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Authors	Bog, Manuela;Sree, K. Sowjanya;Fuchs, Joerg;Hoang, Phuong T.N.;Schubert, Ingo;Kuever, Jan;Rabenstein, Andreas;Paolacci, Simona;Jansen, Marcel A. K.;Appenroth, Klaus-J.
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SYSTEMATICS AND PHYLOGENY

A taxonomic revision of *Lemna* sect. *Uninerves* (Lemnaceae)

Manuela Bog,[1] K. Sowjanya Sree,[2] Joerg Fuchs,[3] Phuong T.N. Hoang,[3,7] Ingo Schubert,[3] Jan Kuever,[4] Andreas Rabenstein,[4] Simona Paolacci,[5] Marcel A.K. Jansen[5] & Klaus-J. Appenroth[6]

1 *University of Greifswald, Institute of Botany and Landscape Ecology, Greifswald, Germany*
2 *Central University of Kerala, Department of Environmental Science, Periye, Kerala, India*
3 *Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany*
4 *Bremen Institute of Material Testing, a division of the Leibniz Institute for Materials Engineering IWT, Bremen, Germany*
5 *University College of Cork, Environmental Research Institute, Cork, Ireland*
6 *University of Jena, Matthias Schleiden Institute – Plant Physiology, Jena, Germany*
7 Present address: *University of Dalat, Faculty of Biology, Dalat City, Lamdong Province, Vietnam*
Address for correspondence: *Manuela Bog*, *manuela.bog@uni-greifswald.de*

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Abstract *Lemna* sect. *Uninerves* Hegelm. consists of three species, *Lemna minuta* Kunth (synonym *L. minuscula*), *L. valdiviana* Phil. and *L. yungensis* Landolt. *Lemna yungensis* was discovered growing on rocks in the Yungas in Bolivia by E. Landolt and was described just 20 years ago. In the original description, Landolt reported that this species is closely related to *L. valdiviana* and that it is difficult to distinguish the three species on a morphological basis. Therefore, the taxonomic position and status of *L. yungensis* remained controversial. Here, we carried out a detailed taxonomic study, integrating approaches that include quantitative morphometry, metabolomic profiling by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) as well as molecular genetic analysis using amplified fragment length polymorphism (AFLP), and barcoding of plastidic sequences. We also investigated genome sizes of clones of the three species. Whereas *L. minuta* can easily be differentiated from *L. valdiviana* and *L. yungensis*, it was not possible to distinguish *L. valdiviana* from *L. yungensis* with any of the methods used. These data imply that *L. yungensis* is identical to *L. valdiviana*. Thus, the name *L. yungensis* should be synonymised with the name *L. valdiviana*, since this is the older name.

Keywords AFLP; barcoding; duckweed; *Lemna yungensis*; MALDI-TOF-MS

Supporting Information may be found online in the Supporting Information section at the end of the article.

■ INTRODUCTION

The genus *Lemna* L. was initially divided into five sections by Landolt (1986); however, this structure was later corrected by reducing the number of sections to four because the *L.* sect. *Hydrophylla* Dumort. (*Lemna trisulca* L.) turned out to be a part of *L.* sect. *Lemna* (Les & al., 2002). The *Lemna* sections, *Lemna*, *Alatae* Hegelm., *Biformes* Landolt, and *Uninerves* Hegelm., represent well-supported monophyletic clades (Les & al., 2002). *Lemna* sect. *Uninerves* includes three species: *L. minuta* Kunth, *L. valdiviana* Phil., and, since 1998 (Landolt, 1998), *L. yungensis* Landolt. *Lemna valdiviana*, which is restricted to warm temperate, subtropical and tropical regions of North and South America (Landolt, 1986), was first described by Rudolph Amandus Philippi (14 September 1808–23 July 1904) honouring the main place of his work, Valdivia, Chile (Philippi, 1864). The taxonomic separation of *L. valdiviana* from its sister species *L. minuta* was reviewed by Reveal (1990) and Crawford & al. (1996). *Lemna minuta*, a species originally distributed throughout the temperate zones and in the mountains of North and South America (Landolt, 1986), but invasive in Europe (e.g., Ceschin & al., 2016a, 2018; Kirjakov & Velichkova, 2016; Paolacci & al., 2018a,b), was already described in *Nova genera et species plantarum* by Karl Sigismund Kunth (Humboldt & al., 1815: 371–372) and was referred to as *L. minuscula* Herter for many years (Landolt, 1986) until Reveal (1990) showed that *L. minuta* is the older, i.e., legitimate name for this species. After investigating 25 clones of *L. minuta* and 26 clones of *L. valdiviana*, Crawford & al. (1996) called the two species sisters because they could only be distinguished, with difficulty, on a morphological basis. Even Elias Landolt, world-renowned Lemnaceae-expert and co-author of Crawford & al. (1996), confessed great problems in distinguishing these two species. It was also Landolt, who established the endemic species *L. yungensis*,

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mainly for ecogeographical reasons, since he found fronds of this species only growing on wet rocks in the province Nor-Yungas, Bolivia (Landolt, 1998), a region belonging to the tropical mountain rain forest, which is rather atypical for members of duckweed. He inserted the species in *L.* sect. *Uninerves* mainly based on its high morphological similarity to *L. valdiviana*. According to Landolt (1998), the main distinguishing features of the two species are the larger size and the more or less symmetric shape of *L. yungensis* compared to *L. valdiviana*. Further, papillae along the midline, which are often present in *L. valdiviana*, are never observed in *L. yungensis*. Finally, the margin of the frond is sometimes slightly reddish violet in *L. yungensis*, which is not the case for *L. valdiviana*. Later, Landolt reported that these markers depend to a large degree on the cultivation conditions and are not very obvious when plants are grown under laboratory conditions (communication of E. Landolt to KJA). The separation of all three species by sequencing of plastidic (Les & al., 2002) or even nuclear DNA fragments (Tippery & al., 2015) has to be considered as not conclusive because only one clone from each species was used. Therefore, we investigated all three species by amplified fragment length polymorphism (AFLP; Bog & al., 2010). In this study, clones of the species *L. minuta* could be distinguished from those of *L. valdiviana* and *L. yungensis*, but *L. valdiviana* could not be distinguished from *L. yungensis*. One of the authors, E. Landolt, was, however, concerned that almost all clones of the species *L. minuta* that were investigated, originated from places were the species was introduced and not native. This might have resulted in a larger genetic similarity between the investigated *L. minuta* clones than it is the case in nature worldwide. To account for this, *L. minuta* clones from South America were included in the current study.

