0.74	253-271
15	10	D10S591	VIC	13.49	0.71	309-339
15	10	D10S587	NED	147.57	0.80	92-114
15	10	D10S537	NED	91.13	0.83	139-165
15	10	D10S189	NED	19	0.72	179-197
15	11	D11S925	NED	118.47	0.84	260-290
16	10	D10S217	FAM	157.89	0.81	96-120
16	11	D11S898	FAM	98.98	0.85	141-165
16	10	D10S548	FAM	45.7	0.70	182-198
16	9	D9S1826	FAM	159.61	0.69	215-231
16	9	D9S290	FAM	140.86	0.83	240-262

16	9	D91817	FAM	59.34	0.88	279-315
16	9	D9S158	FAM	161.71	0.69	330-356
16	10	D10S196	VIC	70.23	0.77	103-115
16	9	D9S1682	VIC	132.09	0.68	147-159
16	11	D11S908	VIC	108.59	0.76	172-190
16	10	D10S1693	VIC	137.39	0.80	213-227
16	10	D10S597	VIC	128.73	0.64	273-297
16	9	D9S283	NED	94.85	0.80	89-115
16	10	D10S1651	NED	168.77	0.80	206-230
16	10	D10S1652	NED	80.77	0.78	269-295
17	12	D12S83	FAM	75.17	0.81	102-122
17	13	D13S218	FAM	32.9	0.66	141-153
17	12	D12S78	FAM	111.87	0.91	174-212
17	13	D13S217	FAM	17.21	0.68	242-262
17	12	D12S1659	FAM	155.94	0.78	290-316
17	13	D13S285	VIC	110.55	0.81	89-115
17	13	D13S170	VIC	63.9	0.90	143-173
17	12	D12S1723	VIC	164.63	0.67	198-216
17	13	D13S175	NED	6.03	0.76	101-119
17	13	D13S263	NED	38.32	0.84	146-174
17	12	D12S346	NED	104.65	0.84	188-214
17	12	D12S1617	NED	44.03	0.80	245-265
18	12	D12S85	FAM	61.34	0.67	99-131
18	12	D12S351	FAM	95.56	0.75	147-169
18	12	D12S368	FAM	66.03	0.81	202-222
18	13	D13S1265	FAM	98.82	0.80	275-305
18	12	D12S86	VIC	134.54	0.89	129-169
18	13	D13S156	VIC	55.85	0.80	277-297
18	12	D12S336	NED	19.68	0.82	111-129
18	12	D12S79	NED	125.31	0.87	160-186
18	12	D12S345	NED	53.09	0.87	211-247
18	12	D12S99	NED	12.6	0.83	264-296
19	13	D13S158	FAM	84.87	0.82	116-133
19	13	D13S159	FAM	79.49	0.90	154-196

19	13	D13S173	FAM	93.52	0.82	232-252
19	12	D12S364	FAM	30.6	0.87	298-326
19	13	D13S265	VIC	98.82	0.70	89-127
19	12	D12S352	VIC	0	0.73	154-174
19	12	D12S326	VIC	86.4	0.80	207-233
19	12	D12S310	VIC	36.06	0.69	244-252
19	13	D13S153	NED	45.55	0.81	89-121
19	13	D13S171	NED	25.08	0.73	177-205
19	12	D12S324	NED	147.17	0.69	233-255
20	14	D14S292	FAM	134.3	0.73	83-100
20	14	D14S275	FAM	28.01	0.70	145-159
20	14	D14S258	FAM	76.28	0.79	192-213
20	14	D14S280	FAM	105	0.68	237-258
20	14	D14S70	VIC	40.11	0.75	98-114
20	14	D14S283	VIC	13.89	0.81	127-158
20	14	D14S63	VIC	69.18	0.76	177-195
20	14	D14S985	VIC	126.61	0.76	240-255
20	14	D14S74	VIC	87.36	0.79	297-321
20	14	D14S65	NED	117.3	0.79	124-156
20	14	D14S288	NED	47.51	0.83	192-215
20	14	D14S276	NED	56.36	0.76	236-249
20	14	D14S261	NED	6.46	0.75	273-305
20	14	D14S68	NED	95.89	0.91	318-346
21	16	D16S3075	FAM	23.28	0.79	76-94
21	16	D16S3136	FAM	62.11	0.69	173-185
21	16	D16S3068	FAM	48.53	0.77	219-235
21	15	D15S130	FAM	100.59	0.66	285-299
21	16	D16S515	FAM	92.1	0.80	325-357
21	15	D15S1002	VIC	14.58	0.78	104-134
21	16	D16S520	VIC	125.82	0.84	149-165
21	15	D15S165	VIC	20.24	0.79	182-213
21	15	D15S131	VIC	71.28	0.83	240-281
21	16	D16S503	VIC	83.55	0.81	299-319
21	15	D15S127	NED	86.81	0.86	118-154

21	16	D16S3091	NED	111.12	0.73	166-182
21	15	D15S153	NED	62.4	0.87	240-274
21	15	D15S117	NED	51.21	0.78	321-339
22	16	D16S3046	FAM	40.65	0.74	83-109
22	15	D15S205	FAM	78.92	0.88	126-166
22	16	D16S415	FAM	67.62	0.72	213-241
22	15	D15S1012	VIC	35.95	0.72	94-112
22	16	D16S423	VIC	10.36	0.73	137-161
22	15	D15S978	VIC	45.62	0.83	184-212
22	16	D16S404	VIC	18.07	0.80	261-281
22	16	D16S3103	VIC	32.07	0.81	315-343
22	15	D15S1007	NED	25.86	0.86	84-108
22	15	D15S120	NED	112.58	0.73	155-183
22	15	D15S128	NED	6.11	0.78	197-215
22	16	D16S516	NED	100.39	0.73	245-267
22	15	D15S994	NED	40.25	0.73	303-315
23	18	D18S70	FAM	126	0.83	111-131
23	17	D17S949	FAM	93.27	0.80	210-228
23	18	D18S478	FAM	52.86	0.64	242-256
23	17	D17S1852	FAM	22.24	0.87	279-310
23	17	D17S831	VIC	6.6	0.82	107-129
23	17	D17S1857	VIC	43.01	0.64	164-174
23	17	D17S799	VIC	31.96	0.68	186-208
23	17	D17S1868	VIC	64.16	0.73	254-268
23	17	D17S798	VIC	53.41	0.80	298-322
23	18	D18S1102	NED	62.84	0.79	90-102
23	17	D17S787	NED	74.99	0.81	138-174
23	18	D18S61	NED	105.03	0.87	209-239
23	17	D17S849	NED	0.63	0.67	253-267
23	18	D18S462	NED	120.05	0.70	296-318
24	18	D18S474	FAM	71.32	0.82	121-143
24	18	D18S53	FAM	41.24	0.79	152-182
24	17	D17S938	FAM	14.69	0.76	238-258
24	18	D18S464	FAM	31.17	0.65	298-314

24	18	D18S63	VIC	8.3	0.79	74-100
24	18	D18S59	VIC	0	0.81	152-174
24	17	D17S921	VIC	36.14	0.72	193-211
24	17	D17S784	VIC	116.86	0.77	230-244
24	18	D18S64	VIC	84.8	0.74	319-345
24	17	D17S928	NED	126.46	0.76	68-102
24	18	D18S452	NED	18.7	0.83	126-144
24	17	D17S785	NED	103.53	0.83	165-193
24	18	D18S1161	NED	114.26	0.82	211-237
24	18	D18S68	NED	96.48	0.68	269-295
24	17	D17S944	NED	82.56	0.75	318-334
25	20	D20S889	FAM	11.2	0.83	87-123
25	20	D20S117	FAM	2.83	0.84	151-187
25	20	D20S112	FAM	39.25	0.81	213-237
25	19	D19S220	FAM	62.03	0.84	267-291
25	19	D19S221	VIC	36.22	0.86	87-110
25	20	D20S171	VIC	95.7	0.78	127-155
25	19	D19S210	VIC	100.01	0.74	172-192
25	20	D20S100	VIC	84.78	0.76	209-235
25	19	D19S420	NED	66.3	0.79	95-117
25	19	D19S414	NED	54.01	0.78	164-194
25	20	D20S115	NED	21.15	0.66	234-246
25	20	D20S196	NED	75.01	0.81	259-295
26	20	D20S119	FAM	61.77	0.82	103-123
26	21	D21S266	FAM	45.87	0.59	156-178
26	20	D20S107	FAM	55.74	0.80	197-221
26	19	D19S902	FAM	72.72	0.79	237-273
26	20	D20S186	VIC	32.3	0.86	113-139
26	22	D22S420	VIC	4.06	0.77	153-169
26	22	D22S280	VIC	31.3	0.82	213-225
26	19	D19S216	VIC	20.01	0.76	256-274
26	22	D22S423	VIC	46.42	0.82	287-309
26	19	D19S884	NED	26.37	0.86	93-113
26	21	D21S1252	NED	35.45	0.80	144-176

26	22	D22S539	NED	14.44	0.58	199-217
26	22	D22S274	NED	51.54	0.77	276-298
27	20	D20S195	FAM	50.81	0.81	128-154
27	22	D22S315	FAM	21.47	0.78	180-210
27	19	D19S209	FAM	10.97	0.77	238-254
27	19	D19S418	VIC	92.56	0.66	87-107
27	20	D20S173	VIC	98.09	0.67	128-182
27	21	D21S263	VIC	27.4	0.75	194-229
27	21	D21S1914	VIC	19.39	0.86	258-280
27	21	D21S1256	NED	9.72	0.65	96-116
27	22	D22S283	NED	38.62	0.89	127-155
27	20	D20S178	NED	66.16	0.83	179-195
27	19	D19S226	NED	42.28	0.85	238-270
27	19	D19S571	NED	84.08	0.81	287-319

Appendix IV: Corrected CEPH sizes for microsatellite markers and corresponding members of RLS3002

Marker	Position (cM)	PATH046	PATH047	PATH048	PATH049	PAYH050	PATH083	PATH199	PATH200	PATH051	PATH052	PATH053	PATH054	PATH055	PATH185	PATH186	PATH187	PATH207	PATH208
D1S468	4.22	183, 189		189, 189	189, 183	183, 183	189, 189	189, 183	183, 189	183, 189	189, 189	189, 183	189, 189	189, 189	189, 189	191, 191	189, 191	189, 191	189, 191
D1S214	14.04	136, 136	136, 141	136, 136	136, 141	136, 141	136, 136	136, 141	136, 136	120, 141	136, 136	120, 136	136, 141	141, 136	136, 136	131, 136	131, 136	136, 136	136, 136
D1S450	20.61	251, 225	251, 279	251, 259	255, 259	255, 259	251, 259	251, 259	251, 255	251, 259	251, 251	251, 251	251, 259	255, 259	251, 255	259, 261	251, 259		
D1S2667	24.68	144, 148	133, 144	144, 148	134, 148	134, 148	144, 148	134, 148	144, 148	142, 148	134, 134	134, 148	134, 142	134, 148	148, 148	140, 146			
D1S2697	37.05	273, 181	277, 181	281, 281	272, 277	277, 281	273, 281	277, 281	273, 281	281, 281	273, 281	273, 281	281, 281	273, 273	273, 273	273, 281	283, 281		
D1S199	45.33	100, 108	110, 114	108, 110	100, 110	108, 114	100, 110	108, 114	100, 110	96,100	98,112	96,112	96,112	96,100	96,100	98,100	96,100	96,08	96,08
D1S1622	56.74	258, 258	258, 279	258, 258	258, 279	258, 279	258, 258			261, 270	258, 270	258, 261	258, 270	258, 270	258, 258	258, 258	258, 258		
D1S255	65.47	72,80	74,74	74,80	74,80	74,74	74,74	74,80	74,80	74,80	74,74	74,74	74,74	74,74	74,80	80,84	80,80		
D1S2797	75.66	168, 168	168, 168	168, 168	168, 168	168, 168	168, 168	168, 168	168, 168	168, 168	168, 170	168, 170	168, 168	168, 168	166, 168	164, 168	166, 168		
D1S2890	85.68	175, 179	177, 179	175, 177	175, 177	179, 179	177, 179	179, 179	175, 179	179, 183	175, 185	179, 185	179, 185	175, 187	175, 187	179, 179	179, 187		
D1S3728	89.49	248, 260	252, 268	248, 252	248, 252	260, 268	252, 260			248, 260	248, 260	260, 260	256, 260	248, 252	248, 252	252, 264	252, 252		

