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Analysis of Chloroplast *rpS16* Intron Sequences in Lemnaceae

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Abstract—Chloroplast *rpS16* gene intron sequences were determined and characterized for twenty-five Lemnaceae accessions representing nine duckweed species. For each Lemnaceae species nucleotide substitutions and for *Lemna minor, Lemna aequinoctialis, Wolffia arrhiza* different indels were detected. Most of indels were found for *Wolffia arrhiza* and *Lemna aequinoctialis*. The analyses of intraspecific polymorphism resulted in identification of several gaplotypes in *Lemna gibba* and *Lemna trisulca*. Lemnaceae phylogenetic relationship based on *rpS16* intron variability data has revealed significant differences between *Lemna aequinoctialis* and other *Lemna* species. Genetic distance values corroborated competence of *Landoltia punctata* separations from *Spirodela* into an independent generic taxon. The acceptability of *rpS16* intron sequences for phylogenetic studies in Lemnaceae was shown.

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Key words: duckweeds, plastid DNA, *rpS16*, polymorphism, phylogeny, haplotype.

The Lemnaceae includes the smallest aquatic flowering plants. The representatives of the family are characterized by extreme reduction of vegetative part to a single leaflike organ (0.3 to 12 mm), referred to as a frond. Miniaturization of organs and cosmopolitan distribution contribute to difficulties in Lemnaceae systematics and taxa delimitation. The fact that *S. punctata* has recently been segregated from the genus *Spirodela* to the separate genus *Landoltia* according to morphological features, allozyme data, and variability of the *rbcL* gene illustrates difficulties in systematics and uncertain taxonomy of Lemnaceae [1]. In addition to the problematic species and genera delimitation Lemnaceae phylogeny is still unclear. Thus, Lemnaceae belong to the most intricate flowering plant families [2].

Molecular methods, including DNA sequence analysis, have wide application in solution of controversial phylogenetic and taxonomic problems. Plant molecular systematics studies often refer to polymorphisms in organelles DNA sequence and in the first place chloroplast DNA (cpDNA) [3-5]. The range of cpDNA polymorphism application is comparable only with that of internal transcribed spacers (ITSs) of the ribosome operon in the nuclear genome. Earlier the nucleotide variability of four chloroplast genome regions (*matK*, *rbcL*, *trnK*, *rpl16* coding and intron sequences) in Lemnaceae has been determined [1, 6]. However, because of ambiguous results additional studies are still need including intraspecies diversity analyses.

For the first time the gene for the small ribosomal subunit S16 protein (*rps16*), containing a group II intron has been used by Oxelman et al. [7] and then frequently has been applied to molecular phylogeny studies of various plant taxa. The aim of this work is to evaluate *rps16* intron variability for clarification of Lemnaceae phylogenetic relationship at different taxonomic levels.

EXPERIMENTAL

The 25 accessions of nine Lemnaceae species belonging to two subfamilies: Lemnoideae (*Spirodela, Landoltia*, and *Lemna*) and Wolffioideae (*Wolffia*) from different ecogeographic zones of Russian Federation have been taken into the analysis. *L. gibba*, geographic range of which in Russia is rather narrow, has been supplemented by accessions from Uzbekistan. As the outgroup taxa accessions of three genera of the related Araceae: *Pistia* (*P. stratiotes*), *Monstera* (*M. deliciosa*), *Anthurium* (*A. andreanum*) have been chosen.

Each accession was obtained by vegetative propagation of a single maternal plant, chosen in accordance with morphological traits of the corresponding genus or species. Total genomic DNA was isolated by standard protocol [8]. Polymerase chain reaction was carried out with primers proposed by Shaw et al. [9].

The reaction was performed in a Gene-Amp PCR System 2700 thermocycler (Applied Biosystems, United States). The PCR program was as follows:

Table 1. Lemnaceae and Araceae accessions analyzed in this study

Species accession (number of population.number of clone)	Acc. no. in GenBank	Sampling locality	
Lemna minor 1.1	EU568894	Moscow	55°50′N, 37°36′E
Lemna minor 6.1	EU568895	Lake Svetloe, Chuvashia	56°15′N, 47°00′E
Lemna minor 10.2	EU568896	Cheboksary, Chuvashia	55°30′N, 47°28′E
Lemna minor 2.3g	EU568892	Sergiev Posad	56°18′N, 38°08′E
Lemna minor 6.1g	EU568893	Volgograd	48°42′N, 44°30′E
Lemna turionifera 6.3	EU568889	Kozmodemyansk, Mari El Republic	52°20′N, 46°33′E
Lemna turionifera 7.3	EU568891	Blagoveshchensk	50°16′N, 127°32′E
Lemna turionifera 8.1	EU568890	Kosh Agach Village, Altai Republic	50°00′N, 88°41′E
Lemna japonica 1.3	EU568887	Tolstovka Village, Amur Region	50°12′N, 127°55′E
Lemna japonica 2.5	EU568888	Volkovo Village, Amur Region	50°15′N, 127°46′E
Lemna trisulca 4.1	EU568882	Moscow	55°50′N, 37°36′E
Lemna trisulca 7.1	EU568885	Vladimir	56°08′N, 40°23′E
Lemna trisulca 9.1	EU568886	Volkovo Village, Amur Region	50°15′N,127°46′E
Lemna trisulca 11.1	EU568884	Lake Balankul, Khakassia	53°42′N, 91°22′E
Lemna trisulca 14.1	EU568883	Voronezh	51°38′N, 39°11′E
Lemna aequinoctialis 5.6	EU568897	St.-Petersburg, Botanical Garden	–
Lemna gibba 1.0	EU568877	Samarkand, Uzbekistan	39°39′N, 66°57′E
Lemna gibba 2.0	EU568878	Tailak, Uzbekistan	39°34′N, 67°11′E
Lemna gibba 3.0	EU568879	Tailak, Uzbekistan	39°34′N, 67°11′E
Lemna gibba 4.0	EU568880	Ivanovo	57°00′N, 41°00′E
Lemna gibba 5.0	EU568881	Taman	45°12′N, 36°43′E
Spirodela polyrhiza 11.6	EU568898	Volkovo Village, Amur Region	50°16′N, 127°32′E
Spirodela polyrhiza 14.9	EU568899	Blagoveshchensk	50°16′N, 127°32′E
Wolffia arrhiza 1.0	EU568901	St.-Petersburg, Botanical Garden	–
Landoltia punctata 2.1	EU568900	St.-Petersburg, Botanical Garden	–
Pistia stratiotes 1.0	EU568902	Moscow	55°50′N, 37°36′E
Monstera deliciosa 1.0	EU568903	Moscow	55°50′N, 37°36′E
Anthurium andreanum 1.0	EU568904	Moscow	55°50′N, 37°36′E

denaturation at 94°C for 4 min followed by 30 cycles: denaturation at 94°C for 30 s, annealing at 53°C for 45 s, and elongation at 72°C for 1 min. Postextension was carried out at 72°C for 7 min. PCR products were purified with a GFX™ PCR purification kit (Amersham, United States) and sequenced with a Big-Dye kit (Applied Biosystems in an ABI 310 capillary DNA Analyzer (Bioengineering Center, Russian Academy of Sciences) using both forward and reverse primers.

The *rpS16* intron sequences have been aligned and analyzed by MEGA 3.0 software [10]. Cladistic reconstruction have been performed by MEGA3.0 using the Neighbor-Joining (NJ, Kimura two-parameter model) [11, 12] and the maximum parsimony (MP) methods. For MP tree search the Close-Neighbor-Interchanges (CNI) algorithm have been used. Bootstrap values were calculated from 1000 replicates. Principal components analysis (PCA) have been performed by STATISTICA 6.0 software based on pairwise distance matrices calculated using MEGA3.0. The sequences have been deposited in GenBank NCBI database under the accession numbers EU568894-EU568904 (Table 1).

RESULTS

PCR amplification with primers proposed by Shaw et al. [9] yielded a fragments of predicted length (about 800 bp) in all samples from nine species of four Lemnaceae genera: *Lemna, Spirodela, Landoltia*, and *Wolffia*.