Little is known on the reproductive biology of the three species, and the information that can be found throughout the literature is not very satisfying. In general, all duckweed species are assumed to reproduce mainly asexually, though flowering in nature is known for all species with a few exceptions for the genus *Wolffia* Horkel ex. Schleid. (Landolt, 1986; Les & al., 1997). The proportion of fruiting samples from herbarium specimens and personal observations by Landolt (1986) were ≤2% for *L. valdiviana* and *L. minuta*. It is not known, whether hybridisation occurs between the three species.

In the present project, after completing a careful quantitative morphological analysis (cf. Ceschin & al., 2016b), we used as many independent molecular taxonomic methods as possible to test whether *L. minuta*, *L. valdiviana* and *L. yungensis* represent three distinct species within *L.* sect. *Uninerves*. Besides fingerprinting by AFLP and barcoding by sequences of plastidic fragments, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS, see Materials and Methods) was applied to these three species. Moreover, the genome size was measured to find additional arguments for or against the species delineation.

■ MATERIALS AND METHODS

Cultivation of plants. — All clones were taken from the stock collection of the University of Jena, Matthias Schleiden Institute – Plant Physiology, and are available as living material from the duckweed stock collections in Jena and at Rutgers State University of New Jersey (New Brunswick, New Jersey, U.S.A.) under the international four-digit code (Zhao & al., 2012) given in Table 1. The clones were cultivated in N-medium (Appenroth & al., 1996) under controlled climatic conditions of 25°C and continuous light at 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR, if not stated otherwise.

For molecular genetic analysis, the plant material was separated by filtration from the nutrient medium after ca. 10 days of cultivation, dried on paper towels, snap frozen in liquid nitrogen and then stored at −70°C until use.

To prepare plant material for MALDI-TOF-MS analysis, plants were conditioned to the N-medium for four weeks (precultivation phase) in order to ensure reproducible results. The nutrient medium was replenished every week to prevent nutrient limitation. Details and the further procedures were described by Ziegler & al. (2015). Thereafter, the plant material (1 g fresh weight) was snap frozen in liquid nitrogen and lyophilised.

For morphometric analysis, four clones per species were cultivated for three weeks at 26°C. Again, the N-medium was replaced every week to prevent nutrient limitation.

Morphometric analysis. — Morphological characters were investigated on 25 fully grown mother fronds per clone. In a first step, the presence of papillae was checked. Then the fronds were bleached in NaOCl (0.28%) for 15 to 30 minutes and subsequently washed in distilled water. For better visualisation of the aerenchymatic part and the nerve, fronds were stained with methanolic carmin according to Schwarz (Schömmer, 1949) for 1.5 days. Subsequently, fronds were washed in distilled water and differentiated in acidic isopropanol (50% isopropanol, 0.5% HCl) for 6 to 8 hours. Preparations were immediately photographed using a Leica DM2500 microscope (Leica Microsystems, Wetzlar, Germany) (Fig. 1). The following frond characters were measured using ImageJ v.1.52a (Schneider & al., 2012): (1) length from base to apex (L), (2) width at the widest part (W) of the fronds, (3) length of nerve from node onwards (LN), (4) distance between node and end of aerenchyma (A) and (5) distance from node to apex (N-A) (see Fig. 1D for schematic overview). We also calculated ratios between some of these characters, e.g., to describe whether or not the nerve reaches beyond the aerenchymatic part, which is a diagnostic character according to Landolt (1980) (Table 2). The averages of all characters were tested for significant differences using the Holm-Sidak test in the programme SigmaPlot v.11.0 (Systat Software, San Jose, California, U.S.A.).

AFLP and sequencing of plastidic fragments. — DNA extraction, AFLP fingerprinting and barcoding were done following protocols as given by Bog & al. (2015) unless otherwise noted below. For scoring of AFLP fragments all

samples, including 14 duplicates, and 216 parameter combinations for automated analysis of electropherograms (GelCompar II v.5.1; Applied Maths, Austin, Texas, U.S.A.) were screened as proposed by Holland & al. (2008). This resulted in an optimal parameter setting of *minimum profiling* set to 1.5, *minimum area* set to 0.3, and *matching tolerance* set to 0.14. After scoring, only one sample of the 14 duplicates was selected randomly for final analysis. Banding patterns were compiled into a 0/1 matrix (suppl. Table S1). Using the programme R v.3.1.3 (R Development Core Team, 2015)

Table 1. Species and clones of *Lemna minuta* Kunth, *L. valdiviana* Phil., and *L. yungensis* Landolt, together with their origin.