D1S230	95.31	177, 179	179, 185	179, 185	179, 185	177, 179	179, 185	177, 185		177, 179	179, 185	177, 179	177, 185	179, 189	179, 189	183, 189	183, 189	189, 189	179, 183
D1S2841	106.45	277, 283	283, 285	277, 283	277, 283	277, 285	277, 283	277, 283	277, 285	277, 283	283, 283	283, 283		275, 281	277, 281	271, 279	271, 281	277, 279	
D1S1728	109.04	162, 166	158, 174	158, 166	158, 166	158, 166	166, 174			162, 166	162, 162	162, 162	162, 162	166, 166	166, 166	162, 166	162, 166		
D1S2868	126.16	146, 180	146, 148	146, 148	146, 148	146, 148	146. 146	146, 148	146, 148	146, 148		144, 148	146, 148	148, 148	146, 148	146, 148	148, 148		146, 148
D1S206	134.20	210, 212	210, 214	210, 212	210, 210	210, 212	212, 214	210, 212	212, 214	210, 212	210, 212	212, 212	210, 210	210, 212	210, 212	206, 206	206, 210		
D1S3723	140.39	154, 198	146, 198	146, 154	146, 154	146, 154	198, 198			154, 198	194, 150	154, 194	150, 198	198, 182	154, 194	190, 194	190, 194		
D1S2726	144.28	276, 280	276, 282	276, 280	276, 280	276, 280	276, 276	276, 282	276, 280	276, 280	278, 280	280, 280	276, 278	276, 280	278, 280	278, 288	278, 280		
D1S498/ D1S252	150.27	189, 193	191, 203	189, 203	193, 203	193, 203	189, 191	189, 203	191, 193	191, 195	191, 195	191, 195	191, 195	189, 193	189, 193	197, 197			193, 197
D1S484	169.69	136, 138	136, 138	136, 136	136, 138	136, 138	136, 136	136, 138	138, 138	136, 142	136, 142	136, 142	136, 136	136, 142	136, 138	136, 136	136, 136		
D1S2878	177.86	169, 177	177, 189	177, 189	169, 189	169, 189	177, 177	177, 189	169, 177	175, 179	177, 183	179, 183	175, 183	169, 177	169, 177	196, 175	169, 169		
D1S196	181.49	267, 277	277, 277	277, 277	277, 277	267, 277	277, 277	277, 277	267, 277	275, 283	267, 275	267, 275		277, 267	277, 267	273, 275	273, 277		
D1S1589		205, 220	205, 205	205, 220	205, 220	205, 220	205, 220			205, 205	208, 208	205, 208	205, 208	205, 220	205, 220	205, 217	205, 217		
D1S413	212.42	246, 246	254, 254	246, 254	246, 254	246, 254	246, 254	246, 254	246, 254	250, 254	254, 254	250, 254	250, 254		246, 250	246, 246	246, 246		246, 250
D1S249	220.65	173, 175	177, 181	174, 177	173, 181	175, 181		173, 181	173, 181	161, 173	171, 177	171, 173	173, 177	173, 175	173, 175	167, 175	173, 175		
GATA124F08	226.16	233, 241	233, 237	233, 237	233, 241	233, 233	233, 241			233, 237	229, 233	229, 233	233, 233	233, 233	233, 237	233, 237	237, 237		

D1S425	231.11	100, 100	96,100	100, 100	96,100	96,100	96,100	96,100	96,100	100, 100	96,96	96,100		92,100	92,102	96,102	96,102	
D1S213	242.23	114, 124	104, 118	104, 114	118, 124	114, 118	104, 114	114, 118	118, 124	108, 124	114, 118	118, 124	114, 124	108, 124	104, 108	108, 114	104, 114	
D1S3462	247.23	257, 263	257, 260	257, 257	257, 257	260, 263	257, 260	257, 260	260, 263	257, 263	257, 260	257, 257	257, 260	257, 263	257, 257	260, 263	260, 257	
D1S2800	252.12	182, 184	182, 184	182, 184	184, 184	184, 184	182, 184	182, 184	184, 184	178, 184	178, 186	178, 186	178, 184	178, 184	182, 190	182, 182	182, 182	
D1S2785	266.27	168, 178	168, 178	168, 168	168, 178	168, 178	178, 178	168, 178	168, 178	174, 178	174, 180	174, 178	174, 178	174, 178		168, 178	168, 174	
D1S547	267.51	290, 290	290, 300	290, 300	290, 290	290, 290	290, 290			286, 290	286, 290	286, 290	290, 290	290, 290	290, 290	290, 290	290, 290	
D1S2842	273.96	227, 227	217, 217	217, 227	217, 227	217, 227	217, 227	217, 227	217, 227	221, 229	221, 221	221, 229		221, 229	227, 229	221, 223	221, 229	
D1S2836	285.75	273, 277	270, 277	273, 277	270, 273		277, 277	270, 273	270, 273	270, 273	270, 270	270, 273		273, 277	270, 277			
D2S2211	15.61	254, 254	258, 258	254, 258	254, 258	254, 258	254, 258	254, 258	254, 258	254, 254	254, 256	254, 256	254, 254	254, 258	254, 254	254, 254	254, 254	254, 254
D2S2952	17.88	185, 189	181, 201	181, 185	181, 185	181, 189	185, 201			185, 189	185, 185	189, 185	185, 185	185, 185	185, 189	185, 185	189, 185	
D2S168	27.06	198, 200	210, 210	200, 210	200, 210	198, 210	200, 210	198, 210	200, 210	198, 200	200, 210	200, 200	200, 200	200, 210	198, 200	198, 210	198, 198	198, 198
D2S405	47.97	245, 245	245, 253	245, 253	245, 253	245, 253	245, 245			245, 249	245, 249	245, 249	249, 249	245, 249	245, 249	245, 253	249, 253	
D2S367	54.96	161, 165	137, 143	143, 161	137, 165	143, 165	137, 165	137, 165	137, 161	163, 165	137, 143	143, 165	137, 165	159, 161	163, 165	161, 165	161, 163	161, 165
D2S2259	64.29	229, 231	227, 229	229, 231	229, 231		227, 229	227, 229	227, 231	229, 229	229, 243	229, 229	229, 243	229, 229	227, 229	227, 231	227, 229	229, 231	227, 229
D2S1356	64.29	237, 240	240, 252	240, 240,	237, 252	240, 240	237, 252			237, 252	252, 252	237, 252	252, 252	237, 252	237, 252	237, 240	237, 252	

D2S391	70.31	144, 150	150, 152	150, 152	144, 150	144, 152	144, 150	150, 150	150, 150	144, 152	144, 144	144, 144	144, 144	144, 144	144, 144	144, 150	144, 144		144, 144
D2S2739	73.61	319, 323	291, 315	315, 319	291, 323	315, 323	391, 323			309, 323	321, 327	321, 323	309, 327	285, 309	309, 323	391, 315	309, 315		
D2S337	80.69	235, 249	233, 235	233, 235	233, 249	235, 249	233, 235			239, 249	247, 253	249, 253	239, 247	239, 247	239, 249	235, 237	237, 239		
D2S290	84.42	205, 215	215, 219	205, 215	215, 215	215, 219	205, 215			205, 215	207, 219	205, 207	205, 219	205, 219	205, 215	217, 219	205, 217		
D2S166	82.42	240, 242	240, 242	240, 240	240, 242	242, 242	240, 240	240, 242	240, 240	240, 242	242, 242	240, 242	240, 242	240, 242	240, 242	240, 244	240, 244		
D2S285	86.02	191, 193	191, 203	193, 203	191, 203	191, 191	193, 203	193, 203	191, 203	191, 193	189, 191	189, 193	191, 193	189, 913	181, 193	189, 199	189, 193		
D2S327	88.15	123, 125	125, 125	123, 125	125, 125	125, 125	123, 125	123, 125	125, 125	123, 125	127, 129	123, 127	125, 129	123, 127	123, 125	125, 129	123, 125		
D2S2113	88.15	194, 194	194, 200	194, 200	194, 200	194, 194	194, 200			194, 194	194, 194	194, 194	194, 194	188, 194	194, 194	194, 200	194, 200		
D2S2110	90.82	139, 141	133, 139	133, 141	133, 141	139, 141	133, 139	133, 139	133, 141	139, 141	133, 139	139, 139	133, 141	133, 139	139, 141	139, 141	133, 141		
D2S286	94.05	136, 144	136, 136	136, 144	136, 144	136, 144	136, 136	136, 136		136, 144	136, 136	136, 136	144, 136	130, 136	136, 144	136, 136	136, 136	136, 144	136, 144
D2S1777	99.41	200, 200	200, 204	200, 204	200, 204	200, 200	200, 200			200, 200	204, 208	200, 208	200, 204	200, 200	200, 200	200, 204	200, 200		
D2S329	101.02	244, 244	236, 238	238, 244	238, 244	236, 244	238, 244	238, 244	238, 244	242, 244	244, 244	242, 244	244, 244	242, 248	244, 244	242, 242	242, 244		
D2S2333	103.16	254, 260	254, 256	254, 256	254, 260	254, 254	256, 260	256, 260	254, 256	254, 256	248, 254	254, 256	248, 254	254, 256	254, 256	254, 258	256, 258	254, 258	254, 254
D2S160	122.96	212, 214	212, 214	214, 214	212, 214	212, 214	212, 214	212, 214	214, 214	212, 214	208, 214	208, 212	208, 214	212, 212	212, 214	210, 210	210, 212		210, 214
D2S1334	145.08	286, 290	286, 298	290, 298	298, 86	286, 286	286, 286			290, 294	286, 298	286, 294	286, 290	286, 298	290, 294	286, 294			

D2S151	152.04	221, 227	221, 221	221, 227	221, 221	221, 221	221, 221	221, 227	221, 227	221, 227	225, 225	221, 225	225, 227	221, 225		221, 227		227, 231	221, 227
D2S142	161.26	254, 254	260, 262	254, 262	254, 260	254, 262	254, 262	254, 260	254, 260	254, 254	260, 260	254, 260		254, 264	254, 262	258, 264	254, 264	258, 262	258, 262
D2S117	194.45	204, 206		206, 206	206, 206	206, 206	204, 208	206, 208	206, 206	192, 204	188, 192	192, 192	192, 204	192, 204	192, 192	206, 208	192, 208	192, 206	192, 206
D2S325	204.53	200, 206		206, 206	204, 206	206, 206	200, 204	204, 206	206, 206	200, 206	198, 198	198, 206	198, 200	200, 200	200, 200	198, 200	200, 200	200, 200	200, 200
D2S2382	213.49	251, 261	251, 259	259, 261	259, 261		251, 251	259, 261	259, 261	261, 273	279, 279	261, 279	273, 279	273, 285	251, 285	251, 256			255, 285
D2S434	215.78	270, 274	266, 278	266, 274	266, 274	274, 278	270, 278			274, 274	270, 282	274, 282	274, 282	274, 274	270, 274	270, 274	274, 274		
D2S126	221.13	141, 151		151, 153	141, 153	141, 151	141, 151	151, 153	141, 153	141, 151	153, 155	151, 155	151, 153	151, 155	141, 151	153, 155	151, 155	151, 155	151, 155
D2S2968	251.94	183, 187	183, 187	183, 187	187, 187	183, 187	183, 187			183, 183	183, 183	183, 183	183, 183	183, 183	183, 187	183, 179	179, 183		
D2S125	260.63	98,98	98,104	98,98	98,98	98,104	98,104	98,98	98,104	104, 106		98,104	98,106	98,106	98,104	92,94	94,104		92,104
D3S1304	22.33	263, 263	267, 267	263, 267	263, 267	263, 267	263, 267			263, 263	265, 267	263, 267	263, 265	263, 263	263, 267	253, 267	253, 267		
D3S1263	36.10	233, 239		239, 247		233, 239	239, 247	239, 247	239, 247	233, 239	233, 237	233, 239	233, 237	233, 239	237, 239	231, 235	235, 237	231, 239	231, 239
D3S2338	42.10	179, 179	183, 189	179, 189	179, 183	179, 183	179, 189	179, 189	179, 189	179, 179	189, 189	179, 189	179, 189		179, 195		193, 195	179, 191	
D3S3038	44.81	199, 203	195, 203	195, 199	203, 203	203, 203	195, 203			199, 215	199, 199	199, 199	199, 215	199, 203	199, 215	199, 199	199, 215		
D3S1266	52.60	291, 291	289, 291	289, 291	291, 291	291, 291	289, 291	289, 291	289, 291	291, 297	289, 297	297, 297	291, 297		291, 291	291, 297	291, 291	291, 297	291, 297
D3S1277	61.52	274, 274	266, 268	268, 274	268, 274	268, 274	266, 274	266, 274	266, 274		268, 268	268, 274	268, 274		274, 274		264, 274	274, 274	274, 274

