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ORIGINAL ARTICLE

Genetic structure of the genus *Lemna* L. (Lemnaceae) as revealed by amplified fragment length polymorphism

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Abstract Duckweeds (Lemnaceae) are extremely reduced in morphology, which made their taxonomy a challenge for a long time. The amplified fragment length polymorphism (AFLP) marker technique was applied to solve this problem. 84 clones of the genus *Lemna* were investigated representing all 13 accepted *Lemna* species. By neighbour-joining (NJ) analysis, 10 out of these 13 species were clearly recognized: *L. minor*, *L. obscura*, *L. turionifera*, *L. japonica*, *L. disperma*, *L. aequinoctialis*, *L. perpusilla*, *L. trisulca*, *L. tenera*, and *L. minuta*. However, *L. valdiviana* and *L. yungensis* could be distinguished neither by NJ cluster analysis nor by structure analysis. Moreover, the 16 analysed clones of *L. gibba* were assembled into four genetically differentiated groups. Only one of these groups, which includes the standard clones 7107 (G1) and 7741 (G3), represents obviously the "true" *L. gibba*. At least four of the clones investigated, so far considered as *L. gibba* (clones 8655a, 9481, 9436b, and Tra05-L), represent evidently close relatives to *L. turionifera* but do not form turions under any of the conditions tested. Another group of clones (6745, 6751, and 7922) corresponds to putative hybrids and may be identical with *L. parodiana*, a species not accepted until now because of the difficulties of delineation on morphology alone. In conclusion, AFLP analysis offers a solid base for the identification of *Lemna* clones, which is particularly important in view of Lemnaceae application in biomonitoring.

Keywords Amplified fragment length polymorphism · Duckweeds · *Lemna* · Lemnaceae

Abbreviations

AFLP	Amplified fragment length polymorphism
NJ	Neighbour-joining

Introduction

Lemnaceae (duckweeds) represent a family of plants ideal for quantitative analysis in plant sciences. Several species of this family are applied in ecotoxicological investigations (e.g. Jansen et al. 1996; Cayuela et al. 2007). Moreover, biomonitoring is well established following the ISO 20079 protocol (Naumann et al. 2007) using *Lemna minor* L., or within the framework of OECD mainly using *L. gibba* L. (Brain and Solomon 2007). The possible value for phytoremediation under several conditions has been reported (Bergmann et al. 2000; Chaiprapat et al. 2005; Ansari and Khan 2008; Ferdoushi et al. 2008). Lemnaceae have also been used to solve questions in basic research, e.g. in

The contributions of M. Bog and H. Baumbach are considered to be equal.

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photosynthesis (Booij-James et al. 2009), phytohormone biosynthesis (Rapparini et al. 1999) or starch degradation (Reimann et al. 2007). Li et al. (2004) and Yamamoto et al. (2001) reported genetic transformation of some duckweed species, although stable transformation still awaits optimization. The possible application of duckweed for the production of pharmaceuticals is obvious (Vunsh et al. 2007; Rival et al. 2008). The complete sequence of chloroplast DNA in *L. minor* was published by Mardanov et al. in 2008. Currently, the Joint Genome Institute (US Department of Energy) is sequencing the genome of the duckweed *Spirodela polyrhiza* (L.) Schleid. to be used as "a biofuels, bioremediation and carbon cycling crop" (press release 2nd July 2009). The availability of genomic information will stimulate and broaden the range of research with this species, not least because the genome of *S. polyrhiza* and other duckweed species is only slightly larger than that of *Arabidopsis thaliana* (Todd Michael, Rutgers University, NJ; personal communication). Smaller genomes have been found in *Genlisea margaretae*, *G. aurea* and *Utricularia gibba* (all Lentibulariaceae; Greilhuber et al. 2006).

There seems to be no doubt that the Araceae including subfamily Lemnoideae is monophyletic (Rothwell et al. 2004; Cabrera et al. 2008). This does not, however, bias the decision upon the taxonomic rank of the Lemnoideae/Lemnaceae. At the moment we consider this as an open question and use the term Lemnaceae. Within the Lemnaceae, 37 species are recognized and assigned to 5 genera (Landolt 1986; Les and Crawford 1999; Lemon and Posluszny 2000): *Landoltia* Les & D. J. Crawford, *Spirodela* Schleid., *Lemna* L., *Wolffiella* Hegelm., and *Wolffia* Schleid. Les et al. (2002) listed 38 species. *Lemna ecuadoriensis* Landolt had been combined before with *L. obscura* (Landolt 2000; cf. also *L. yungensis* in Landolt, 1998). Following Les et al. (2002), the genus *Lemna* consists of four well-supported sections: *Lemna*, *Alatae*, *Biformes*, and *Uninerves* (Table 1). The section *Hydrophylla* (Dumortier 1827) with *Lemna trisulca* as the only member was confirmed by Landolt (1986) but rejected by Les et al. (2002) and integrated in section *Lemna*.