Species	Clone	Origin	Morphometry	AFLP	Barcoding *psbK-psbI* / *atpF-atpH*	Proteomic profiling	Genome size in Mbp/1C (CV%)
L. minuta	5571	Ireland		+	MK516234* / MK516253*		
	5572	Ireland		+	MK516235* / MK516254*		
	5573	Ireland		+	MK516236* / MK516255*		
	5575	Germany				+	
	6717	Guatemala	+	+	MK516224* / MG775393	+	367 (0.23)
	7612	Peru	+	+	MK516225* / MK516245*	+	362 (0.22)
	8699	Japan		+	MK516226* / MK516246*		
	9226	England		+	MK516227* / MK516247*		
	9414	Italy		+	MK516228* / MK516248*		
	9473	Netherlands		+	MK516229* / MK516249*		
	9474	Netherlands		+	MK516230* / MG775394		
	9476	England		+	MK516231* / MK516250*		
	9484	Greece	+	+	MK516232* / MK516251*	+	366 (0.60)
	9581	Greece	+	+	MK516233* / MK516252*	+	365 (0.44)
L. valdiviana	8685	Chile	+				559 (0.38)
	8845	Brazil					371 (2.45)
	9228	Brazil	+	+	MK516215* / MK516239*	+	370 (0.37)
	9229	Ecuador		+	MK516216* / MK516238*		
	9233	Ecuador		+	MK516217* / MK516240*		
	9267	U.S.A.		+	MK516218* / MG775406		
	9395a	Venezuela		+	MK516219* / MK516241*		
	9401	Venezuela	+	+	MK516220* / MK516242*	+	395 (0.11)
	9442	Brazil	+	+	MK516221* / MK516243*	+	392 (0.09)
	9443	Brazil		+	MK516222* / MG775407		
	9475	Brazil		+	MK516223* / MK516244*		
L. yungensis	9207	Bolivia	+	+	KJ136052 / KJ136035	+	371 (0.23)
	9208	Bolivia	+	+	MK516213* / KJ136033	+	375 (0.71)
	9209	Bolivia	+	+	MK516214* / MK516237*	+	370 (0.33)
	9210	Bolivia	+	+	KJ136051 / KJ136034	+	370 (0.53)
Outgroup							
L. aequinoctialis	6746	U.S.A.			GU454312 / GU454216		
L. tenera	9020	Australia			KJ136049 / MG775399		

The methods applied for identification are indicated by +. For AFLP, the 0/1 matrix is given in the supplementary material (Table S1), the accession numbers of the barcoding fragments *psbK-psbI* and *atpF-atpH* are given in this Table. * Sequences generated during this study.
CV%, coefficient of variation in %.

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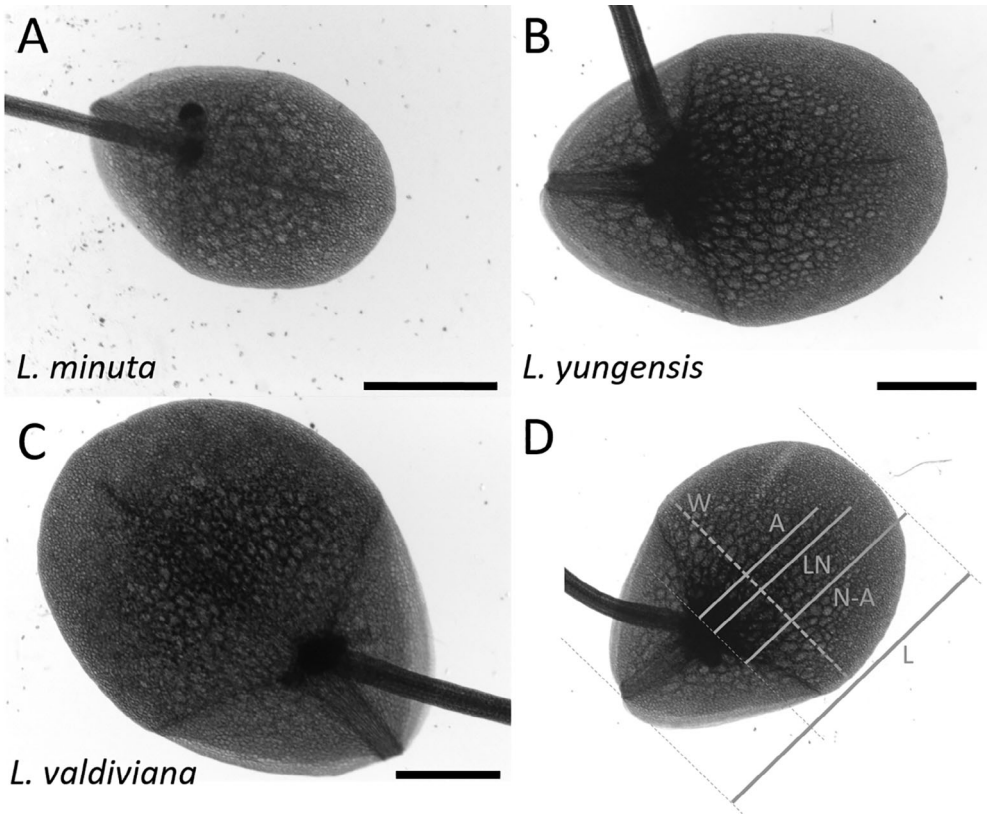


Fig. 1. Ventral view of the investigated duckweed species. **A,** *Lemna minuta* clone 9581; **B,** *L. yungensis* clone 9208; **C,** *L. valdiviana* clone 8685; **D,** Overview of the measured characters for the morphological analysis. Fronds were prepared using a methanolic carmin staining method. L = length from base to apex, W = width at the widest part of the fronds, LN = length of nerve from node onwards, A = distance between node and end of aerenchyma, N-A = distance from node to apex. Scale bar represents 1 mm.

and the packages *ecodist* v.1.2.9 (Goslee & Urban, 2007) and *ape* v.3.2 (Paradis & al., 2004), we conducted a principal coordinate analysis (PCoA) based on Bray-Curtis distances to investigate the structure of the dataset.