D3S1289	71.41	211, 215	205, 215	205, 211	211, 215	211, 215	205, 215	205, 211	205, 215	209, 217	209, 209	209, 209	209, 217	211, 215	211, 217		211, 217	211, 217	217, 217
D3S1300	80.32	229, 241	235, 237	235, 241	239, 237	239, 237	237, 241	239, 235		235, 237	217, 217		217, 235	229, 241	229, 235	237, 237	235, 237	235, 237	235, 237
D3S4542	89.91																		
D3S1285	91.18	232, 232	238, 238		232, 238	232, 238	232, 238	232, 238	232, 238	232, 238	238, 238	238, 238	232, 238	232, 232	232, 240	232, 238		232, 240	232, 240
D3S2406	102.64	346, 350	314, 318	314, 350	314, 350	318, 250	318, 350			346, 350	306, 330	330, 346	330, 350	334, 350	334, 346	306, 330	330, 334		
D3S3681	109.22																		
D3S1271	117.76	156, 158	156, 156	156, 158	156, 158	156, 158	156, 158	156, 156	156, 156	156, 158	156, 156	156, 156	156, 158		156, 158	154, 156	154, 156	154, 158	
D3S3045	124.64	187, 195	183, 191	191, 195	191, 195	183, 195	183, 195			191, 195	179, 195	191, 195	195, 195	195, 191	187, 195	203, 203	187, 203		
D3S1278	129.73	203, 217	209, 225	203, 225	203, 225	203, 209	203, 209	217, 225	203, 225	203, 227	207, 207	207, 227	203, 207		203, 217	221, 221	217, 221	203, 221	203, 221
D3S1303	136.32	196, 202	202, 202	202, 202	202, 202	202, 202	202, 202			202, 210	196, 202	202, 210	202, 202	202, 210	196, 202	206, 214	196, 214		
D3S1267	139.12	153, 167	145, 149	145, 153	149, 153	145, 167		149, 167	149, 167	149, 153	155, 157	149, 155	153, 155	149, 153	153, 167	147, 149	149, 167	147, 167	147, 153
D3S1292	146.60	160, 166	144, 156	156, 166	144, 160	156, 160	156, 166	156, 160	144, 160	160, 166	156, 156	156, 160	156, 166	156, 166	148, 160	144, 156	156, 160	144, 160	148, 156
D3S1569	158.38	283, 287	277, 291	283, 291	277, 287	277, 283	283, 291	283, 291	277, 287	277, 291	281, 287	277, 287	281, 291		277, 287		277, 277	277, 277	277, 289
D3S3053	181.87	234, 242	226, 234	226, 242	226, 242	226, 234	226, 242			226, 242	226, 238	226, 238	226, 226	234, 238	226, 238	226, 238	238, 238	234, 238	
D3S1565	186.04	239, 241	239, 245	239, 241	239, 241	239, 239	239, 241	239, 241	239, 239	239, 241	241, 241	239, 241	239, 241	239, 241	239, 241	241, 241	241, 241	239, 241	241, 241

D3S1262	201.14	112, 112	118, 128	112, 128	112, 128	112, 128	112, 128			116, 116	116, 122	116, 122	116, 116	112, 116	116, 124	116, 124	112, 124		
D3S1580	207.73	139, 155	139, 143	139, 155	139, 155	139, 155	139, 155	143, 155		145, 145	153, 155	145, 155	145, 153	139, 155	145, 155	143, 157	143, 155	143, 145	143, 155
D3S3023	(deCode)	234, 234	234, 246	234, 234	234, 234	234, 246	234, 246			234, 234	243, 246	234, 246	234, 246	234, 234	234, 324	234, 240	234, 240		
D4S412	4.74	245, 245	237, 237	237, 245	237, 245	237, 245	237, 245	237, 245	237, 245	243, 245	245, 245	245, 245			245, 245	245, 247	245, 247	245, 245	245, 247
D4S403	25.90	225, 227	217, 219	219, 225	219, 225	217, 227	217, 225			217, 227	217, 227	217, 217	227, 227	217, 225	217, 227	225, 227	227, 227		
D4S3248	75.52	241, 245	241, 237	241, 241	241, 245	237, 245	237, 241			241, 241	237, 237	241, 237	241, 237	237, 245	241, 245	237, 245	237, 241		
D4S3243	88.35	174, 174	170, 174	170, 174	170, 174	174, 174	174, 174			162, 170	162, 174	170, 174	162, 170	174, 174	174, 174	174, 178	178, 174		
D4S1534	95.09	146, 156	158, 156	146, 148	148, 156	146, 156	156, 156	148, 156	146, 148	146, 156	146, 146	146, 156		156, 156	146, 156	154, 158	154, 156	156, 158	154, 156
D4S414	100.75	231, 236	236, 241	231, 241	231, 241	236, 241	236, 236	236, 241	231, 241	241, 246	234, 236		234, 241	231, 236	231, 236	236, 238	236, 238	236, 236	236, 238
D4S1647	104.94	140, 148	148, 148	148, 148	148, 148	148, 148	140, 148			144, 148	144, 132	144, 144	144, 132	140, 148	140, 148	132, 140	140, 140		
D4S1572	107.95	147, 147	141, 143	143, 147	143, 147	141, 147	141, 147	143, 147	143, 147	141, 147	147, 149	141, 147	141, 149	147, 147	147, 147	147, 147	147, 147	147, 147	147, 147
D4S407	117.06	117, 125	111, 121	111, 117	111, 117	117, 121	121, 125	111, 117	111, 117	127, 127	117, 129	127, 129	117, 127	117, 125	125, 127	119, 125	125, 125	125, 125	125, 125
D4S2989	203, 211	207, 209	207, 211	207, 211	207, 211	209, 211	203, 209	207, 211		205, 209	201, 207	205, 207	201, 205	203, 211	203, 209	201, 213	203, 213	203, 213	203, 213
D4S406	117.06	234, 246	248, 250	234, 248	234, 248	234, 250	246, 250	234, 248	234, 248	250, 254	234, 252	234, 250	250, 252	234, 246	246, 254	248, 250	246, 248	246, 248	246, 248
D4S402	124.45	289, 309	287, 319	309, 319	309, 319		289, 319	309, 319	309, 319	289, 309	287, 315		289, 315	289, 309	289, 319	297, 315	289, 315	289, 315	289, 315

D4S1575	132.05	221, 223	223, 225	223, 225	221, 223	223, 223	221, 225				219, 221	221, 221	221, 223	221, 221	219, 221	221, 221	221, 221	219, 221	219, 221
D4S424	144.56	188, 190	180, 190	188, 190	188, 190	180, 188	180, 190	190, 190	190, 190	188, 190	182, 190	190, 190	190, 190	199, 190	199, 190	190, 190	188, 190	190, 190	190, 190
D4S1629	157.99	141, 149	145, 157	141, 145	145, 149	141, 147	149, 157			141, 149	145, 153	145, 149	141, 153	145, 153	141, 149	141, 153	141, 153		
D4S413	157.99	128, 166	130, 140	140, 166	128, 140	130, 166	128, 130	128, 140	128, 140	128, 166				128, 166	128, 166	134, 140	134, 166	128, 140	128, 134
D4S1539	176.19	225, 227	223, 225	223, 225	223, 227	225, 225	225, 225	223, 227	223, 227	225, 227	225, 227	225, 227	225, 225	223, 227	225, 227	225, 225	225, 225	225, 227	225, 225
D4S415	181.36	174, 196	174, 194	194, 196	194, 196	174, 174	174, 174	194, 196	194, 196	174, 196	174, 198	196, 198	174, 198	196, 196	174, 196	194, 194	174, 194	194, 196	194, 196
D4S2417	181.93	255, 255	263, 263	255, 263	255, 263	255, 263	255, 263	255, 263	255, 263	255, 255	263, 263	255, 263	255, 263	259, 263	255, 255	255, 259	255, 259		
D4S2417	181.92	255, 255	263, 263	255, 263	255, 263	255, 263	255, 263			255, 255	263, 263	255, 263	255, 263	259, 263	255, 255	255, 259	255, 259		
D4S1535	195.06	185, 185	183, 185	183, 185	183, 185	185, 185	185, 185	183, 185	183, 185	185, 185	185, 185	185, 187	185, 187				187, 191		187, 191
D4S408	195.06	229, 229	233, 242	229, 243	229, 243	229, 233	229, 233			229, 229	229, 233	229, 229	229, 229	233, 233	229, 233	229, 239	229, 233		
D4S426	206.98	179, 181	177, 191	177, 181	181, 191	181, 191	179, 191	177, 179	177, 181	181, 191	179, 191		191, 191	189, 191		181, 187	187, 189	187, 189	187, 189
D5S1981	1.72	262, 266	262, 268		262, 266	262, 268	266, 268	262, 268	262, 266	268, 272	262, 266	262, 268	262, 268	262, 272	262, 266	268, 278	266, 268	266, 278	266, 278
D5S406	11.85	178, 180	168, 178		168, 178	178, 180	178, 178	178, 180	168, 178	178, 180	168, 180	168, 178	178, 180	178, 180	178, 180	168, 180	178, 180	178, 180	168, 178
D5S630	19.67	231, 248	254, 258	248, 254	248, 254	231, 258	248, 254	231, 258	248, 254	248, 258	248, 254	248, 254	248, 248	231, 248	231, 248	248, 262	248, 248	231, 248	248, 248
D5S817	22.88	264, 268	264, 268	268, 268	268, 268	264, 264	268, 268			268, 272	260, 268	260, 268	268, 268	264, 268	264, 268	260, 272	268, 272		

D5S416	28.76	284, 286	292, 292		284, 292	286, 292	284, 292	286, 292	284, 292	282, 284	284, 288	284, 284	284, 286	284, 286	284, 286	284, 290	284, 284		284, 284
D5S419	39.99	212, 222	218, 222	212, 218	212, 218		222, 222	218, 222	212, 222	206, 212	222, 224	212, 222	212, 224		206, 216			216, 216	206, 216
D5S1470	45.34	173, 197	177, 197	197, 197	197, 197	173, 197	197, 177			177, 197	181, 197	197, 197	181, 197	173, 181	177, 181	189, 193	181, 189		
D5S426	51.99	183, 193	183, 199	193, 199	193, 199	183, 199	183, 193	183, 199	183, 199	193, 203	201, 201	193, 201	193, 201	183, 201	201, 203	183, 199	199, 201	183, 203	199, 201
D5S418	58.55	215, 217	213, 215	213, 215	213, 215	213, 217	215, 215	213, 217	215, 215	215, 221	215, 219	215, 215	215, 219	213, 217	213, 221	215, 219	213, 215	219, 221	213, 219
D5S2500	69.23	157, 161	153, 153	153, 161	153, 161	153, 157	153, 161	153, 161	153, 157	161.173	161, 161	161, 161	161, 161	149, 173	149, 173	153, 153	153, 149		
D5S2072	73.35	159, 163	159, 163	163, 163	163, 163	163, 163	159, 163			159, 163	161, 163	161, 163	161, 163	159, 163	159, 163	159, 167	159, 163		
D5S647	74.07	140, 158	140, 142	142, 158	142, 158	142, 158	140, 158			138, 158	134, 136	134, 158	136, 158	136, 158	136, 156	138, 142	138, 156		
D5S2036	74.68	113, 123	121, 123	113, 121	113, 121	113, 121	113, 123	113, 123	113, 123	113, 113		113, 123		113, 121	113, 121	113, 123	113, 121		
D5S2003	78.31	185, 189	187, 177													175, 189			
D5S424	81.95	130, 130	128, 130	130, 130	130, 130		128, 130	130, 130	128, 130	130, 130	124, 130	130, 130	124, 130	126, 130	126, 130	130, 132	126, 132	130, 130	130, 130
D5S641	92.38	267, 269	267, 269	267, 269	267, 269	267, 269	267, 269	267, 267	269, 269	267, 269	265, 267	265, 267	267, 267	267, 275	265, 275	267, 271	265, 267	271, 275	271, 275
D5S428	95.40	245, 247	241, 247	245, 241	245, 247	241, 245	247, 247		247, 247	245, 247	241, 249	241, 245	245, 249	245, 249		241, 247	241, 241	247, 249	247, 249
D5S1725	97.82	188, 192	188, 200	188, 188	192, 200	188, 192	188, 200			188, 192	196, 204	192, 204	192, 196	192, 208	188, 208	188, 188	188, 188		
D5S644	104.76	97,99	97,107	97,99	97,107	97,97	99,107	97,97	99,107	95,97	101, 107	97,101	97,107	97,99		95,99	83,99	95,99	95,99