Duckweeds are extremely reduced in morphology and present a developmental hybrid of leaf and stem origin (Lemon and Posluszny 2000). The extreme reduction in plant stature, miniaturization of organs, and its worldwide distribution, combined with high phenotypic plasticity in response to environmental conditions (Kandeler 1975; Vaughan and Baker 1994), have made taxonomy of Lemnaceae a challenge for scientists over the past 200 years. Even status and systematic position of the family has been disputed since long time ago. Morphological classification was mainly confirmed by biochemical markers (Crawford et al. 1996, 1997, 2001, 2005; Les et al. 1997). Still, even for specialists, the identification of species not to mention clones remains extremely difficult. In consequence, a robust phylogeny and systematic treatment of Lemnaceae require genetic markers. The paper of Les et al. (2002) can be regarded as a landmark since it was the first phylogeny of Lemnaceae based on cp-DNA sequences. However, in the final evaluation of the data, Les et al. (2002) used only one clone per species. On the other hand, physiological experiments revealed considerable intraspecific genetic variation in duckweeds (Appenroth 2003). Jordan et al. (1996) compared the *rpl*16 region but only for nine clones of *L. minor* and one clone of *L. valdiviana*, all of them from North America. Rothwell et al. (2004) investigated the *trn*L–*trn*F intergenic spacer but used only five clones as representatives of the five genera. A larger number of clones were studied by Martirosyan et al. (2008, 2009), representing, however, only six *Lemna* species. Moreover, our preliminary experiments with sequences of the plastidic regions *rpl*16, *trn*L–*trn*F and *trn*S–*trn*G of some clones did not provide sufficient interspecific variation (K Kuehdorf, M Bog and K-J Appenroth, unpublished results). We, therefore, decided to apply the molecular marker technique amplified fragment length polymorphism (AFLP). AFLP is currently the technique of choice when there is no solid knowledge of sequence variation or developed markers for a group of plants (Vos et al. 1995; Lara-Cabrera and Spooner 2004; Meudt and Clarke 2007). AFLPs provide much more resolution than cp-DNA restriction site data and nuclear ribosomal internal transcribed spacer data at the genus level (Despres et al. 2003). One important advantage of this technique is the fact that species rather than gene trees are

Table 1 Classification of accepted sections and species of the genus *Lemna*

Section	Species	No. of clones investigated
Lemna L.	*L. disperma* Hegelm.	2
	L. gibba L.	16
	L. japonica Landolt	3
	L. minor L.	27
	L. obscura (Austin) Daubs	7
	L. turionifera Landolt	5
Hydrophylla Dumortier	*L. trisulca* L.	3
Alatae Hegelm.	*L. aequinoctialis* Welw.	4
	L. perpusilla Torr.	2
Biformes Landolt	*L. tenera* Kurz	3
Uninerves Hegelm.	*L. minuta* Kunth	5
	L. valdiviana Phil.	4
	L. yungensis Landolt	3

The section *Hydrophylla* is not accepted by all authors. The number of clones investigated in the present paper is also given

generated because target loci are distributed all across the genome (Bänfer et al. 2004). AFLP genotyping is often chosen due to the high number of polymorphic markers generated in a single PCR experiment and the high reproducibility (Gemeinholzer and Bachmann 2005; Bonin et al. 2007). Markers obtained by AFLP analysis complement the results of Les et al. (2002) for Lemnaceae since most of them represent polymorphisms in nuclear DNA.

In the present paper, we focused on the genus *Lemna*. In order to define relationships within and among the species of this genus, 84 clones were investigated representing all 13 accepted species (Table 2). One particular interest that we focused on in this study was whether morphological classification is congruent with clusters derived from molecular data. Publications about the four other Lemnaceae genera are in progress.

Materials and methods

Taxon sampling and cultivation of clones

The AFLP analysis of the genus *Lemna* covered 84 clones representing all 13 accepted species (Table 2). All these clones were cultivated under axenic conditions in the following medium: 8 mM KNO_3, 0.06 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM $Ca(NO_3)_2$, 5 µM H_3BO_3, 0.4 µM Na_2MoO_4, 13 µM $MnCl_2$, 25 µM Fe(III)NaEDTA (Appenroth et al. 1996). They were kept in continuous white light (100 µmol m^{-2} s^{-1}) at 25 ± 1°C. In most cases, plants were harvested after 7 days and frozen in liquid nitrogen.

Turion formation

Turions are vegetative bud-like organs of perennation and play an important role in the survival strategy of several duckweed species (Appenroth and Nickel 2010). The capacity to form turions was tested for the clones 94328, 9482 (both *L. gibba* III), 8655a, 9436b and 9481 (*L. gibba* IV). As positive control, *L. turionifera* (9434) was investigated. Four three-frond colonies were inoculated from axenic cultures in 50 ml of the above-described autoclaved nutrient medium and three parallel samples were investigated. The following turion formation inducing factors (Landolt 1986; Dudley 1987; Appenroth 2002; Appenroth and Nickel 2010) were tested: addition of glucose (50 mM), application of short-day conditions (8 h light/16 h darkness), addition of abscisic acid (3 µM) and cultivation at lower temperature (18°C). Light and temperature conditions were the same as during the cultivation as described above. Possible formation of turions was checked 50 days after the start of the experiments using a magnifying glass (5×).