For barcoding, the markers *atpF-atpH* and *psbK-psbI* (Wang & al., 2010; Borisjuk & al., 2015) were used. The annealing temperature for the PCR amplification of both plastidic markers was 55°C. All sequencing steps after purification of PCR products were done by Macrogen (Amsterdam, Netherlands). Accession numbers of the sequenced fragments are given in Table 1. The alignment (suppl. Appendices S1 and S2) of the two markers was edited manually using BioEdit v.7.2.5 (Hall, 1999). Indels of the concatenated cpDNA sequences were coded using SeqState v.1.4.1 (Müller, 2005) according to the method of Simmons & Ochoterena (2000). Subsequently, TCS v.1.21 (Clement & al., 2000) was used to build a haplotype network from which we selected unique haplotypes to run jModeltest v.2.1.10 (Darriba & al., 2012). The sequences and indel-coded partitions were tested independently to select the best-fit models of nucleotide substitution. For phylogenetic tree reconstruction, we used the programme BEAST v.1.10.4 (Suchard & al., 2018). The xml input file was created using the BEAST companion programme BEAUti v.1.10.4 with the following parameters: substitution model for sequence partition – GTR + I with estimated base frequencies; substitution model for indel partition – HKY with estimated base frequencies; molecular clock model – strict clock; tree prior – Yule process; parameter priors – default. The length of the MCMC was set to 10 million steps with sampling of every 1000th step leading to 10,001 trees. The analysis was run three independent times. All three runs were examined for congruence and effective sampling sizes of ESS >200 using TRACER v.1.7.1 (Rambaut & al., 2018). Finally, the runs were processed with two further BEAST companion programmes. They were combined using LogCombiner v.1.10.4 with the first 10% discarded as burn-in, respectively, and the tree information was summarised onto a single target tree using TreeAnnotator v.1.10.4 with the following parameters: burn-in = 0, posterior probability limit = 0.5, target tree type = maximum clade credibility, node heights = median heights. As a second phylogenetic tree inference, we used the maximum likelihood (ML) optimality criterion as implemented in the programme RAxML-NG v.0.9.0 (Kozlov & al., 2019). Since RAxML-NG did not run with the same partition scheme as we used for BEAST, we ran the sequence and indel partitions as unpartitioned data under the GTR + I substitution model. We started the ML tree search with 50 random +50 parsimony-based trees and ran

Table 2. Measurements of morphology for the three investigated species.

	Length (L)	Width (W)	Length of Nerves (LN)	Length Node-Aerechnchym (A)	Length Node-Apex (N-A)	L : W ratio	LN : A ratio	LN : N-A ratio
6717	2.9 ± 0.2	2.0 ± 0.1	1.3 ± 0.2	1.3 ± 0.2	2.0 ± 0.2	1.45 ± 0.06	1.00 ± 0.07	0.65 ± 0.06
7612	3.7 ± 0.3	2.4 ± 0.2	1.8 ± 0.2	2.0 ± 0.2	2.5 ± 0.2	1.54 ± 0.09	0.93 ± 0.04	0.73 ± 0.04
9484	2.1 ± 0.2	1.6 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.4 ± 0.1	1.29 ± 0.05	1.01 ± 0.04	0.72 ± 0.06
9581	2.4 ± 0.1	1.8 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.6 ± 0.1	1.35 ± 0.07	1.03 ± 0.05	0.75 ± 0.04
L. minuta	**2.8 ± 0.7a**	**1.9 ± 0.3b**	**1.3 ± 0.4b**	**1.4 ± 0.4a**	**1.9 ± 0.50a**	**1.41 ± 0.11a**	**0.99 ± 0.04b**	**0.71 ± 0.04b**
8685	4.3 ± 0.2	3.3 ± 0.2	2.6 ± 0.1	2.3 ± 0.1	3.0 ± 0.1	1.29 ± 0.06	1.12 ± 0.05	0.86 ± 0.02
9228	4.1 ± 0.2	3.1 ± 0.2	2.3 ± 0.2	2.0 ± 0.2	2.8 ± 0.2	1.35 ± 0.06	1.16 ± 0.08	0.84 ± 0.02
9401	4.1 ± 0.3	3.0 ± 0.2	2.4 ± 0.2	1.9 ± 0.2	2.9 ± 0.2	1.37 ± 0.06	1.28 ± 0.14	0.82 ± 0.03
9442	3.0 ± 0.2	2.4 ± 0.2	1.6 ± 0.1	1.5 ± 0.1	2.1 ± 0.1	1.24 ± 0.05	1.08 ± 0.07	0.77 ± 0.04
L. valdiviana	**3.9 ± 0.6a**	**2.9 ± 0.4a**	**2.2 ± 0.4a**	**1.9 ± 0.3a**	**2.7 ± 0.44a**	**1.31 ± 0.06a**	**1.16 ± 0.09a**	**0.82 ± 0.04a**
9207	4.0 ± 0.2	3.0 ± 0.2	2.3 ± 0.2	1.9 ± 0.2	2.7 ± 0.1	1.33 ± 0.07	1.20 ± 0.10	0.83 ± 0.04
9208	4.0 ± 0.2	3.0 ± 0.1	2.2 ± 0.1	1.9 ± 0.2	2.7 ± 0.1	1.32 ± 0.05	1.15 ± 0.07	0.83 ± 0.02
9209	3.1 ± 0.2	2.3 ± 0.2	1.8 ± 0.2	1.6 ± 0.1	2.2 ± 0.2	1.32 ± 0.08	1.12 ± 0.06	0.80 ± 0.04
9210	3.4 ± 0.3	2.4 ± 0.2	1.9 ± 0.3	1.7 ± 0.3	2.3 ± 0.3	1.40 ± 0.08	1.08 ± 0.11	0.79 ± 0.03
L. yungensis	**3.6 ± 0.5a**	**2.7 ± 0.4a**	**2.0 ± 0.3a**	**1.8 ± 0.2a**	**2.5 ± 0.3a**	**1.34 ± 0.04a**	**1.14 ± 0.05a**	**0.81 ± 0.02a**

For each clone, 25 adult fronds were measured. Mean values ± standard deviation are given. Distances in mm. Significance was tested using the Holm-Sidak test. Identical letters demonstrate no significant differences between the means of the clones.