D5S2027	119.50	149, 157	151, 157	149, 157	149, 151	157, 157	151, 157	157, 157	149, 151	157, 157	157, 157	157, 157	157, 157	149, 157	151, 157	151, 157	151, 157	151, 157	151, 157
D5S1505	129.83	255, 259	267, 259	255, 267	255, 259	255, 267	259, 259			251, 259	267, 271	251, 267	259, 267	255, 259	251, 259	259, 271	259, 259		
D5S471	129.83	242, 248	242, 246	242, 246	242, 246	242, 246	246, 248	246, 248	242, 246	236, 248	236, 248	236, 248	236, 248	242, 248	236, 246	236, 246	236, 246	236, 246	236, 246
D5S2115	138.64	271, 273	259, 271	271, 273	259, 273		271, 273	271, 271	259, 273	271, 273	271, 271	271, 273	271, 271	273, 279	275, 279	271, 271	271, 275	271, 279	271, 279
D5S436	147.49	238, 248	236, 246	246, 248	236, 238	236, 238	236, 248	246, 248	236, 248	238, 248	236, 248	248, 248	236, 238	236, 246	236, 246	236, 240	236, 240	236, 240	236, 240
D5S1480	147.49	218, 236	239, 236	236, 239	236, 236	218, 236	236, 236			218, 236	218, 218	218, 236	218, 218	218, 236	218, 236	239, 239	218, 239		
D5S410	156.47	161, 169		161, 163	161, 169	161, 169	161, 163	163, 169	161, 163	163, 169	159, 169	159, 163	169, 169	163, 163	163, 163	159, 163	159, 163	163, 163	159, 163
D5S400	174.80	228, 234	228, 230	228, 230	228, 230	228, 234		228, 228	228, 230	228, 228	224, 230	224, 228		228, 234	228, 230	224, 228	228, 228	228, 228	228, 228
D5S211	182.89	192, 200	192, 192	192, 192	192, 200	192, 192	192, 200			192, 200	200, 200	200, 200	200, 192	192, 202	192, 202	200, 202	202, 202		
D5S408	195.49	257, 257	257, 257		257, 257	257, 257	257, 257			257, 257	255, 257	255, 257	255, 257	255, 257	255, 257	257, 259	255, 257	255, 259	257, 257
D6S1574	9.81	166, 166	174, 178	166, 174	166, 178	166, 174	166, 174	166, 178	166, 178	174, 176	160, 172	160, 174	172, 176	166, 166	166, 174	166, 172	166, 166	166, 172	166, 166
D6S470	18.22	126, 138	128, 130	126, 130	128, 138	126, 130		128, 138	126, 128	128, 130	126, 128	126, 130	128, 128	126, 138	130, 138	128, 130	130, 138	128, 138	130, 138
D6S2434	25.08	230, 230	233, 227	230, 233	227, 230	230, 233	230, 233			227, 230	224, 230	230, 230	224, 227	230, 230	230, 233	227, 233	233, 233		
D6S289	29.93	219, 221	221, 223	219, 221	221, 223	219, 221	221, 221	221, 223	219, 223	217, 229	217, 223	217, 229		219, 221		219, 219		219, 221	221, 219
D6S422	35.66	191, 197	191, 207	191, 197	197, 207	191, 191	191, 197	197, 207	191, 207	191, 197	197, 211	191, 211	197, 197	191, 197	191, 195	191, 195	191, 195	195, 195	195, 195

D6S2439	42.27	250, 250	230, 242	242, 250	230, 250	242, 240	242, 240			250, 250	234, 242	242, 250	250, 234	242, 242	242, 250	238, 242	238, 242		
D6S1051	50.75	231, 231	231, 235	231, 231	231, 235	231, 231	231, 231	231, 235	231, 235	227, 231	227, 235	227, 227	231, 235	231, 231	231, 231	231, 235	231, 235		
D6S1610	53.81	133, 139	133, 139	139, 139	133, 139	133, 139	139, 139	133, 139		133, 139	135, 137	133, 135	137, 139	133, 139	133, 139	133, 133	133, 133	133, 133	133, 133
D6S1607	59.34	285, 279	277, 279	279, 279	279, 279	279, 285	279, 279			277, 279	283, 283	277, 283	279, 283	279, 285	281, 285	275, 287	281, 287		
D6S2410	73.13	244, 248	240, 252	244, 252	240, 248	248, 252	244, 252			244, 244	240, 240	240, 244	240, 244	244, 248	244, 248	236, 244	240, 244		
D6S257	79.92	174, 178	178, 182	178, 182	174, 178	174, 182	178, 182	174, 178	174, 178	176, 176	176, 178	176, 178	176, 178	174, 176	174, 176	174, 180	174, 176	174, 174	174, 180
D6S460	89.93			150, 160	144, 158	150, 160	150, 158	144, 158	144, 158	144, 144	144, 152	144, 144	144, 152	144, 158	144, 158	154, 158	144, 154	154, 158	158, 158
D6S462	99.01	285, 287	285, 285	285, 287	285, 285	285, 287	285, 285	285, 285	285, 285	285, 287	285, 287	825, 287	285, 287	285, 285	285, 285	285, 287	285, 285	285, 285	285, 287
D6S287	121.97	159, 167	161, 167	167, 167	161, 167	159, 167	161, 167	167, 167	161, 167	161, 167	161, 163	163, 167	161, 161	159, 167	159, 167	141, 161	141, 167	159, 161	159, 161
D6S1040	128.93	273, 277	269, 277	269, 277	273, 277	269, 277	273, 277			269, 277	269, 277	277, 277	269, 269	277, 277	277, 277	277, 269	269, 277		
D6S262	130.00	177, 179	171, 179	171, 179	171, 179	171, 179	177, 179	171, 177	177, 179	177, 179		177, 183		169, 179	169, 179				169, 179
D6S292	136.97			149, 149	151, 157		151, 157	149, 157	151, 157	157, 157	153, 153	153, 157	153, 157	149, 157	149, 157		153, 157	149, 153	149, 153
D6S308	144.46	199, 199	199, 199	199, 199	199, 199	199, 199	199, 199	199, 199	199, 199	199, 199		193, 199	193, 199	199, 199	199, 199	197, 197	197, 199	197, 199	197, 199
D6S441	154.10	172, 180	166, 180	166, 172	172, 180	166, 172		166, 180	172, 180	168, 172	172, 174	172, 172	168, 172	172, 180		168, 172	172, 180	168, 172	172, 172
D6S2436	154.64	121, 141	121, 145	121, 141	141, 145	131, 141	121, 145			121, 139	121, 149	121, 133	121, 121	121, 141	121, 141	121, 149	121, 149		

D6S1581	164.78	219, 219	219, 221	219, 219	219, 221	219, 219	219, 221	219, 219	219, 221	219, 227	229, 229	219, 229		219, 219	219, 219	215, 219	215, 219	219, 219	215, 219
D6S264	179.07	114, 122	108, 108	108, 114	108, 114	108, 114		108, 114	108, 114	114, 114	114, 114	114, 114	114, 114	114, 122	114, 122	108, 114	108, 122	114, 122	114, 122
D6S446	189.00	201, 201	203, 209	201, 209	201, 203	201, 209	201, 203	201, 203	201, 203	201, 201	201, 207	201, 207			201, 201	201, 205		201, 201	201, 201
D6S281	190.14		209, 209	207, 209	207, 209	207, 209	207, 209	207, 209	207, 209	207, 207	203, 207		203, 207	203, 207	203, 207	203, 221	203, 207	203, 203	203, 203
D7S517	7.44	247, 255	245, 245	245, 247	245, 255	245, 247	245, 255	245, 255	245, 255	245, 249	251, 255		245, 255	245, 247	247, 255	251, 257	247, 257	247, 257	255, 257
D7S513	17.74	183, 195	181, 195	181, 183	195, 195	195, 195	181, 195			195, 199	195, 195	195, 199	195, 195	183, 195	183, 191	181, 195	183, 195		
D7S3051	29.28	144, 170	166, 166	144, 166	166, 170	170, 166	166, 170			170, 170	144, 170	170, 170	170, 170	170, 144	144, 170	170, 170	170, 170		
D7S493	34.69	210, 214	214, 220	210, 214	214, 214	214, 214	214, 220	214, 220	214, 214	194, 214	210, 214	194, 210	194, 214	210, 212	210, 214	210, 216	210, 214	210, 210	210, 214
D7S516	41.69	266, 266	256, 260	260, 266	256, 266	256, 266	260, 266	260, 266	256, 266	254, 260	254, 262	254, 262	254, 254	260, 266	266, 266	260, 266	260, 266	260, 266	260, 266
D7S484	53.70	99,105	105, 105		99,105	105, 105	105, 105	105, 105	105, 105	105, 105	103, 105		105, 105	99,105	99,105	105, 111	105, 105		105, 111
D7S2846	57.79	180, 184	180, 184	180, 184	180, 180	180	180, 184			180, 184	188, 188	180, 188	180, 188	180, 188	180, 188	180, 180	180, 188		
D7S519	69.03		256, 272	266, 272	266, 272	262, 272	256, 262	256, 266	262, 272	262, 266	256, 262	256, 266	262, 266	262, 266	262, 266	266, 272	262, 266	262, 266	262, 272
D7S3046	78.65	338, 342	330, 334	334, 338	334, 338	334, 338	339, 338			332, 338	334, 338	334, 338	334, 338	322, 338	322, 338	322, 346	322, 346		
D7S669	90.42	123, 137	131, 139	131, 137	131, 137	131, 137	137, 139	137, 139	123, 131	123, 137			123, 137	123, 137	123, 137	131, 141	123, 141	123, 141	123, 131
D7S821	109.12	258, 266	254, 266	258, 266	266, 266	266, 266	254, 266			262, 266	258, 266	262, 266	258, 266	250, 262	250, 258	258, 266	258, 266		

D7S530	134.55	112, 114	108, 114		114, 144	108, 112	108, 114	114, 114		106, 112	110, 114	112, 114	112, 114	106, 112	112, 112	112, 112	112, 112	112, 112	112, 112
D7S1804	136.95	258, 266	258, 262	258, 266	258, 262	262, 266	262, 266			254, 266	262, 258	262, 266	258, 266	254, 266	266, 266	258, 278	258, 266		
D7S640	137.83	116, 122	122, 124	122, 122	116, 122	122, 124	116, 124	116, 122	122, 122	122, 128		116, 128	116, 128	122, 128	122, 128	116, 122	122, 122	122, 122	116, 122
D7S684	147.22	171, 175		171, 171	175, 175	171, 175	171, 171	171, 175	175, 175	171, 183	171, 179	171, 183	179, 183	171, 183		171, 171	171, 175	171, 175	171, 175
D7S661	155.10	260, 268	266, 274		260, 274	260, 266	266, 268	268, 274	260, 274		270, 272	269, 272	264, 270	264, 268	260, 264	266, 268	260, 266	260, 266	260, 268
D7S1823/ D7S3058	173.71	211, 223	211, 227	211, 211	223, 227	211, 223	223, 227	211, 211		223, 223	227, 231	223, 227	223, 231	223, 223	223, 223	211, 231	211, 223		
D8S264	0.73	131, 131	129, 141		131, 141	131, 141	129, 131	129, 131	129, 131	131, 131	129, 139		129, 131	131, 133	131, 133	137, 139	131, 139	131, 139	131, 137
D8S1130	22.41	132, 144	144, 160	144, 132	132, 160	132, 160	132, 144			132, 144	140, 152	144, 152	132, 152	144, 144	144, 144	140, 148	144, 148		
D8S549	31.73		168, 170	170, 170	168, 170	168, 170	170, 170	170, 170	168, 172	168, 170	168, 170		170, 170	170, 170	170, 170	170, 170	170, 170	170, 170	170, 170
D8S258	41.55	152, 152	148, 150	150, 152	148, 152	148, 152	150, 152	150, 152	150, 152	150, 152	148, 152	150, 152	152, 152	150, 152	148, 150	148, 152	148, 152	148, 150	148, 150
D8S136	43.96	74,74	82,82	74,82	74,82	74,82	74,82			74,74	74,82	74,82	74,82	74,74	74,74	82,86	74,86		
D8S1771	50.05	226, 232	224, 226	224, 226	226, 232	226, 232	224, 226	226, 232	224, 226	226, 228	226, 228	226, 228		226, 228		226, 226	226, 232	226, 226	226, 226
D8S505	60.87	207, 211	203, 213	211, 213	203, 211		211, 213	207, 213	211, 213		203, 205	205, 207	205, 211	207, 211	207, 207	205, 207	205, 207	207, 207	207, 207
D8S532	64.75	239, 253	251, 247	247, 253	251, 253	239, 251	247, 253			241, 251	243, 251	241, 243	243, 251	241, 251	239, 241	245, 251	239, 251		
D8S285	71.00	118, 120	108, 122	120, 122	108, 120	120, 122	120, 122			108, 120	108, 118	108, 118	120, 118	108, 120	108, 118	120, 120	120, 118		