DNA isolation and AFLP analysis

The amount of 500 mg fresh plant material was stored at −20°C until DNA extraction. Total genomic DNA was isolated immediately after grinding in liquid nitrogen using the Qiagen (Hilden, Germany) plasmid mini kit following the protocol of Hellwig et al. (1999). Extracted DNA was quantified photometrically at 260 nm. The complete AFLP procedure can be taken from Vos et al. (1995) but we used IRD-labelled primers for the selective PCR amplification and an automated DNA sequencer (model 4000L; Li-Cor Biosciences, Bad Homburg, Germany) for electrophoretic separation of generated fragments as described in Baumbach and Hellwig (2007). Twenty primer combinations were screened and checked for band polymorphism, band brightness, band/background contrast, number of amplification products and reproducibility of band patterns. Four primer combinations were finally selected for AFLP analysis: (1) *Eco*RI-ATT/*Mse*I-CAC (76 loci, 47–451 bp), (2) *Eco*RI-ATT/*Mse*I-CAT (58 loci, 49–484 bp), (3) *Eco*RI-ATT/*Mse*I-CCA (44 loci, 50–340 bp), (4) *Eco*RI-ATT/*Mse*I-CTA (77 loci, 53–391 bp). They yielded a total of 255 well-amplified and highly reproducible polymorphic bands. Every single clone could be distinguished as separate AFLP phenotype. AFLP patterns were compiled into a 0/1-matrix ("1" for presence, "0" for absence of a band), assuming that bands of equal fragment size are homologous and represent independent loci. This matrix for the investigated 84 clones of the genus *Lemna* is available as Supplementary material of the present paper.

Data analysis

For phenetic analyses in the genus *Lemna*, we used neighbour-joining (NJ) cluster analysis (computed with Treecon; Van de Peer and De Wachter 1994) based on genetic distances (Nei and Li 1979) between clones. To infer the approximate number of genetic clusters present in the data set (K) and to assign individuals to these clusters, we used Structure version 2.2 (Pritchard et al. 2000; Falush et al. 2007). For determining K, we used Pritchard et al.'s (2000) ad hoc method giving the program a range of values for K as priors and determining which K gave the highest estimated log-probability of the data. With the complete data set (13 species), we computed five independent runs for each possible K from 1 to 13 using a burn in of 50,000 followed by 50,000 data collection repetitions. All iterations were run with the admixture model, which assumes that individuals may have mixed ancestry. We also selected to model allele frequencies as correlated. With a reduced data set (*L. aequinoctialis*, *L. disperma*, *L. perpusilla*, *L. tenera*, *L. trisulca* only), we computed five independent runs for each possible K from 1 to 5 with the same parameters.

Table 2 Clones analysed by AFLP

1	6746	*Lemna aequinoctialis*	Plainsburg, Merced Co., CA, USA
2	7339	*Lemna aequinoctialis*	Bubanza, Bujumbura, Burundi
3	7608	*Lemna aequinoctialis*	Heliofila, Espirito-Santo, Brazil
4	9433 (Map06-L)	*Lemna aequinoctialis*	Rio Limpopo, Marracuene, Mozambique
5	7223	*Lemna disperma*	Murray's Dairy, Canberra, Australia
6	7268	*Lemna disperma*	Lewisham, Tasmania, Australia
7	6745	*Lemna gibba*	Jacksonville, Tuolumn Co., CA, USA
8	6751	*Lemna gibba*	Watsonville, Monterey Co., CA, USA
9	7107 (G1)	*Lemna gibba*	Schildow/Berlin, Tegeler Fließ, Germany
10	7705	*Lemna gibba*	Anand, Khaira, Gujarat, India
11	7741 (G3)	*Lemna gibba*	Catania, Sicilia, Italy
12	7922	*Lemna gibba*	Chascomas, Arroyo Vitel, Buenos Aires, Argentina
13	8655a	*Lemna gibba*	Not known
14	9248	*Lemna gibba*	Trento, Alto Adige, Italy
15	9255	*Lemna gibba*	Pukila, Uusimaa, Finland
16	9352	*Lemna gibba*	Marburg, Germany
17	9435 (Alb06-2)	*Lemna gibba*	Saveri, Distr. Lushnja, Albania
18	9436b (Alb06-4)	*Lemna gibba*	Sulzotaj Divjaka, Distr. Lushnja,, Albania
19	9438 (Kas06-L)	*Lemna gibba*	Bohemia, Kaspercki Hory, Czech Republic
20	9481 (Mon05-L)	*Lemna gibba*	Isle Møn, Denmark
21	9482 (Bari06-1)	*Lemna gibba*	Bari, Italy
22	Tra05-L	*Lemna gibba*	Tramin, South Tyrol, Italy
23	6728	*Lemna japonica*	Baltimore, Baltimore Co., MD, USA
24	8695	*Lemna japonica*	Yodo, Kyoto, Japan
25	9250	*Lemna japonica*	Vesijärvi-Lake, Suoth-Häme, Finland
26	6580	*Lemna minor*	Harrington, Bergen Co., NJ, USA
27	7015	*Lemna minor*	Southern Island, Christchurch, New Zealand
28	7018	*Lemna minor*	Erzincan, Anatatolia, Turkey
29	7022	*Lemna minor*	Cordoba, Andalusia, Spain
30	7123	*Lemna minor*	Saskatoon, Saskachewan, Canada
31	7194	*Lemna minor*	Masaka, Uganda
32	7210	*Lemna minor*	Rockeby, Grahamstown, Cape, South Africa
33	7264	*Lemna minor*	Serrae, Macedonia, Greece
34	7283	*Lemna minor*	Ammiq, El Beqaa, Lebanon
35	7295	*Lemna minor*	Wadi Derna, Libya
36	7753	*Lemna minor*	Djinbar-Wans, Hara, Semien, Ethiopia
37	7755	*Lemna minor*	Floriana, Malta
38	7766	*Lemna minor*	Southern Island, Richmond, New Zealand
39	7868	*Lemna minor*	Ballsbridge, Dublin, Ireland
40	8292	*Lemna minor*	Ghassem Abbath, Ramsar, Mazandaran, Iran
41	8389	*Lemna minor*	Albany District, Transvaal, South Africa
42	8623	*Lemna minor*	Aalborg, Ijland, Denmark
43	8625	*Lemna minor*	Honefoss, Oslo, Norway
44	8676	*Lemna minor*	Kashmir, India
45	8744	*Lemna minor*	Lezha, Albania
46	8784	*Lemna minor*	Simrishamn, Sweden
47	9240	*Lemna minor*	Near Glazov, Udmurt Republic, Russia
48	9436 (Alb06-3)	*Lemna minor*	Semeni, Saveri, Distr. Lushnja, Albania
49	9437 (San05-L)	*Lemna minor*	Sankt Josef, South Tyrol, Italy