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1000 bootstrap replicates. For both tree reconstruction methods, the trees were rooted using the species *Lemna aequinoctialis* Welw. and *L. tenera* Kurz.

To estimate the number of species that can be found on the basis of the concatenated and indel-coded cpDNA sequence data, we used two popular methods for single-locus species delimitation. First, we used the Generalised Mixed Yule Coalescent (GMYC) model (Pons & al., 2006) as implemented in the R package *splits* v.1.0-19/r52 (Ezard & al., 2017). Second, we used the multi-rate Poisson Tree Process (mPTP) method (Kapli & al., 2017), which is accessible via a web service at https://mptp.h-its.org/. GMYC requires as input file an ultrametric tree like the one that was reconstructed by BEAST. The application mPTP is less restrictive and was run with both the tree inferred by BEAST and the tree with the best ML score from RAxML-NG. In both species delimitation calculations, the two outgroup species were discarded.

MALDI-TOF-MS. — Over the last decade, matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) has emerged as a powerful tool for the species-level identification of bacteria (Mellmann & al., 2008), archaea (Krader & Emerson, 2004), yeasts (Marklein & al., 2009) and filamentous fungi (Bader, 2013). The method enables the highly reproducible detection of protein mass patterns (proteomic profiles) that are obtained by analysing whole cells, cell lysates or crude cell extracts. The majority of the signals in the MALDI-TOF mass spectra can be assigned to ribosomal and other highly abundant proteins. These protein patterns have shown to be highly conserved and consistently expressed under a range of growth conditions (Meyer & al., 2017). Our own preliminary tests have shown that species of duckweed can also be successfully differentiated by this method. For proteomic profiling of species of the genus *Lemna*, MALDI-TOF-MS was carried out with the MALDI Biotyper (Bruker Daltonik, Bremen, Germany) using freeze-dried plant material as described by Blättel & al. (2013). MALDI-TOF-MS was performed on a Microflex LT spectrometer using the software flexControl v.3.0 (Bruker Daltonik). Main Spectrum Profiles (MSP) of each of the clones were created from 20 to 24 single mass spectra. To check the quality of the mass spectra the flexAnalysis software v.3.3 (Bruker Daltonik) was used. Mass spectra with anomalies were excluded. To evaluate the similarity of the clones investigated, a score-oriented dendrogram was created using the dendrogram creation standard method of the Biotyper v.3.1 software (Bruker Daltonik) which calculates the distance correlation of the different MSP by the average group linkage algorithm.

Flow cytometric genome size estimation. — Genome size measurements were performed using a CyFlow Space flow cytometer (Sysmex Partec, Görlitz, Germany) according to Doležel & al. (2007). Appropriate amounts of fresh fronds were chopped with a sharp razor blade together with corresponding amounts of young leaf material of *Raphanus sativus* L. convar. *sativus*, accession "Voran" (IPK gene bank

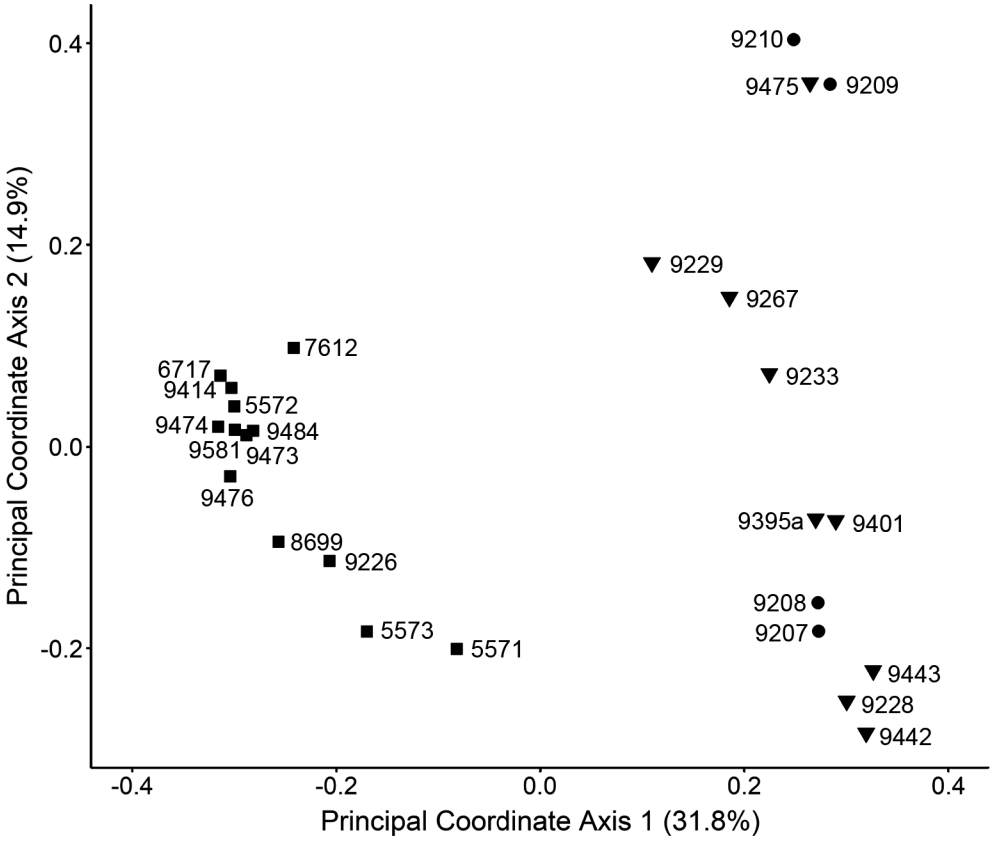


Fig. 2. Principal coordinate analysis of the AFLP data. The proportions of explained variation were given on the axes 1 and 2 in brackets. Square – *L. minuta*, triangle – *L. valdiviana*, circle – *L. yungensis*.