D8S260	79.36	209, 205	203, 207	205, 203	205, 207	203, 205	203, 205			199, 203	187, 205	199, 205	187, 199	199, 205	209, 203	199, 205	205, 209		
D8S2324	94.08	200, 216	204, 204	204, 216	204, 216	204, 216	200, 204			208, 216	196, 212	196, 216	208, 212	204, 208	200, 204	200, 204	200, 204		
GAAT1A4	110.20	156, 156	140, 152	140, 156	152, 156	152, 156	152, 156			156, 156	156, 148	156, 156	156, 148	156, 156	156, 156	152, 156	156, 156		
D8S1784	118.15		163, 167	163, 167	163, 163	163, 163	163, 163	163, 167	163, 167	163, 163	157, 163	157, 163	163, 163	163, 163	163, 163	157, 165	163, 165		163, 165
D8S514	130.00	215, 217	213, 215	215, 215	213, 217	213, 217	213, 215	215, 217	215, 217	217, 223	215, 223		215, 223	215, 215	215, 215	213, 217	213, 215	213, 215	213, 215
D8S1179	135.08	182, 190	182, 182	182, 182	182, 190	182, 190	182, 182			186, 190	174, 178	174, 190	178, 186	182, 182	182, 182	182, 182	182, 182		
D8S284	143.82	273, 275	243, 253	253, 273	243, 273	243, 273	243, 273	253, 275		243, 257	253, 255	243, 253	255, 257	243, 273	243, 273	243, 253	253, 273	243, 253	243, 253
D8S256	148.12	210, 220	210, 228	210, 210	210, 228	210, 228	210, 228			210, 210	226, 228	210, 228	210, 228	210, 210	210, 210	224, 224	224, 210		
D8S272	154.02	227, 227	237, 227	227, 237	227, 237	227, 237	227, 237	227, 237	227, 237	227, 237	223, 229	229, 237	223, 227	223, 227	227, 237	227, 235	227, 227	235, 237	235, 237
D9S917	0.00	145, 145	133, 142	133, 145	142, 145	142, 145				145, 145	133, 133	133, 145	145, 133	145, 145	142, 145	142, 148			
D9S1813	9.83	226, 246	226, 226	226, 226	226, 246	226, 246	226, 246			226, 246	226, 226	226, 226	226, 226	226, 246	226, 250	252, 252	250, 252		
D9S288	9.83		130, 132	130, 130	132, 134	132, 134	130, 134	130, 130	130, 130	130, 134		130, 136		130, 134	128, 130	126, 132	128, 132	128, 132	126, 128
D9S1810	12.78		204, 212		204, 212	204, 214				204, 212		204, 212	212, 212	204, 212	204, 204		204, 204		
D9S286	18.06	139, 159	153, 153	153, 159	139, 153	139, 153	139, 153	153, 159	153, 159	139, 159	139, 145	145, 159	139, 145	139, 159	139, 159	139, 155	139, 139	139, 139	155, 159
D9S274	28.42	163, 171					163, 171								163, 165	163, 171	171, 165		

D9S285	29.52	123, 125	123, 123	123, 125	123, 123	123, 123	123, 123	123, 125	123, 125	123, 125	113, 113	113, 125	113, 123	123, 123	107, 123	125, 125	123, 125	123, 125	123, 125
D9S157	32.24	133, 137	137, 145	137, 137	133, 137	133, 137	133, 145	137, 137	137, 145	133, 137	137, 139	137, 139	133, 139	133, 133	133, 145	145, 145	133, 145	133, 145	133, 145
D9S1679	44.28	135, 137	137, 141	135, 141	135, 141	137, 141	135, 137	137, 141	137, 137	135, 137	139, 141	137, 141	135, 141	135, 135	141, 135	139, 141	135, 139		
D9S1121	44.28	184, 184	204, 212	184, 212	212, 184	184, 212	184, 204			184, 184	184, 184	184, 184	184, 184	184, 212	184, 212	184, 212	184, 212		
D9S169	49.20	269, 271	269, 277	271, 277	271, 277	269, 277	269, 271	269, 269	269, 277	279, 271	271, 263	263, 269	263, 271	271, 273	269, 273	265, 267	267, 273		
D9S161	51.87	121, 131	121, 133	121, 131	121, 131	121, 121	121, 131	121, 121	121, 133	121, 131	121, 131	121, 131	121, 131	121, 131	121, 133	131, 133	121, 133	121, 131	121, 131
D9S1118	58.26	141, 169	161, 161	161, 169	141, 161	161, 169	161, 169			141, 169	141, 141	141, 141	141, 169	157, 169	157, 173	157, 165	157, 157		
D9S273	65.79	211, 211	209, 215	209, 211	209, 211	209, 211	209, 211	209, 211		211, 211	209, 211	211, 211	211, 211	209, 211	205, 209	205, 209	209, 209	205, 209	205, 209
D9S175	70.33	220, 226	208, 218	218, 220	218, 220	218, 226	218, 220	218, 220	208, 226	220, 226	208, 210	210, 226		220, 226	224, 226		224, 226	208, 226	208, 226
D9S1122	75.88	203, 203	191, 199	199, 203	203, 199	191, 203	199, 203			203, 203	203, 195	203, 203	203, 203	203, 203	203, 203	195, 199	199, 203		
D9S167	83.41	266, 270	276, 278	266, 276	266, 276	266, 278	266, 276	270, 276	266, 278	266, 270	270, 270	270, 270	266, 270	266, 270	270, 284	264, 268	264, 270	264, 270	268, 270
D9S257	91.87	261, 263	261, 259	259, 261	259, 261	261, 261	261, 289			261, 263	261, 267	267, 263	267, 261	263, 267	267, 263	263, 259	259, 263		
D9S287	103.42	168, 170	160, 170	170, 170	170, 170	168, 170	170, 170	168, 170	168, 170	168, 172	170, 170	168, 170	170, 172	168, 170	168, 170	170, 174	168, 170	168, 170	168, 174
D9S1690	106.63	231, 233	225, 227	227, 231	227, 231	225, 231	227, 231	227, 233	225, 231	233, 237	225, 233	225, 237	233, 237	231, 233	231, 233	225, 237	225, 233	233, 237	233, 237
D9S1677	117.37	251, 263	251, 255	251, 263	251, 251	255, 263	251, 251	251, 263	251, 255	263, 263	251, 265	251, 263	263, 265	251, 263	251, 263	259, 263	259, 263	259, 263	263, 263

D9S1776	123.33		113, 121	113, 117	117, 121	113, 117	117, 121	117, 121	113, 117	121, 123	111, 123	111, 123		117, 121	117, 121	111, 113	111, 121	111, 121	113, 121
D9S1682	132.09	204, 204	202, 204	202, 204	204, 204	202, 204	204, 204	204, 204	204, 204	202, 204	204, 204	202, 204	202, 204	202, 204	204, 204	202, 204	204, 204	204, 204	204, 204
D9S282	136.47	233, 237	233, 237	233, 237	233, 237	233, 237	233, 233			235, 235	233, 235	233, 235	233, 235	235, 237	235, 237	235, 242	235, 242		
D9S290	140.86	158, 160	152, 158	152, 158	158, 160	152, 158	158, 158	158, 158	152, 160		152, 158	158, 160	158, 158	158, 160	158, 160	152, 156	152, 158	156, 160	152, 158
D9S1826	159.61	133, 135	133, 147	133, 147	133, 135	135, 147	135, 147	133, 147	133, 147		135, 139	135, 135	135, 139	135, 135	135, 135	135, 145	135, 135	135, 145	
D10S249	2.13	120, 126	118, 118	118, 126	118, 120	118, 120	118, 126	118, 120		126, 130	118, 122	118, 126	122, 126	120, 126	120, 126	122, 130	120, 122	122, 126	120, 130
D10S189	19.00	186, 188	186, 182	186, 186	182, 186	186, 188	182, 186			182, 188	186, 186	186, 188	186, 188	186, 188	182, 186	180, 186	180, 182		
D10S547	29.15	236, 238	236, 242	236, 238	236, 242		236, 242	236, 236	236, 238	236, 236	236, 246	236, 236	236, 246	238, 246	236, 236	236, 238	236, 238	236, 236	236, 236
D10S1653	40.36	203, 205	203, 207	203, 205	203, 203	203, 203	203, 207	203, 205	203, 205	203, 203	203, 203	203, 203	203, 203	203, 205	203, 203	203, 203	203, 203	203, 203	203, 203
D10S548	45.70	140, 144	140, 142	140, 142	142, 144	140, 140	140, 140	142, 144	142, 144	140, 140	142, 146	140, 142	140, 142	140, 144	140, 140	140, 146	140, 146	140, 140	140, 140
D10S1423	46.23	234, 238	230, 234	230, 234	230, 238	234, 238	234, 238			230, 238	226, 226	226, 238	226, 238	230, 234	230, 238	234, 226	230, 234		
D10S197	52.10	107, 171	171, 171	171, 171	167, 171	167, 171	167, 171	171, 171	171, 171	167, 173	171, 171	171, 173	171, 173	167, 171	167, 173	171, 171	167, 171	167, 171	171, 173
D10S1208	63.30	179, 179	179, 194	179, 179	179, 179	179, 194	179, 194			179, 179	191, 197	179, 197	179, 191	179, 179	179, 179	194, 194	194, 179		
D10S196	70.23	105, 109	103, 105	105, 105	105, 109	103, 109	103, 105	105, 105	105, 109	103, 105	103, 105	105, 105	105, 105	105, 109	103, 105	103, 103	103, 103	103, 103	103, 105
Gata121A08	88.41	188, 192	188, 196	188, 188	188, 192	192, 196	196, 192			188, 192	188, 184	188, 188	188, 184	188, 188	188, 192	184, 188	184, 192		

D10S537	91.13	146, 160	154, 160	146, 160	154, 160	160, 160	160, 160	146, 160		154, 160	154, 160			146, 160	154, 160	144, 144	144, 154	144, 154	144, 160
D10S2327	100.92	204, 220	204, 220	204, 220	204, 220	204, 204	204, 204			204, 220	224, 224	204, 224	220, 224	204, 220	204, 220	204, 204	220, 204		
D10S1686	105.04	186, 202	184, 200	184, 202	200, 202	184, 202	200, 202	186, 200	200, 202	186, 200	184, 196	196, 200	184, 186	186, 200	186, 200	186, 204	186, 204	200, 204	186, 204
D10S185	116.34		149, 159	149, 159	149, 149	149, 159	149, 149	149, 151	149, 149	151, 165	147, 159	159, 165		157, 165	151, 165	147, 147	147, 151	147, 165	147, 151
D10S192	124.27		192, 198	196, 198	192, 196	196, 198	192, 196	192, 196	192, 196		196, 200	196, 196	196, 200	186, 196	196, 196	196, 196	196, 196	196, 196	196, 196
D10S1239	125.41	160, 180	176, 180	176, 180	180, 180	176, 180	180, 180			160, 180	160, 176	176, 180	160, 160	180, 180	160, 176	176, 176	176, 160		
D10S597	128.73	216, 216	216, 218	216, 218	216, 216	216, 216	216, 216	216, 216	216, 216	216, 216	216, 218	216, 216	216, 216	216, 220	216, 218	218, 218	216, 218	218, 218	216, 218
D10S1693	137.39	257, 259	255, 259	257, 259	255, 257	257, 259	255, 259		255, 259	257, 259	255, 255	255, 257	255, 259	255, 257	257, 259	253, 255	255, 259	253, 257	255, 259
D10S1230	142.78	126, 132	126, 132	126, 126	132, 132	132, 126	132, 132			123, 126	126, 129	129, 126	123, 126	123, 126	126, 132	132, 132	132, 132		
D10S587	147.57	174, 176	176, 184	176, 176	174, 184	174, 176	174, 184	176, 184	174, 184	174, 176	176, 180	176, 176	174, 180	174, 176	174, 176	174, 180	174, 174	176, 180	174, 180
D10S217	157.89	200, 202	200, 214	200, 214	200, 214	200, 202	202, 214	200, 214	202, 214	202, 202	206, 206	202, 206	202, 206		202, 202	200, 200	200, 202	200, 202	200, 202
D10S1651	168.77	190, 190	182, 196	190, 196	182, 190	190, 196		190, 196	182, 190	180, 180	182, 190	180, 190	180, 182		180, 190	182, 196	190, 196	180, 182	180, 182
D10S212	170.94	195, 199	195, 189	189, 195	195, 195	189, 195	195, 195			189, 191	191, 195	191, 191	195, 189	189, 199	191, 195	195, 195	195, 195		
D11S4046	2.79	187, 187	195, 197	187, 197	187, 197	187, 197	187, 197	187, 197	187, 197	187, 195	187, 197	195, 197	187, 197	183, 187	183, 195	183, 183	183, 195	183, 183	183, 183
D11S1338	12.92	259, 265	257, 259		257, 265	257, 259	257, 259		257, 265	263, 265		259, 265	257, 263	259, 263	263, 265	257, 259	257, 265	257, 263	257, 263