Table 2 continued

50	9440 (Neu02-L)	*Lemna minor*	Neukirchen/Eisenach, Germany
51	9441 (St)	*Lemna minor*	Marburg, Germany
52	Lot02-L	*Lemna minor*	Lotschen/Jena, Stadtroda, Germany
53	6600	*Lemna minuta*	Fales Hot Springs, Mono Co., CA, USA
54	7724	*Lemna minuta*	Lac Marion, Biarritz, France
55	9260	*Lemna minuta*	Botanical Garden, Catania, Sicily, Italy
56	Bari06-2	*Lemna minuta*	Botanical Garden, Bari, Italy
57	LLJ	*Lemna minuta*	Botanical Garden, Jena, Germany
58	7182	Lemna obscura	Unknown
59	7325	*Lemna obscura*	Pearl City, Oahu, HI, USA
60	7856	*Lemna obscura*	L.S.U., East Baton Rouge, Par., LA, USA
61	7982	*Lemna obscura*	Nauwoo, Hancock Co., IL, USA
62	8896	*Lemna obscura*	Guayaquil, Ecuador
63	9235	*Lemna obscura*	Duran-Grl. J. Montera, Guayas, Ecuador
64	9342	*Lemna obscura*	Lake Maracaibo, Venezuela
65	8612	*Lemna perpusilla*	Princetown, Mercer Co., NJ, USA
66	P146	*Lemna perpusilla*	Chapel Hill, Orange County, NC, USA
67	9020	*Lemna tenera*	Condorl Water Hole, North Territory, Australia
68	9024	*Lemna tenera*	Nancar Billabong, North Territory, Australia
69	9243	*Lemna tenera*	Kien Giang, U Minh, Vietnam
70	7192	*Lemna trisulca*	Kabali, Kigezi, Uganda
71	Bari06-3	*Lemna trisulca*	Botanical Garden, Bari, Italy
72	Jena69-L (TJ)	*Lemna trisulca*	Jena, Germany
73	6573	*Lemna turionifera*	Highway 2, Lincoln Co., MT, USA
74	7683	*Lemna turionifera*	Sosa, Kyonggi Prov., Korea
75	9109	*Lemna turionifera*	Topilo, Bialowieza, Poland
76	9434 (She06-L)	*Lemna turionifera*	Near river Olkha, Shelekhov, Russia
77	Lab08-L	*Lemna turionifera*	Unknown
78	7005	*Lemna valdiviana*	Crawfordville, Wakulla Co., FL, USA
79	7356	*Lemna valdiviana*	Charlesburg, Paramaribo, Suriname
80	9206	*Lemna valdiviana*	El Tacal, Cundinamarca, Columbia
81	9233	*Lemna valdiviana*	La Y de la Laguna, Esmerelda, Ecuador
82	9207	*Lemna yungensis*	NNE of Sacramento, La Paz, Bolivia
83	9208	*Lemna yungensis*	NNE of Sacramento, La Paz, Bolivia
84	9210	*Lemna yungensis*	Velo de la Novia, La Paz, Bolivia

Terms in parentheses are additionally those used before

Results

NJ cluster analysis

NJ cluster analysis clearly recognized 10 out of the 13 *Lemna* species. With few exceptions, the 84 investigated clones were clustered according to their species affiliation (Fig. 1).

All 27 *L. minor* clones grouped within one main cluster quite distant from the other clones. Within the second main cluster, the species *L. obscura*, *L. turionifera*, *L. japonica*, *L. disperma*, *L. trisulca*, *L. tenera*, *L. aequinoctialis*, *L. perpusilla* and *L. minuta* are clearly identified with high bootstrap support (95–100%). The clones of *L. obscura* are separated from all other clones of this second main cluster.