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accession number RA 34; 2C = 1.11 pg; Schmidt-Lebuhn & al., 2010) or *Glycine max* (L.) Merr. convar. *max* var. *max*, accession "Cina 5202" (IPK gene bank accession number SOJA 392; 2C = 2.23 pg; Borchert & al., 2007) as internal reference standard. For nuclei isolation, the DNA staining kit "CyStain PI Absolute P" (Sysmex Partec, Görlitz, Germany) was used. Usually, 10,000 events per sample were analysed, and at least four independent measurements per clone were performed. The absolute DNA contents (pg/2C) were calculated based on the mean values of the G1 peak and the corresponding genome sizes (Mbp/1C) according to Doležel & al. (2003). Data were evaluated statistically using the programme R v.3.3.3 (R Development Core Team, 2017). We first checked for normality using the Shapiro-Wilk test (Shapiro & Wilk, 1965). Then we performed one-way ANOVA accounting for unequal variances as implemented in the package *userfriendlyscience* v.0.7.0 (Peters, 2017), since the integrated Levene test showed heteroscedasticity. Data were further investigated using the pairwise Games-Howell post-hoc test. Subsequently, species that showed no significant differences in genome size were combined and tested against the remaining species. Since this dataset did not meet the assumptions of normality and homoscedasticity, a Mann-Whitney U test was used to check whether the two groups exhibit stochastic equality.

■ RESULTS

Morphometric analyses. — Quantitative morphometric analyses (Table 2) were carried out based on characters used to distinguish species by Landolt (1980, 1998). The parameters used are schematically shown in Fig. 1D. The parameters W and LN as well as the ratios LN : A and LN : N-A were significantly different for *L. minuta* compared to the other two species, *L. valdiviana* and *L. yungensis*. In no case were significant differences observed between *L. valdiviana* and *L. yungensis*. No other parameters revealed any significance between species. Also, the reported differences in the symmetry of the base of the fronds (Landolt, 1980, 1998) were not evident between the species. When the datasets for *L. valdiviana* and *L. yungensis* were combined and tested against that

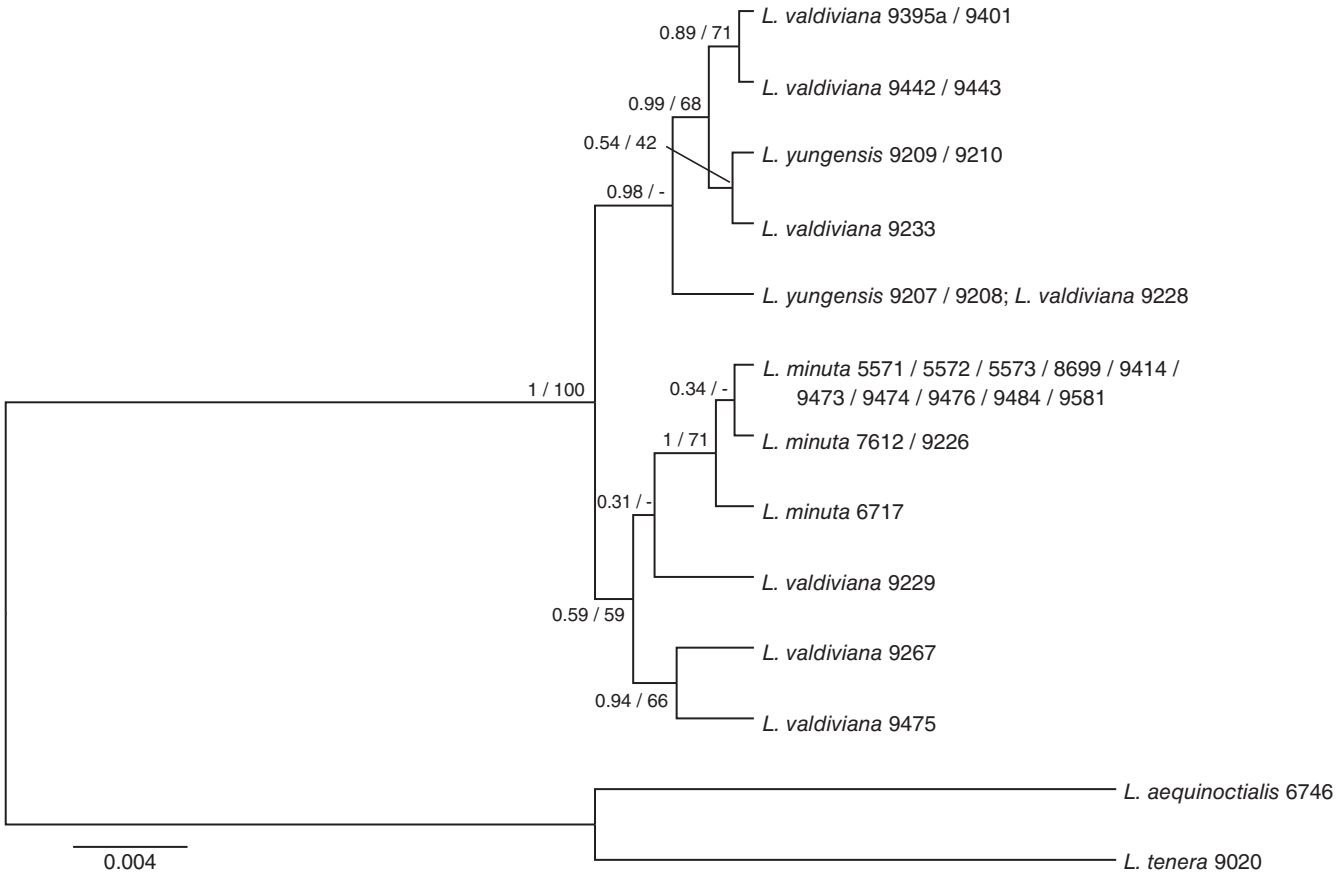


Fig. 3. Maximum clade credibility tree based on the concatenated cpDNA *psbK-psbI* + *atpF-atpH* dataset as inferred by the programme BEAST v.1.8. Numbers on the branches are posterior probability (PP) from the Bayesian inference and bootstrap support (BS) values from the maximum likelihood analysis (PP/BS). A dash means that the node is absent from the respective analysis. *Lemna aequinoctialis* 6746 and *L. tenera* 9020 were used as outgroups.