D11S902	21.47	149, 161	151, 155	149, 155	149, 151	151, 161	151, 161	149, 151		147, 157	155, 157	155, 157	155, 157	149, 157	147, 157	151, 153	153, 157	153, 157	
D11S1981	21.47	162, 162	154, 162	162, 162	154, 162	154, 162	154, 162			174, 158	162, 162	162, 174	162, 174	162, 174	174, 158	158, 166	158, 174		
D11S904	33.57	193, 201	185, 201	201, 201	185, 201	185, 193	185, 201	185, 201		191, 199	191, 201	191, 191	191, 201	191, 201	193, 199		185, 193	187, 193	187, 199
D11S935	45.94		204, 206	206, 206	196, 204	196, 204	196, 204	204, 206		206, 206	206, 206	206, 206	206, 206	196, 206		196, 204	204, 206	196, 206	196, 206
D11S905	51.95	210, 210	208, 224	210, 224	210, 224	208, 210		208, 210	208, 210	208, 210	212, 224	208, 212	208, 224	208, 210	210, 210	208, 224	210, 224	208, 210	210, 224
D11S4191	60.09		111, 131	111, 115	113, 131	113, 131	113, 131	115, 131	113, 131		111, 113		111, 119	115, 119	113, 115	111, 113	113, 115	113, 115	111, 113
D11S987	67.48	110, 116	110, 122	110, 122	110, 116	110, 116	110, 110	110, 110		110, 116	112, 116	110, 116	110, 112	108, 112	110, 116	116, 120	110, 116	110, 116	
D11S1314	73.64	225, 225	209, 215	209, 225	215, 225	215, 225	215, 225	215, 225		225, 225	209, 223	209, 225	223, 225	215, 221	225, 225	209, 223	223, 225	223, 225	223, 225
D11S2371	76.13	201, 209	201, 201	201, 201	201, 209	201, 209	201, 201			201, 209	201, 201	201, 201	201, 201	201, 201	201, 209	197, 213	201, 213		
D11S937	79.98	256, 260	248, 264	260, 264	248, 256	248, 256	248, 260	248, 260		256, 260	250, 254	254, 260	250, 260	246, 256	256, 260		258, 260	258, 260	256, 258
D11S901	85.48	176, 176	168, 178	168, 176	176, 178	176, 178	176, 178	176, 178	176, 178	176, 176	168, 180	176, 180	168, 176	180, 188	176, 176		176, 176	176, 180	176, 176
D11S4175	91.47	151, 193	151, 187	151, 151	187, 193	187, 193	151, 187	151, 187		151, 193	171, 189	151, 189	151, 189	175, 189	151, 193	151, 151	151, 151	151, 151	151, 193
D11S898	98.98	146, 152	146, 150	146, 146	146, 152	146, 152	146, 146	146, 146	146, 152	146, 152	146, 146	146, 146	146, 146	152, 152	146, 152	142, 150	142, 146	142, 152	142, 152
D11S2000	100.62	219, 221	215, 235	219, 215	215, 219	215, 219	215, 221			219, 219	215, 219	215, 219	215, 219	215, 221	219, 219	219, 225	219, 219		
D11S4090	105.74	173, 197	183, 189	183, 197	183, 197	197, 183	173, 183			173, 197	179, 189	179, 173	173, 179	173, 191	177, 197	183, 191	177, 191		

D11S908	108.59	145, 147	147, 149	145, 149	145, 149	145, 149	147, 149	147, 149	145, 149	145, 147	147, 151	147, 147	147, 147	145, 147	145, 145	145, 147	145, 147	145, 147	145, 147
D11S1998	113.13	153, 153	149, 153	149, 153	149, 153	149, 153	149, 153			153, 153	149, 153	149, 153	149, 153	153, 153	149, 153	149, 157	149, 149		
D11S925	118.47		191, 195	195, 195	195, 195	195, 195	195, 195	187, 195	195, 195	187, 195	173, 189	187, 189	187, 189	187, 195	173, 195	173, 195	173, 195		173, 195
D11S912	131.26	109, 105	103, 109	109, 103	103, 109	103, 109	103, 105			101, 105	107, 111	101, 111	105, 107	105, 109	101, 105	107, 107	101, 107		
D11S1320	141.91	227, 233	227, 227	227, 227	227, 227	227, 227	227, 233	227, 227	227, 233	233, 233	231, 231	231, 233	231, 233	227, 233	233, 233	227, 227	227, 233	227, 233	227, 233
D11S968	147.77		147, 147	147, 147	147, 147	147, 147	147, 147	147, 147	147, 147		145, 149	147, 149	145, 147	147, 147	147, 147	143, 149		147, 149	147, 149
D12S99	12.60	210, 210	208, 212	208, 210	208, 210	208, 210	210, 212	210, 212	210, 212	210, 212	212, 212	212, 212	212, 212	212, 212	212, 212		208, 212	208, 212	210, 212
D12S1617	44.03		257, 257	257, 259	257, 259	257, 261	257, 259	257, 261	257, 259	255, 259	247, 257	247, 255	247, 255	259, 261	259, 261	247, 261	247, 261	261, 261	247, 259
D12S345	53.09	212, 226	224, 228	212, 224	226, 228	226, 228	224, 226	224, 226	212, 228	210, 216	224, 224	210, 224	210, 224	212, 226	212, 226	210, 230	210, 226	210, 226	212, 230
D12S398	68.16	132, 140	132, 128	128, 140	140, 132	132, 132	128, 140			128, 132	128, 132	132, 132	132, 132	132, 140	128, 132	128, 140	128, 140		
D12S83	75.17	87,93	85,93	85,93	93,93	87,93		85,93	87,93	85,87	85,91	87,91	87,91	93,102	85,101	87,89	85,87	85,87	89,101
D12S1300	104.12	123, 123	119, 123	123, 123	119, 123	119, 123	119, 123			107, 123	119, 127	107, 119	107, 127	123, 123	107, 123	119, 119	119, 123		
D12S346	104.65		172, 174	154, 174	154, 172	162, 172	162, 172	162, 174	154, 174	160, 170	164, 172	160, 172	160, 164	154, 162	160, 170	172, 176	170, 172	170, 176	170, 176
D12S2070	125.30	89,98	89,101	101,89	89,89	89,89	89,89			89,89	98,98	89,98	89,98	89,98	89,98	89,98	89,89		
D12S79	125.31	159, 179	161, 171	159, 171	159, 161	159, 161	159, 161	159, 171	159, 171	159, 165	181, 183		165, 181	165, 179	165, 179		159, 165	165, 179	

D12S2082	130.94	286, 290	290, 294	286, 290	286, 294		290, 294	286, 290	286, 290	286, 294	290, 298	286, 298	294, 298	290, 294	290, 294	294, 294	294, 294	294, 294	
D12S366	133.33	193, 195	195, 195	195, 195	195, 195		193, 195	195, 195	195, 195	189, 193	191, 195	189, 189		189, 193	189, 193		189, 189	189, 189	189, 189
D12S86	134.54	140, 146	128, 144	138, 140	138, 140	140, 144	138, 146	140, 144	140, 144	142, 152	128, 132	132, 142	132, 142	146, 152	146, 152	144, 146	144, 152		
D12S2078	149.60	258, 270	274, 274	274, 258	270, 274	274, 258	274, 270							258, 270	258, 270	258, 262	258, 270		
D12S1659	155.94	105, 109	109, 109		109, 109	105, 109	109, 109	105, 109	105, 109	101, 109	109, 109		101, 109	105, 109	105, 109	109, 109	109, 109		105, 109
D12S1723	164.63	167, 169	167, 167	167, 169	167, 169	167, 169	167, 169	167, 169	167, 167	167, 169	173, 175	167, 175	167, 175	167, 169	167, 169	167, 175	169, 175	167, 175	167, 175
D13S175	6.03	103, 105	101, 103	103, 103	101, 105		103, 105	101, 103	103, 103	105, 109	107, 107	107, 109	107, 109	103, 105	103, 103	103, 103	103, 103	103, 103	103, 103
D13S1493	25.80	231, 243	223, 235	231, 235	223, 231	235, 243	235, 243			231, 243	223, 243	243, 243	243, 243	231, 243	231, 243	231, 243	231, 243		
D13S218	32.90	191, 191	191, 197	191, 197		191, 197	191, 197	191, 191	191, 191	191, 191	189, 189	189, 191	189, 191	189, 191	191, 191	191, 195		191, 195	191, 195
D13S800	55.31	299, 303	307, 311	299, 307	303, 311	303, 311	299, 311			303, 311	307, 307	303, 307	307, 311	307, 311	303, 303	311, 323	303, 323		
D13S156	55.85	280, 280	280, 282	280, 280	280, 282	280, 282	280, 282	280, 280		276, 278	278, 280	278, 280	278, 280	276, 278	278, 280		280, 282	280, 282	278, 280
D13S170	63.90	125, 127	127, 127	127, 127	125, 127	125, 127	127, 127	127, 127	125, 127	117, 121	123, 125		121, 123	121, 125	117, 121	125, 131	117, 125	117, 125	
D13S793	76.26	257, 261	257, 265	257, 257	261, 265	257, 261	257, 265			257, 265	265, 269	265, 265	265, 265	265, 257	261, 265	257, 261	261, 261		
D13S779	82.93	189, 195	195, 180	180, 189	195, 195	180, 195	189, 195			189, 189	189, 195	189, 195	189, 195	180, 195	189, 195	192, 192	192, 195		
D14S283	13.89	137, 145	131, 147	131, 145	131	145, 147	145, 147	145, 147	145, 147	137, 145	133, 133	133, 145	133, 137	125, 137	145, 145	143, 145	143, 145	143, 145	143, 145

D14S70	14.11	210, 210	214, 214	210, 214	210, 214	210, 214	210, 214			210, 214	210, 214	210, 210	210, 214	210, 212	210, 212	212, 216		210, 216	210, 216
D14S608	28.01	200, 200	200, 220	200, 220	200, 220	200, 200	200, 220			200, 200	200, 224	200, 224	200, 200	200, 224	200, 200	200, 216	200, 200		
D14S599	40.68	90,90	90,84	90,90	90,90	90,84	90,90			90,90	84,90	90,84	90,90	90,90	90,90	96,96	90,96		
D14S587	55.82	274, 274	250, 254	254, 274	250, 274	250, 274	254, 274			254, 274	250, 254	254, 250	254, 254	254, 274	254, 266	254, 266	266, 254		
D14S592	66.80	228, 237	237, 240	237, 237	237, 240	237, 240	237, 237			228, 237	231, 234	234, 237	228, 231	228, 237	228, 234	234, 237	234, 237		
D14S63	69.18	205, 209	205, 215	205, 215	205, 205	205, 215	205, 205	205, 205	205, 205	209, 211		211, 215	209, 215	205, 209		205, 207	207, 207	205, 211	205, 211
D14S588	75.61	125, 133	121, 125	121, 125	125, 125	125, 125	121, 133			129, 133	121, 129	129, 129	121, 133	133, 125	129, 137	121, 125	121, 137		
D14S258	76.28	170, 178	172, 174	170, 174	170, 172	170, 172	174, 178	170, 172	172, 178	174, 178	172, 178	174, 178	172, 178	170, 178	174, 174	170, 178	170, 174		174, 178
D14S53	86.29	149, 151	149, 151	149, 151	151, 151	151, 151	149, 149			149, 151	149, 151	149, 151	149, 149	149, 153	143, 151	143, 151	143, 151		
D14S1279	95.89	131, 135	131, 135	131, 135	131, 135	135, 135	131, 131			131, 135	131, 135	135, 135	131, 135	131, 135	135, 127	131, 131	127, 131		
D14S65	117.30	135, 145	147, 149	145, 147	135, 147		145, 149	135, 149		145, 147	141, 147	145, 147	141, 147	143, 147	135, 145	141, 141	135, 141	141, 145	141, 145
D14S985	126.61	136, 142	138, 142	136, 138	136, 142	136, 138	136, 142	142, 142	138, 142	136, 142	134, 136	134, 136	136, 142	142, 142		138, 138	138, 142		138, 142
D15S128	6.11	201, 203	199, 201	201, 203	199, 203	199, 201	199, 203	199, 201	201, 201	199, 203	201, 201	201, 203	201, 203	201, 203	201, 201	201, 207		201, 201	201, 207
D15S1002	14.58	105, 107	119, 119	105, 119	107, 119	107, 119	105, 119	107, 119	107, 119	107, 111	119, 121	107, 119	107, 121	107, 111	107, 107	107, 119	107, 119	107, 119	107, 119
D15S1007	25.86	175, 179	177, 185	175, 185	179, 185	179, 185		179, 185	177, 179	165, 177	173, 177	165, 173	173, 177	165, 177		179, 187	179, 179	179, 187	177, 179