The species *L. valdiviana*, *L. yungensis* and *L. gibba* cannot be clearly recognized. It was not possible to distinguish between the species *L. valdiviana* and *L. yungensis*. Although the clones of both species appear within a well-supported cluster (88% bootstrap) together with *L. minuta*, only the clones of the latter species are clearly separated whereas the *L. valdiviana* and *L. yungensis* clones do not cluster according to their species affiliation. The branch lengths indicate high dissimilarity among the clones of *L. valdiviana* and *L. yungensis*. The clones of *L. gibba* can be found in two large clusters divided into two subclusters each. Operationally, we therefore defined four groups of *L. gibba* (I–IV, Table 3). *L. gibba* I and II are well supported. *L. gibba* II contains the standard clones 7107 (known as G1) and 7741 (known as G3). The second main cluster is

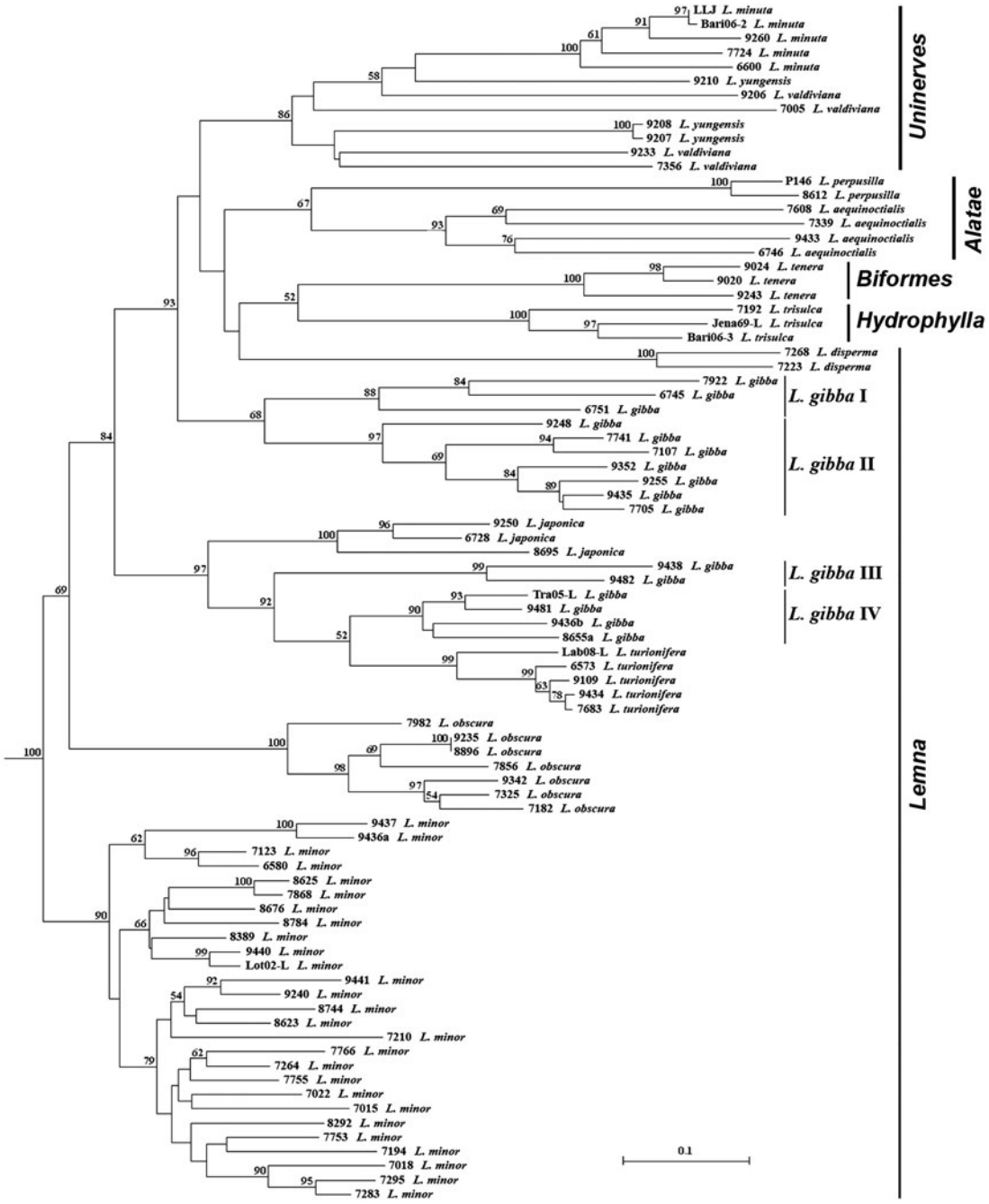

Fig. 1 Dendrogram resulting from a neighbour-joining analysis based on genetic distances (Nei and Li 1979) of the clones of the genus *Lemna*. Bootstrap values (1,000 replicates, computed with Treecon) are given above the branches

split into a subcluster of *L. gibba* III on the one hand and a subcluster of *L. gibba* IV and *L. turionifera* on the other hand. Both subclusters cluster together with *L. japonica*. In order to exclude the possibility that *L. gibba* III and IV contain misidentified *L. turionifera* clones, we tested the clones 8655a, 9436b, 9438, 9481 and 9482 from these two groups, together with *L. turionifera* 9434 as a positive control, for the ability to form turions (Table 4). Possible turion formation was studied by addition of glucose and/or the phytohormone abscisic acid, by decreasing the temperature (18°C) and cultivation under short-day conditions for 50 days. In no case turions were formed (Table 4).