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of *L. minuta*, differences in L, A and N-A became significant in addition to those mentioned above.

Amplified fragment length polymorphisms. — Clones of *L.* sect. *Uninerves* were analysed by AFLP, and the data were evaluated by PCoA (Fig. 2). Thirteen clones of *L. minuta*, nine clones of *L. valdiviana* and four clones of *L. yungensis* were investigated (Table 1). All clones of *L. minuta* were clearly separated from all clones of *L. valdiviana*/*L. yungensis* by principal coordinate axis 1, which explains 31.8% of the total variation. Clones of *L. valdiviana* and *L. yungensis* were distributed along axis 2 (14.9%), but no clear separation between the two species could be seen.

Barcoding by plastidic sequences. — The concatenated cpDNA sequence alignment of *psbK-psbI* and *atpF-atpH* consisted of 1162 bp and additionally 32 recoded indel sites. All 28 investigated clones, including two outgroup specimens, comprised in total 13 unique haplotypes. Figure 3 shows the maximum clade credibility tree of the Bayesian inference by BEAST. The three *L. minuta* haplotypes formed one cluster with high posterior probability (PP = 1). A second cluster is comprised of a mixture of five *L. valdiviana* and *L. yungensis* haplotypes with a high support value (PP = 0.98). Nevertheless, *L. valdiviana* represents a paraphyletic group, because three haplotypes build a separate cluster with *L. minuta*, though with low support (PP = 0.59). The bootstrap scores (BS) of the ML analysis are low to moderately high (BS < 75), and the tree topology is very similar compared to the Bayesian tree. Running the two species delimitation programmes GMYC and mPTP resulted in one proposed species for either programme. There was also no difference in which tree was used as input data for mPTP. These results suggest that the chloroplast fragments have no discriminatory power for the species under investigation.

MALDI-TOF-MS. — Specific and reproducible reference spectra could be generated for each of the clones with the MALDI Biotyper. The similarity analysis of the MALDI-TOF MSP of the different *Lemna* clones showed clearly two clusters (Fig. 4); one included all selected clones of *L. minuta* (five clones); the other one included all selected clones of *L. valdiviana* (three clones) and *L. yungensis* (four clones) together. The existing substructure of the second cluster did not separate clones of *L. valdiviana* and *L. yungensis* but rather comprised a mixture of the species.

Genome sizes. — We also tested whether the three species under discussion can be distinguished based on their genome sizes since Landolt (1986) mentioned a correlation between progress in evolution (in the sense of reduction of morphological structures) with increasing genome size (cf. Wang & al., 2011). Four clones of each species were tested by flow cytometry (Table 1) to establish genome size. An additional clone of *L. valdiviana* (8685) showed a strikingly higher genome size (559 Mbp/1C) than the other clones of the same species and was therefore not included in statistical testing. Genome sizes were measured as follows: *L. minuta* (mean ± standard deviation) 365 ± 2 Mbp/1C, *L. valdiviana* 382 ± 13 Mbp/1C, and *L. yungensis* 372 ± 2 Mbp/1C. One-way ANOVA revealed significant differences between the tested species ($F = 4.69$, $P = 0.04$), which were further investigated by the Games-Howell post hoc test. Solely for the comparison of *L. minuta* with *L. yungensis* the difference in genome size was significant ($t = 4.04$, $P = 0.016$). No significant differences were observed for the other pair-wise comparisons, i.e., for the other two pairs: *L. minuta* vs. *L. valdiviana* $t = 2.52$, $P = 0.160$ and *L. valdiviana* vs. *L. yungensis* $t = 1.55$, $P = 0.383$. When the values for *L. valdiviana* and *L. yungensis* were combined, and tested against *L. minuta*, a Mann-Whitney U test showed that the values for the combined group were significantly different from those of *L. minuta* ($W = 0$, $P = 0.008$).

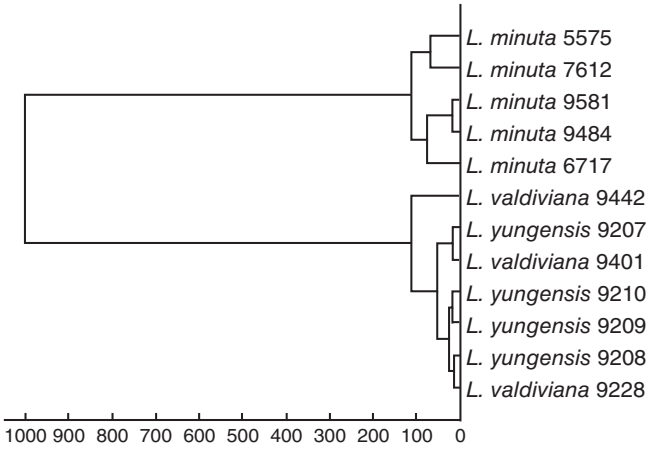


Fig. 4. Dendrogram resulting from a similarity analysis calculating the distance correlation of the *Lemna* clone MALDI-TOF-MSPs by the average group linkage algorithm.