D15S994	40.25	206, 218	210, 212	206, 210	212, 218	212, 218	212, 218			204, 218	204, 206	206, 218	204, 206	204, 218	204, 210		204, 210	206, 210	204, 210
D15S978	45.62	221, 237	221, 225	221, 221	225, 237	225, 237	225, 237	225, 237	221, 221	225, 237	225, 237		225, 225	225, 237	225, 231	225, 241	225, 241	225, 231	
D15S117	51.21	146, 148	140, 148	146, 148	140, 148		140, 148	140, 148	140, 146	134, 148	134, 146	146, 148	134, 134	134, 138	134, 138	138, 138	134, 138	138, 138	134, 138
D15S1507	60.17	208, 212	204, 208	204, 212	208, 208	208, 208	208, 204			204, 208	208, 212	204, 212	204, 208	204, 220	204, 220	204, 208	204, 208		
D15S1015	66.90	103, 105	103, 103	103, 105	103, 103	103, 103	103, 103			103, 103	103, 105	103, 105	103, 103	103, 103	103, 103	103, 103	103, 103		
D15S153	68.10	216, 222	212, 214	212, 222	214, 216	214, 216	214, 216			216, 218	218, 216	216, 218	218, 218	212, 218	212, 218	212, 216	212, 218		
D15S131	71.28	260, 262	260, 260	260, 260	260, 260	260, 260	260, 262			256, 260	240, 258	240, 256	256, 258	240, 256	240, 262	254, 262	262, 262		
D15S211	75.85	225, 231	229, 235	231, 235	229, 231	229, 231	225, 229			227, 231	229, 252	227, 252	227, 229	225, 227	225, 225	240, 247	225, 247		
D15S205	78.92	156, 160		156, 156	156, 160	156, 160	160, 160	156, 160	160, 160	156, 166	150, 156	156, 166	150, 166		160, 160	158, 160		160, 160	158, 160
D15S127	86.81	130, 146	135, 144	144, 146	135, 146		130, 135	135, 146	130, 135	130, 146	114, 144	114, 130	130, 144	128, 134	130, 146	130, 146		140, 146	130, 130
D15S816	100.59	128, 136	132, 136	132, 136	136, 136	136, 136	136, 128			128, 136	128, 128	128, 136	128, 136	128, 136	136, 136	132, 132	132, 136		
D15S130	100.59	222, 222	222, 222	222, 222	222, 222	222, 222	222, 222	222, 222	222, 222	222, 222	222, 222	222, 222	222, 222	220, 222	222, 222	220, 230	222, 230		
D15S120	112.58	170, 172	160, 170	170, 170	160, 170	160, 170	160, 170	170, 170		170, 170		170, 170	170, 170	170, 170	170, 170	170, 170	170, 170	170, 170	
D16S404	18.07	131, 135	131, 135	135, 135	135, 135	131, 131	131, 131	135, 135	131, 131	131, 133	129, 133	131, 133	133, 133	135, 133	131, 135	131, 133	131, 133	131, 131	131, 133
D16S3075	23.28	206, 208	208, 212	208, 212	208, 212	206, 208	208, 208	208, 208	208, 208	206, 212	206, 212	206, 206	206, 212	206, 212	206, 208	212, 214		206, 214	206, 214

D16S3046	40.65	84,102	102,104	102,104	102,104	84,104	102,102	102,104	102,102	100,102	100,104	100,100	100,100	100,102	84,102	102,104	84,102	84,104	84,104
D16S403	43.89	140,140	138,152	140,152	140,152	140,152	140,138			140,140	144,152	140,144	140,144	140,140	142,150	138,138	138,150		
D16S3068	48.53	137,147	139,143	139,147	139,147	139,147	143,147	139,147	143,147	133,147	141,143	133,143	133,143	133,147	137,141	139,139	137,139	137,139	137,139
D16S3136	62.11	203,203	201,205	201,203	201,203	201,203	203,205	201,203	203,205	203,203			203,205	203,203	201,203	201,205	201,203	201,203	201,203
D16S415	67.62	220,226	222,226	220,226	220,226	226,226	222,226	226,226	220,226	220,224	220,224	224,224	224,224	220,224	224,226	224,224	224,226	224,226	
D16S3253	71.77	179,187	179,187	179,187	187,187	179,187	179,179			187,175	187,187	187,175	175,187	175,187	179,187	179,183	179,183		
D16S503	83.55	223,223	219,229	223,229	223,229	223,229	219,223	223,229	219,223	223,223	229,229	223,229		223,229	223,223	223,229	223,229	223,229	223,223
D16S515	92.10	226,230	230,232	226,232	230,230	226,232	226,230		230,230	230,230	230,238	230,230	230,230	230,230	226,230	234,236	226,236	226,234	226,236
D16S516	100.39	170,170		170,170	170,170	170,170	170,170	170,170	170,170	168,170	170,174	168,178	168,170	170,170	170,170	170,170	170,170	170,170	
D16S3091	111.12	117,119	119,119	117,119	119,119	117,119	117,119	117,119	119,119	119,119	125,125	119,125	119,125	117,123	117,119	119,127	119,119		
D16S520	125.82	185,197	193,195	185,193	195,197			185,193	185,195	189,191	189,189	189,191	189,191	185,197	185,189	187,191			189,191
D17S849	0.63	255,257	253,253	253,255	253,257	253,255	253,257	253,255	253,257	255,257	253,257		253,257	255,257	253,257	253,253	253,257	253,253	
D17S831	6.60	224,224	230,230	224,230	224,230	224,230	224,230	224,230	224,230	224,228	224,234		224,228	224,224	228,236	230,230	236,230	228,230	228,230
D17S938	14.69	180,182	180,180	180,180	180,182	180,180	180,182	180,180	180,182	180,182	166,178	178,182	178,180	166,180			166,178		
D17S974	22.24	213,217	209,123	213,217	213,217	109,123	109,217			205,217	205,213	205,213	205,213	205,205	295,213	209,205	295,213		

D17S1852	22.24	210, 210	200, 202	202, 210	202, 210	200, 210	200, 210	202, 210	200, 210	200, 210	202, 212		200, 212	200, 204	204, 210		202, 210	204, 208	
D17S799	31.96	192, 196	192, 200	192, 196	192, 196	192, 192	192, 196	192, 192	192, 196	192, 196	186, 200		186, 192	192, 196	192, 196	192, 196	192, 196		192, 196
D17S921	36.14	175, 183	175, 183	175, 175	175, 175	183, 183	175, 175	175, 183	175, 183	175, 183	181, 183		181, 183	183, 183	183, 183		183, 183	183, 183	183, 183
D17S1857	43.01	181, 181	177, 181	181, 181	181, 181	177, 181	181, 181	181, 181	177, 181	181, 181	177, 181		181, 181	181, 185	181, 185	179, 181	179, 185	181, 185	181, 181
D17S2196	44.62	139, 151	155, 147	139, 155	139, 155	139, 155	139, 155			139, 155	151, 155	159, 159	159, 159	147, 151	147, 151	147, 151	147, 147		
D17S1294	50.74	252, 256		252, 252	252, 252	256, 256	252, 252		252, 256	252, 252	260, 264	252, 260	252, 260	252, 256	252, 260		252, 256	252, 256	256, 260
D17S798	53.41	223, 229	223, 223	223, 229	223, 229	223, 223	223, 229	223, 223	223, 229	209, 229	209, 225	209, 209	209, 209	223, 223	223, 223	209, 217	209, 223	209, 223	217, 223
D17S1868	64.16	190, 196	188, 198	188, 196	188, 190	190, 198	188, 196	188, 190	196, 198	190, 196	196, 202	190, 196	190, 196	192, 196	190, 196	192, 192	190, 192	192, 196	190, 192
D17S2180	66.85	122, 122	119, 119	119, 122	119, 122	119, 122	119, 122			122, 122	119, 119	119, 122	119, 122	122, 122	119, 122	116, 122	116, 122		
D17S787	74.99	142, 152	138, 138	138, 152	138, 142	138, 142	138, 152	138, 142	138, 142	138, 152	140, 142		138, 142	142, 152	138, 144	142, 152	138, 142	144, 152	138, 152
D17S944	82.56	212, 216	212, 216	212, 216	216, 216	212, 216	212, 212	216, 216	216, 216	212, 212	216, 218		212, 216	212, 216	212, 212	216, 222	212, 216	212, 222	212, 222
D17S2193	89.32	141, 153	141, 147	141, 141	141, 153	141, 153	141, 147			141, 141	141, 147	141, 141	141, 147	141, 153	141, 141	147, 156	141, 147		
D17S949	93.27	207, 221	215, 223	215, 221	207, 215	207, 223	221, 223	207, 215	207, 215	211, 221	217, 221		217, 221	207, 221	211, 221	219, 221	211, 221	219. 221	
D17S785	103.53	185, 189	189, 189	189, 189	189, 189	185, 189	185, 189	185, 189	185, 189	185, 195	189, 197		189, 195	189, 195	189, 195		189, 197	195, 197	189, 197
D17S784	116.86	230, 234	228, 232	232, 234	228, 234	232, 234	230, 232			230, 234	234, 236	230, 236	234, 234	232, 234	232, 234	232, 234	232, 234		

D17S928	126.46	149, 149	149, 153	149, 149	149, 153	149, 149	149, 149	149, 153	149, 153	145, 149	151, 155		145, 151	145, 149	145, 149		145, 153	145, 153	149, 153
D18S63	8.30	255, 273	277, 277	273, 277	255, 277		255, 273	255, 255	255, 273	255, 273	255, 273	255, 277	255, 273	269, 271	255, 271	255, 277	273, 277		
D18S53	41.24	171, 171	171, 173	171, 173	171, 173	171, 171	171, 171	171, 171	171, 171	171, 175	167, 175	175, 175	167, 175	167, 171	171, 171	171, 173	171, 171	171, 171	171, 171
D18S452	18.70	135, 135	133, 139	135, 139	135, 139	135, 139	133, 135	131, 133		131, 135	133, 135	133, 135	133, 135	131, 133	131, 133	133, 135	133, 135	131, 133	133, 135
D18S464	31.17	289, 291	287, 289	287, 291	287, 289	289, 291	289, 291	289, 291	289, 289	289, 291	289, 289	289, 291	289, 291	289, 291	289, 289	289, 291	289, 291	289, 291	289, 289
D18S53	41.24	171, 171	171, 173	171, 173	171, 173	171, 171	171, 171	171, 171	171, 171	171, 175	167, 175	175, 175	167, 175	167, 171	171, 171	171, 173	171, 171	171, 171	171, 171
D181102	62.84	214, 218	210, 210	210, 218	210, 214	210, 218	216, 216		210, 216	216, 218	214, 218	210, 218	214, 216	210, 214	214, 214		210, 218	214, 216	214, 214
D18S1134	88.62	215, 215	211, 213	211, 215	211, 215	213, 215	213, 215	213, 215	213, 215	211, 215	211, 213	213, 215	213, 215	211, 215	211, 215	209, 211	209, 211		
D18S1148	90.60	144, 150	148, 150	148, 150	148, 150	144, 150	150, 150	144, 150	144, 150	148, 150	142, 150	150, 152	150, 152	148, 150	144, 150	148, 150	148, 150		
D18S1147	90.60	226, 226	210, 226	226, 226	226, 226	210, 226	210, 226	210, 226	210, 226	216, 226	212, 226	212, 226	212, 226	216, 226	212, 226	210, 220	212, 220		
D18S1148	90.60	144, 150	148, 150	148, 150	148, 150	144, 150	150, 150	144, 150	144, 150	148, 150	142, 152	150, 152	150, 152	148, 150	144, 150	148, 150	148, 150		
D18S1147	90.60	226, 226	210, 226	226, 226	226, 226	210, 226	210, 226	210, 226	210, 226	216, 226	212, 226	212, 226	212, 226	216, 226	212, 226	210, 220	212, 220		
D18S68	96.48	284, 288	272, 288	272, 284	272, 284		284, 284	272, 280	280, 284	280, 284	284, 284	284, 288	284, 288	272, 284	272, 284	288, 288	288, 288	284, 288	284, 284
D18S465	100.11	239, 239	239, 241	239, 241	239, 241	239, 239	239, 239	239, 239	239, 239	239, 237	243, 245	237, 245	237, 245	237, 245	239, 239	239, 239	239, 239		
D18S61	105.03	175, 179	159, 175	175, 175		159, 179	159, 179	175, 175	159, 175	159, 175	175, 175	159, 175	159, 175		159, 175	159, 179	159, 179	177, 179	159, 177