Structure analysis

Using genetic similarity as evaluated by AFLP analysis, all investigated clones of the genus *Lemna* were grouped using structure analysis. The maximum ln $P(D)$ value was

Table 3 Operational assignment of clones to four groups of *Lemna gibba*

L. gibba group	Clones assigned
I	6745, 6751, 7922
II	7107, 7741, 7705, 9248, 9255, 9352, 9435
III	9438, 9482
IV	8655a, 9436b, 9481, Tra05-L

Table 4 Test of different turion-inducing factors on the turion formation capacity of clones of two groups of *Lemna gibba*

Conditions	Clone 9434 (positive control)	Clones 8655a, 9436b, 9438, 9481, 9482
cW, N	0.05 ± 0.01	0
cW, Z	0.86 ± 0.04	0
SD, N	0.21 ± 0.01	0
SD, Z	1.41 ± 0.07	0
cW, N, ABA	0.98 ± 0.05	0
cW, N, 18°C	n.d.	0

The following clones were investigated 9438, 9482 (group *L. gibba* III), 8655a, 9436b, 9481 (group *L. gibba* IV), and 9434 (*L. turionifera*; used as positive control)

cW continuous white light (24 h per day), *SD* short day (8 h light, 16 h darkness), *N* Lemnaceae nutrient medium without sugar, *Z* 50 mM glucose added to medium N, *ABA* abscisic acid added to medium N, *18°C* 18°C used instead of 25°C as in all other parts of these experiments, *n.d.* not determined

obtained at $K = 6$ forcing the species into six groups of similar clones (Fig. 2). The following species were safely separated: *L. minor*, *L. obscura*, and *L. minuta*. When considering the complete data set, the five species *L. aequinoctialis*, *L. disperma*, *L. perpusilla*, *L. tenera*, and *L. trisulca* were not separated by structure analysis. Nevertheless, a subsequent structure analysis of the reduced data set which comprised only these species clearly revealed five species clusters (highest ln $P(D)$ at $K = 5$; Fig. 3).

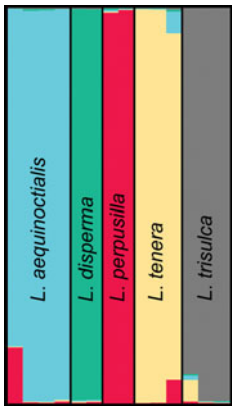

Fig. 3 Assignment test results of the reduced data set: percentage assignment of each clone of *L. aequinoctialis*, *L. disperma*, *L. perpusilla*, *L. tenera*, and *L. trisulca* (represented by *vertical bars*) to each of the $K = 5$ genetic clusters (represented by *different colours*) inferred by the program Structure (Pritchard et al. 2000)

Clones of *L. gibba* do not cluster together but are clearly grouped into four clusters (Fig. 2). Most strikingly, at least four of the clones of *L. gibba* (*L. gibba* IV) are closely connected to the *L. turionifera* cluster and cannot be distinguished from *L. turionifera* by this method. *L. gibba* III, consisting of only two clones, has an approximately 50% admixture from the not further resolved cluster of *L. aequinoctialis*, *L. disperma*, *L. perpusilla*, *L. tenera* and *L. trisulca*. Also with structure analysis, *L. gibba* II (the "true" *L. gibba* cluster) formed one separated group, including the standard clones 7107 (G1) and 7741 (G3). *Lemna gibba* I on the one hand resemble *L. gibba* II but on the other hand also have affinities to one of the five species *L. aequinoctialis*, *L. disperma*, *L. perpusilla*, *L. tenera*, and *L. trisulca*.

The genetic structure of *L. valdiviana* is obviously complex. Moreover, *L. valdiviana* as well as *L. yungensis* are remarkably similar to *L. minuta*.