■ DISCUSSION

In a previous paper, Crawford & al. (1996) stressed that it was very difficult to distinguish the sibling species *Lemna minuta* and *L. valdiviana* of *L.* sect. *Uninerves* based on morphological characters. These authors used an allozyme-based approach, which enabled them to distinguish most of the clones of these two species. While it has been difficult to distinguish *L. minuta* and *L. valdiviana*, the more recently discovered *L. yungensis* has even higher similarity to *L. valdiviana* (Landolt, 1998). As Ceschin & al. (2016b) demonstrated the power of quantitative morphometry by distinguishing the invasive species *L. minuta* from the native species *L. minor* in Italy, our investigations of *L.* sect. *Uninerves* were started with quantitative morphometry. Especially the length of the nerve was a useful parameter for our study species. With this method, it was possible to distinguish *L. minuta* from *L. valdiviana* and *L. yungensis*, but no significant differences could be discovered between *L. valdiviana* and *L. yungensis*. Basal symmetry of fronds was mentioned as an important character to distinguish *L. valdiviana* from *L. yungensis* by Landolt (1998), but this could not be proven

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by our observations. Representative fronds from both species showed either more or less symmetric or asymmetric bases.

Subsequently, we applied as many independent molecular methods of taxonomy as possible. AFLP uses DNA restriction fragments of nuclear DNA and, to a lesser degree, of plastids that function as markers (Bänfer & al., 2004; Gemeinholzer & Bachmann, 2005; Bonin & al., 2007). This method has been referred to as a fingerprinting method (Bog & al., 2018). PCoA evaluation demonstrated that all clones of *L. minuta* were separated from all clones of *L. valdiviana* and *L. yungensis*. The combined group of *L. valdiviana* and *L. yungensis* showed some heterogeneity, but a clear separation into defined groups was not possible, which implies the lack of separation into two species.

As a second method, we used DNA-barcoding by PCR amplification and sequencing of the plastidic intergenic spacers *psbK-psbI* and *atpF-atpH*. Although clones of the species *L. minuta* were separated with high probability from those of the two other species, *L. yungensis* could not be separated from *L. valdiviana*, and *L. valdiviana* even appeared as a paraphyletic group. It cannot be clearly stated yet, whether the insufficient sequence differentiation is due to hybridisation events with *L. minuta* as pollen recipient from *L. valdiviana* or incomplete lineage sorting, because of the limited knowledge of the species' reproduction biology. Nevertheless, the investigated plastidic fragments are not suitable for species delimitation. This is in agreement with our experience that for different genera of the family Lemnaceae Martinov (or Araceae Juss. subfam. Lemnoideae Bab.) different plastidic fragments should be used as markers to obtain optimal results (Bog & al., 2013, 2015, 2018). Comparing the results of the plastidic fragments (i.e., a few single nucleotide polymorphisms [SNPs] from two chloroplast loci) to the genotyping-by-sequencing results of Bog & al. (2020: fig. 2; i.e., thousands of SNPs from thousands of loci from the whole genome), there is a clear separation between *L. minuta* and *L. valdiviana/L. yungensis*, but not between the latter two species, supporting the synonymisation of *L. valdiviana* and *L. yungensis*.

So far, the method of MALDI-TOF-MS has not yet been used to characterise the identity of angiosperm species or clones. However, this proteomic method has already been used for fast and reliable identification of a variety of bacteria, moulds, and yeast species (Marklein & al., 2009; Stevenson & al., 2010; Jensen & Arendrup, 2011). Results can be obtained that are not influenced by the age of the culture, the medium used, or the growth conditions (Mellmann & al., 2008; Jensen & Arendrup, 2011). MALDI-TOF-MS turned out to be an easy, fast and reliable tool to characterise and identify plant species. As with the molecular methods, *L. minuta* could be separated from *L. valdiviana* and *L. yungensis*, but no differentiation between the latter two species was obvious. The same was true for differences in genome size. We found significant differences between *L. minuta* and the combined clones of *L. valdiviana* and *L. yungensis*. Yet, these results should be treated with caution as the sample size was rather small, and it was shown by Wang & al. (2011) that the intraspecific variation within *Lemna* can be high. Thus, the genome size might be less useful in the determination of species of *L.* sect. *Uninerves*. We also found evidence that this might be true also for the investigated species, as one clone (cf. clone 8685) had a considerably higher genome size than all the others. The main process of this intraspecific variation is not fully clear yet, but it is assumed that polyploidisation is one important driver and is known to have occurred several times in duckweed evolution (Landolt, 1986; Wang & al, 2011).

■ TAXONOMIC TREATMENT

Taken together, the morphological method as well as the different molecular and proteomic methods demonstrate the clear separation of the species *L. minuta* from the two other species *L. valdiviana* and *L. yungensis*. No indication was available to separate the latter two species from each other with any of the methods used. Therefore, we propose not to treat them as distinct species but to synonymise the name *L. yungensis* with the name *L. valdiviana*. As the description of *L. valdiviana* Phil. is the older (Philippi, 1864) compared to *L. yungensis* Landolt (Landolt, 1998), *L. valdiviana* Phil. should be the correct name for clones defined before as *L. valdiviana* and *L. yungensis*. The number of accepted plant species in the family Lemnaceae (or Araceae subfamily Lemnoideae) is thus reduced to 36 (cf. Sree & al., 2016) by this proposal.

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■ AUTHOR CONTRIBUTIONS

Research conception and design: KJA, KSS, MB; providing of clones: KJA; acquisition of morphometric data and analysis: MB; acquisition and analysis of AFLP and barcoding data: MB, SP, MAKJ; acquisition and analysis of MALDI-TOF-MS data: AR, JK; acquisition and analysis of flow cytometric data: JF, PTNH, MB, IS; drafting of the manuscript: KJA, MB, KSS; final critical revision: all authors. — MB, https://orcid.org/0000-0002-9979-8336; KSS, https://orcid.org/0000-0002-1356-3406; JF, https://orcid.org/0000-0003-4171-5371; PTNH, https://orcid.org/0000-0002-7418-9091; IS, https://orcid.org/0000-0002-6300-2068; SP, https://orcid.org/0000-0003-0057-623X; MAKJ,

https://orcid.org/0000-0003-2014-5859; KJA, https://orcid.org/0000-0002-6385-6645

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