D18S1161	114.26	82,94	94,94	82,94	94,94		94,104	94,94	94,104	94,104	82,94	94,94	94,104	82,90	90,104	94,94	94,94	82,94	90,104
D18S462	120.05	187,193	185,185	185,193	185,187	185,187	185,187	181,187	181,185	185,187	187,193	185,187	185,187	183,187	187,187	185,187	185,187	185,187	183,185
D18S70	126.00	112,122	106,114	106,112	106,122	114,122	112,122	114,120	114,122	120,122	112,112	114,122	112,122		112,122	114,122	106,122	112,112	120,122
CA19S322	0.00	326,326	326,326	326,326	326,326	326,326	326,326	326,326	326,326	326,326	326,326	326,326	326,326	324,326	324,326	324,326	326,326	326,326	326,326
D19S886	0.00	156,158	140,160	156,160	158,160	156,160	140,156	158,160	140,158	156,158	154,156	156,156	156,156	156,158	156,158	156,156	156,156	156,156	156,156
GT19S949	0.00	272,274	256,276	272,276	274,276	272,276	256,272	274,276	256,274	272,274	270,272	272,272	272,272	272,274	272,274	272,272	272,272	272,272	272,272
ATA19S1001	0.69	408,411	402,411	408,411	411,411	408,411	402,408	411,411	402,411	408,411	393,405	393,408	393,408	402,408	402,408	389,408	408,408	408,408	389,408
GT19S949	5.54	272,274	256,276	272,276	274,276	272,276	256,272	274,276	256,274	272,274	270,272	272,272	272,272	272,274	272,274	272,272	272,272	272,272	272,272
ATA19S1001	5.54	408,411	402,411	408,411	411,411	408,411	408,411	411,411	402,411	408,411	393,405	393,408	393,408	402,408	402,408	389,408	408,408	408,408	389,408
CA19S2310	6.57	270,276	272,274	274,276	270,274	274,276	272,276	270,274	270,274	270,276	266,276	276,276	276,276	266,276	266,276	268,272	268,276	268,276	266,272
D19S878	6.57	212,218	214,216	216,218	212,216	216,218	214,218	212,216	212,216	212,218	208,218	218,218	218,218	208,218	208,218	210,214	210,218	210,218	208,214
D19S565	6.57	166,178	186,192	178,186	166,186	178,186	178,186	166,186	166,186	166,178	178,178	166,178	178,178	176,178	176,178	176,186	176,178	176,178	176,178
GT192518	6.57	293,305	315,321	305,315	293,315	305,315	305,321	293,315	293,315	293,305	305,305	293,305	305,305	303,305	303,305	303,315	303,305	303,305	303,315
D19S591	9.84	104,108	104,108	104,104	108,108	104,108	104,108	104,108	104,108	104,108	104,108	104,108	104,104	100,104	100,104	100,104	104,104	104,104	100,100
D19S424	10.97	147,155	155,155	147,155	155,155	147,155	147,155	155,155	155,155	147,155	153,155	155,155	147,155	147,147	147,147	147,157	147,147	147,147	147,157

D19S209	10.97	262, 268	264, 266	262, 266	266, 268	262, 266	262, 264	266, 268	266, 268	262, 268	270, 270	268, 270	262, 270	262, 272	262, 272	262, 264	262, 262	262, 262	264, 272
D19S894	15.55	144, 154	158, 160	144, 160	154, 160	144, 160	154, 158	154, 160	154, 160	144, 154	160, 162	154, 160	144, 160	144, 144	144, 144	144, 146	144, 146	144, 146	144, 144
D19S216	20.01	185, 187	179, 187	185, 187	187, 187	185, 187	179, 187	187, 187	187, 187	185, 187	185, 191		185, 191	179, 185	179, 185	179, 185	185, 185	185, 185	185, 185
D19S1034	20.75	226, 234	234, 234	234, 234	226, 234	234, 234	226, 234	226, 234	226, 234	226, 234	230, 234	226, 234	234, 234	230, 234	230, 234	234, 238	234, 238	234, 238	234, 234
D19S884	26.37	218, 226	224, 224	224, 226	218, 224	224, 226	218, 224	218, 224	218, 224	226, 226	226, 234	226, 226	226, 226	218, 236	218, 226		226, 234	222, 226	218, 234
D19S221	36.22	207, 207	205, 207	205, 207	205, 207	205, 207	207, 207	205, 207	205, 207	207, 207	201, 203		203, 207	207, 207	207, 207	207, 207	207, 207		207, 207
D19S226	42.28	253, 243	241, 243	235, 243	243, 243	243, 235	241, 243	243, 243	243, 243	253, 235	233, 235	235, 253	233, 235	243, 245	235, 243	243, 245	235, 243		
D19S410	45.48	156, 170	170, 170	170, 170	156, 170	170, 170	156, 170	156, 170	156, 170	156, 170	156, 156	156, 156	156, 170	156, 156	156, 170	156, 170	156, 170		
D19S414	54.01	163, 177	179, 181	177, 181	181, 163	181, 163	163, 179	163, 181	163, 179	177, 163	181, 181	181, 163	177, 181	163, 163	163, 163	163, 185	163, 163		
D19S220	62.03	273, 277	275, 277	273, 275		275, 277	277, 277	275, 277	277, 277	273, 281	275, 279	273, 279	273, 275	267, 277	267, 277		277, 279		277, 279
D19S420	66.30	255, 265	259, 265	259, 265	255, 259	255, 259	255, 265	255, 259	255, 265	257, 265	263, 263	257, 263	263, 265	255, 263	255, 263	257, 263	255, 257	255, 263	255, 257
D19S902	72.72	209, 217	203, 209	203, 217	203, 209	203, 209	203, 209	203, 209	209, 209	213, 217	209, 211	209, 213	211, 217	209, 217	209, 213		209, 209	209, 211	209, 209
D19S210	100.01	169, 175	175, 175	169, 175	169, 175	175, 175	175, 175	175, 175	175, 175	169, 169	165, 169		165, 169	169, 175	169, 175	165, 165	165, 175	165, 169	165, 175
D20S864	0.00	224, 226	224, 26	224, 226	224, 226	224, 226	224, 226			224, 228	226, 228	226, 228	226, 228	224, 226	226, 228	226, 228	226, 228		
D20S199	6.25	108, 120	108, 120	108, 108	108, 108	108, 108	108, 120			112, 116	108, 120	108, 116	108, 116	108, 120	112, 116	112, 114	112, 112		

D20S113	8.97	178, 178	178, 178	178, 178	178, 178	178, 178	178, 178			178, 178	178, 186	178, 186	178, 178	178, 178	178, 178	178, 178	178, 178		
D20S842	8.97	164, 168	160, 164	160, 168	160, 168	160, 164	160, 168			164, 172	172, 174	172, 174	164, 172	166, 172	164, 168	156, 176	156, 168		
D20S181	9.53	156, 156	164, 164	156, 164	156, 164	156, 164	156, 164			156, 160	164, 164	160, 164	156, 164	156, 160	156, 156	164, 164	156, 164		
D20S193	9.53	150, 154	150, 158	154, 158	154, 158	150, 158	154, 158			150, 150	150, 150	150, 150	150, 150	150, 150	150, 154	150, 154	150, 154		
D20S116	11.20	109, 109	115, 119	109, 119	109, 119	109, 119	109, 110			109, 121	109, 111	109, 121	109, 111	113, 121	109, 109	109, 117	109, 117		
D20S889	11.20	266, 280	272, 278	266, 278	266, 278	278, 280	266, 278	278, 280	266, 272	280, 292	272, 282		272, 280	290, 292	266, 280		266, 272		280, 284
D20S849	13.98	214, 230	214, 216	214, 214	214, 214	214, 230	214, 214			214, 230	214, 230	214, 230	214, 230	214, 230	214, 230	214, 230	214, 230		
D20S895	13.98	216, 224	216, 224	216, 216	216, 216	216, 216	216, 216			224, 224	216, 218	216, 224	218, 224	224, 224	216, 216	220, 226	216, 220		
D20S882	15.05	120, 126	120, 122	120, 122	120, 120	120, 126	120, 120			120, 126	120, 120	120, 120	120, 126	120, 126	120, 126	120, 126	120, 120		
D20S115	21.15	234, 236	232, 236	236, 236	232, 236	232, 234	232, 236	232, 234	232, 236	234, 236	234, 234		234, 236	234, 236	234, 236	236, 236	236, 236	234, 236	234, 236
D20S851	24.70	132, 132	128, 136	138, 132	132, 136	132, 136	132, 136			128, 132	132, 146	132, 146	128, 132	128, 132	132, 132	132, 134	132, 134		
D20S186	32.30		127, 131	121, 131	121, 127	125, 127	121, 127	125, 127	121, 127	113, 131	113, 119		113, 131	125, 131	121, 125		113, 121	121, 125	121, 125
D20S112	39.25	199, 211	199, 207	207, 211	199, 211	199, 199	199, 199	199, 211	199, 211	207, 213	209, 219		209, 213	207, 213	199, 211		209, 211	199, 209	199, 209
D20S107	55.74	266, 274	272, 274	266, 274	266, 274	266, 272	266, 272	266, 272	266, 272	266, 270	268, 270		26,270	266, 270	266, 270		266, 270		266, 270
D20S119	61.77	108, 118	104, 110	110, 118	110, 118	104, 108	104, 118	104, 118	104, 118	112, 118			112, 114	112, 118	108, 118	112, 116		108, 116	108, 112

D20S178	66.16		248, 252	252, 252	252, 252	248, 252	248, 252	252, 252	248, 252	250, 252	248, 248	248, 252	248, 250	250, 252	244, 252		244, 250	244, 254	244, 254
D20S100	84.78		206, 208	208, 208	208, 208	206, 208	206, 208	208, 208	206, 208	208, 210	206, 206		206, 210	208, 208	208, 208		206, 208	208, 214	208, 214
D20S171	95.70	135, 139	135, 141	139, 141	139, 141	135, 135	135, 139	139, 141	135, 139	137, 137	135, 137	135, 137		135, 137	135, 137		137, 143	135, 137	137, 137
D20S173	98.09	185, 185	175, 191	185, 191	185, 191	185, 185	185, 185	185, 191	185, 185	185, 187	181, 185	175, 187	181, 185	185, 187	185, 187		187, 187		185, 187
D21S1914	19.39	203, 207	201, 205	205, 207	201, 207	201, 207	201, 203		201, 203	203, 207	299, 209	203, 209	207, 209	197, 203	197, 207		197, 205	197, 203	197, 203
D21S263	27.40	175, 179	179, 181	175, 179	175, 179	175, 179	179, 179	179, 179	179, 179	175, 179	175, 181	175, 175	175, 175	179, 179	179, 199			175, 199	175, 199
D21S1252	35.45	231, 247	231, 239	239, 247	239, 247	239, 247	231, 239	231, 239	231, 239	231, 247			247, 249	231, 245	231, 245		245, 249	231, 231	245, 249
D21S2055	40.49	127, 153	127, 153	127, 153	127, 153	127, 153	153, 153			127, 193	127, 153	127, 127	127, 127	177, 193	177, 193	115, 185	193, 185		
D21S266	45.87	157, 171	157, 169	169, 171	169, 171	169, 171	157, 169	157, 169	157, 169	157, 171		171, 171	171, 171	157, 157	157, 157		157, 157	157, 157	157, 157
D22S420	4.06	152, 158	148, 156	148, 152	148, 152	148, 158	152, 156	148, 158	152, 156	150, 152	150, 152	150, 150	150, 152	150, 152	150, 152		152, 156		152, 156
D22S539	14.44	123, 123	123, 131	123, 123	123, 123	123, 123	123, 123	123, 123	123, 131	123, 123	123, 133	123, 133	123, 133	123, 123	123, 123		123, 123	123, 123	123, 123
GCT10C10	18.10	195, 201	192, 195	192, 201	192, 195	192, 201	195, 201			195, 201	210, 195	195, 201	201, 201	195, 195	195, 201	195, 198	195, 201		
D22S315	21.47	193, 195	195, 195	195, 195	195, 195	195, 195	195, 195	195, 195	195, 195	195, 195	195, 195	195, 195	195, 195	193, 197	193, 197		193, 197	193, 197	197, 197
D22S280	31.30	216, 216	210, 216	216, 216	216, 216	216, 216	210, 216	216, 216	216, 216	216, 216	216, 218	216, 216	216, 216	214, 214	212, 214		212, 216	212, 216	214, 214
D22S283	38.62	130, 154	146, 148	146, 154	146, 154	130, 146	130, 148	146, 154	146, 154	130, 154	144, 148	144, 154	144, 154	140, 150	140, 150		140, 142	140, 142	140, 150

D22S423	46.42	152, 158	148, 156	148, 152	148, 152	148, 158	152, 156	148, 158	152, 156	150, 152	150, 152	150, 150	150, 152	150, 152	150, 152		152, 156		152, 156
D22S274	51.54	206, 206	200, 214	206, 214	206, 214	206, 214	200, 206	206, 214	206, 214	206, 206	204, 206	204, 206	204, 206	206, 206	202, 210		202, 202	202, 202	206, 210