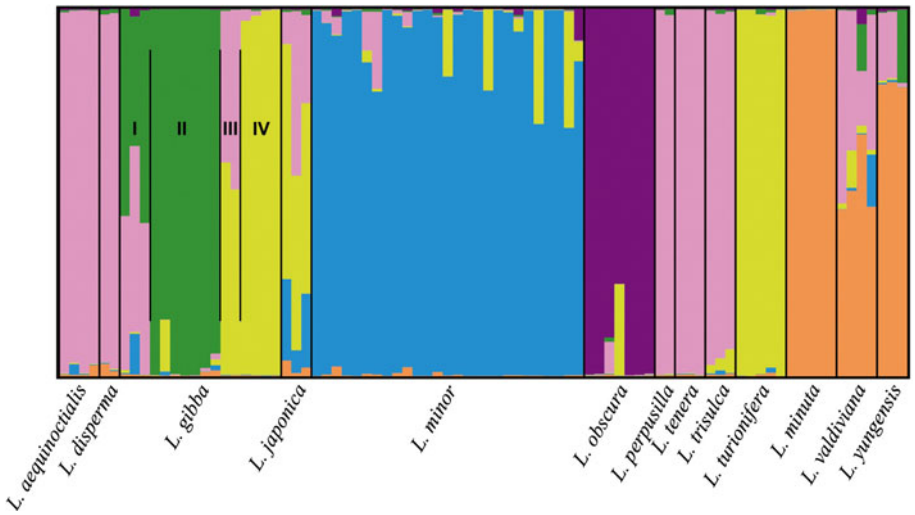

Fig. 2 Assignment test results of the complete data set: percentage assignment of each *Lemna* clone (represented by *vertical bars*) to each of the $K = 6$ genetic clusters (represented by *different colours*) inferred by the program Structure (Pritchard et al. 2000). The four different groups of *L. gibba* are marked

Discussion

In the present paper, AFLP was used to analyse the genetic structure of the genus *Lemna* (Lemnaceae, duckweed family) including a large number of clones representing all described species for the first time. This work would not have been possible without the experience of collecting and cultivating duckweeds for over more than 50 years by one of the authors (E.L.) which resulted in his classifications of the family on the basis of morphological and biochemical markers (Landolt 1986).

The available data enabled us to check whether recognition of four sections within the genus *Lemna* can be confirmed (cf. Table 1). The largest section, *Lemna*, does not form a cluster and cannot be separated from the other sections. Within section *Lemna*, there are six rather well-supported groups formed by (1) *L. minor*, (2) *L. obscura*, a group (3) of *L. turionifera*, *L. gibba* IV and *L. gibba* III, and *L. japonica*, (4) *L. gibba* I, (5) *L. gibba* II, and (6) *L. disperma*. Section *Alatae* which comprises the well-supported species *L. aequinoctialis* and *L. perpusilla* is separated but with low bootstrap support. Likewise, the monotypic section *Biformes* (*L. tenera*) and section *Hydrophylla* (*L. trisulca*; cf. below for discussion) are supported by our data. The remaining well-supported group *Uninerves* includes *L. yungensis*, *L. valdiviana* and *L. minuta*. This section was also well separated by sequence analysis of four plastidic DNA regions (Les et al. 2002) but this previous analysis was based on only one clone per species.

In summary, the three known sections *Alatae*, *Biformes* and *Uninerves* (Crawford et al. 2001) can be recognized in the AFLP-based dendrogram albeit with low bootstrap support in the case of *Alatae*. These three sections have several characteristics in common which separate them from *Lemna* and *Hydrophylla*: orthotropous ovules, no anthocyanin, only 1–2 layers of aerenchymatic cells, roots not longer than 3 cm and an open spathe. Intermediate forms are not known.

L. trisulca is considered to be a member of the section *Lemna* by some authors (Les et al. 2002), whereas others accommodate this species in its own section, i.e. *Hydrophylla* (Landolt 1986). The sections *Lemna* and *Hydrophylla* consist of a large group of species that can be separated from all other species of the genus on a morphological basis (Crawford et al. 2001). This group is characterized by anatropous or amphitropous ovules, the presence of anthocyanin, at least three layers of aerenchymatic cells (except in *L. trisulca*), longest roots between 3 and 15 cm and a closed spathe. *L. trisulca* (Hydrophylla), however, has less than three layers of aerenchymatic cells, often only one leaf nerve, dentate rims at the tip of the frond and often more than 1 cm long stipe-like, green connecting elements at the leaf basis. Thus, morphological markers suggest to separate *L. trisulca* from section *Lemna*. This is also in accordance with the results of Martirosyan et al. (2008) using random amplification of polymorphic DNA (RAPD) markers. These authors investigated 16 different clones of *L. trisulca*. Therefore, we suggest to accept the section *Hydrophylla* to accommodate *L. trisulca*—in accordance with previous results (Landolt 1986, p. 425). With respect to remaining section *Lemna*, our data give no clear clue. There is no justification to unite the six supported clusters to one single section while at the same time the other sections are kept apart (for details, see discussion below).

Using NJ cluster analysis at the level of species, there is a clear separation for 10 of the 13 known species. Separation of *L. perpusilla* from *L. aequinoctialis* by Kandeler and Hügel (1974) (at that time known as *L. paucicostata* Hegelm.) was supported in the present analysis.

There remain, however, some problems. First of all, the species of section *Uninerves* are not completely separated from each other. *L. minuta* appears to be a clear cut taxon well apart from the two other species (*L. valdiviana* and *L. yungensis*) within the section. There is, however, a major caveat here, since with the exceptions of clone 6600 (California) and clone 7724 (France), all other clones of *L. minuta* are collected from Botanical Gardens in Europe. They may come from rather similar populations (e.g. propagated and distributed from 7724 because this clone comes from the first *L. minuta* population discovered in Europe) and thus are genetically more similar than clones collected in different places. Crawford et al. (1996) distinguished *L. valdiviana* and *L. yungensis* by their allozyme patterns. Morphologically, they are almost indistinguishable (Les et al. 1997). The present analysis shows that they are genetically not sufficiently differentiated to prevent that clones from both species are mixed up in one cluster. Whether both species should be kept apart must be further tested by a larger number of clones and/or by including sequence data.

The most serious but highly interesting problem is posed by *L. gibba*. All our data analyses demonstrate *unisono* that the clones of this species may be assigned to four groups (cf. Table 3). Structure analysis provided further insight. *L. gibba* I may represent a hybrid between *L. gibba* II and another species (i.e. *L. aequinoctialis*, *L. disperma*, *L. perpusilla*, *L. tenera* or *L. trisulca*). The best candidate for the formation of this hybrid is *L. disperma* because both species show relatively high flowering frequency and occupy overlapping areas of distribution (Landolt 1986). Moreover, both species share special morphological features (i.e. smaller size, red spots near the tip of the upper surface and only 1–2 seeds per fruit) as described by Giardelli (1937). These clones were formally described as *L. parodiana* but the taxon was rejected because no clear morphological characterization was possible (Landolt 1986, p. 475). The relationship between *L. gibba* and *L. disperma* was investigated

by Crawford et al. (2005) on the basis of allozyme variation using 26 clones of *L. gibba* (including two clones suggested here to be *L. parodiana*) and 12 clones of *L. disperma*. The authors distinguished between the two species but did not find reasons for recognizing *L. parodiana*. They concluded that *L. disperma* originated via dispersal of *L. gibba* or of a common ancestor of the two species. Whether the intermediate position of *L. parodiana* is due to hybridism or incomplete genetic separation of two related species remains an open question at this point. Assuming that the hybrid theory is correct, *Lemna parodiana* should be accepted as a nothospecies. *L. gibba* II is well supported in NJ and structure analyses. Since this group includes the best known clones of *L. gibba* 7107 and 7741 (G1 and G3, compare Tab. 2), we suggest to name it the "true *L. gibba*". However, formal taxonomic revision is beyond the scope of this study paper. The remaining two groups of *L. gibba* III and IV clustered together with *L. turionifera* and *L. japonica* but were well separated in subclusters. *L. gibba* III has certain affinities to one or several of the five species (*L. aequinoctialis*, *disperma*, *perpusilla*, *tenera*, *trisulca*) mentioned above. The remaining four clones (*L. gibba* IV) are indistinguishable from *L. turionifera* using structure analysis but were clearly differentiated by cluster analysis. Moreover, in no case, turion formation was observed as should be expected if these clones of *L. gibba* III and IV were in reality *L. turionifera*. There is, however, no doubt that *L. gibba* IV is different from the other groups of *L. gibba*.

Several questions concerning the systematics of Lemnaceae that remained unanswered for decades can now be answered on the basis of the molecular analysis. As an important example, delimitation between *L. minor* and *L. gibba* is an extremely difficult problem using only morphological data (De Lange 1975; Kandeler 1975; Landolt 1975), since the important character "gibbosity" depends on external factors (Vaughan and Baker 1994). One other reason is the heterogeneity of the species *L. gibba*. As already mentioned by Kandeler (1975) who cited Hegelmaier (1868), "fronds of *L. gibba* can vary in a very pronounce manner and that there exist flat forms of *L. gibba* of great similarity with *L. minor*". The distinction, however, is very important because *L. minor* is used for biotests on the basis of the ISO 20079 protocol (Naumann et al. 2007) whereas in the OECD-based investigations often *L. gibba* is preferred (Brain and Solomon 2007). Using AFLP markers, the identification of *L. minor*, however, represents not a problem at all. This was not possible in the RAPD analysis of many clones of *L. minor* and *L. gibba* by Martirosyan et al. (2008). Another example is the existence of a species described as *Lemna symmeter* by Giuga (1973) which was supposed by Kandeler (1975) to be a hybrid between *L. minor* and *L. gibba*. We did not find any indications for the existence of such hybrids. Problems also existed to distinguish *L. disperma* and *L. obscura*. Kandeler (1975) pointed out that considerable investigation is warranted for the detection of suitable characters. Application of AFLP markers allows for secure identification of both species.

Crawford et al. (2001) investigated the species *L. aequinoctialis*, *L. perpusilla* and *L. tenera*, because there existed difficulties in delimitations, too. Our results support the conclusion that all three species are well separated; especially that *L. aequinoctialis* and *L. perpusilla* (section *Alatae*) are well separated from *L. tenera* (section *Biformes*). In agreement with their conclusions, we do not find any indication for the hypothesis that *L. perpusilla* might be a hybrid of *L. aequinoctialis* and *L. turionifera*.

In conclusion, our AFLP analysis results offer a solid basis to aid in future identification of *Lemna* clones through established molecular markers, which is highly desirable for unambiguous selection of duckweed clones to be deployed in biomonitoring applications. Further exploration of molecular data may improve species delimitations. This requires extended sampling of clones, especially in *L. gibba* and the critical section *Uninerves*. Special attention should be paid to *L. disperma* which might also help to understand the evolution of *L. gibba*.

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