The *rpS16* intron amplicons vary in length from 834 bp in *S. polyrhiza* to 884 bp in *W. arrhiza*. The total length of the aligned Lemnaceae sequences is

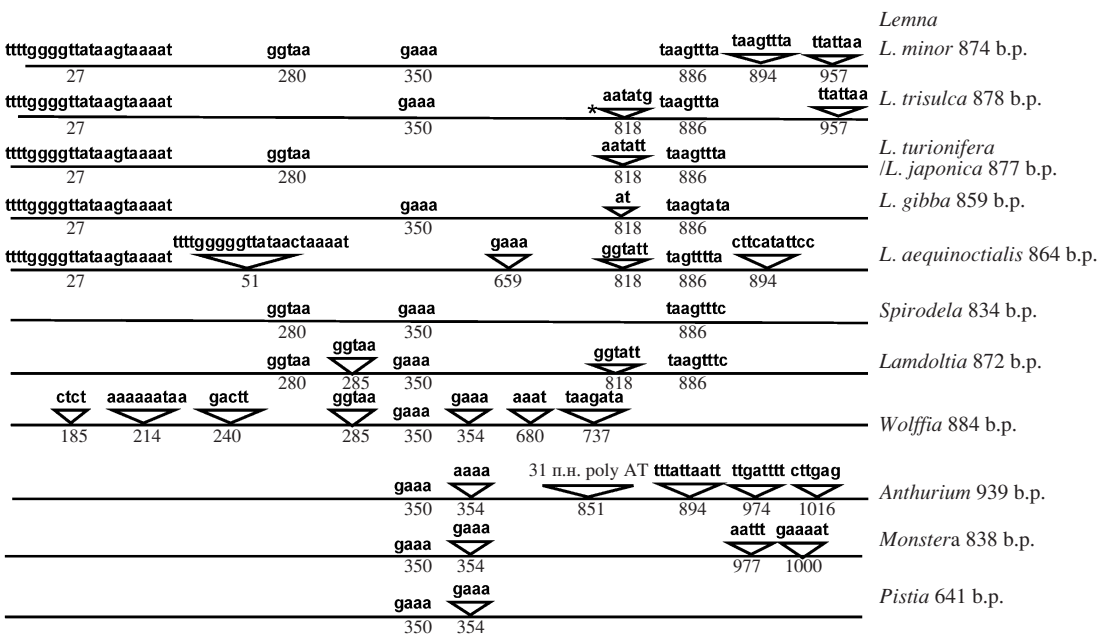

Fig. 1. Analysis of the intron sequence in the *rpS16* gene. Triangles indicate inserts, and the asterisk indicates the sequence duplicated by inserts.

1050 bp. It should be noted that in Araceae the intron length varies in a broader range, from 641 bp in *Pistia stratiotes* to 939 bp in *Anthurium andreanum*.

Analysis of the amplified Lemnaceae cpDNA sequences indicates that they are AT-rich (A, 37.1%, and T, 35.5%). These data are in agreement with nucleotide compositions of the *rpS16* intron in other plant taxa [13, 14]. The set of analyzed sequences contains 413 variable nucleotide sites (39.3% of the whole alignment), and 205 nucleotide sites (19.5%) are parsimony informative.

It was shown that different Lemnaceae genera possess specific sets of indels and substitutions. The majority of indels (seven) was found in *W. arrhiza*. Five of them are unique, being absent in other Lemnaceae sequences (Fig. 1).

Lemna species are characterized by the presence of relatively few species-specific indels. The *rpS16* intron in five out of six *Lemna* species (*L. turionifera, L. japonica, L. trisulca, L. gibba* and *L. minor*), differ mainly in having single nucleotide substitutions (Fig. 1), whereas the *L. aequinoctialis* intron contains, in addition to single nucleotide substitutions, a relatively great number of indels, both species-specific and synapomorphic to other Lemnaceae genera (Fig. 1).

For example, the *L. aequinoctialis* intron contains a 6-bp GG(AA)TATG insertion (818–823), that is also present at the same sequence position in several other *Lemna* species (*L. turionifera, L. japonica,* and *L. trisulca*) and in a specie of another genus: *Landoltia punctata*. In *L. gibba* this insertion is reduced to two nucleotides (AT) and is absent in *L. minor* sequences.

The *rpS16* intron sequence of the *L. minor* contains two insertions, one of which has a species-specific duplication (894–901 bp). Another is also present in *L. trisulca* accessions (Fig. 1). In addition, the *L. minor* intron has two deletions (225–230 and 800–806 bp). In intron sequences of *L. gibba, L. trisulca, L. turionifera*, or *L. japonica* no species-specific indels was found and only specific nucleotide substitutions were detected.

As mentioned above, the analyzed accessions of three Araceae genera differ significantly in the intron length due to numerous deletions in the *Pistia stratiotes* intron. In contrast, the *Anthurium* intron contains specific inserts, one of them run up 31 bp (Fig. 1).

Analysis of the intraspecific variability of the *rpS16* intron reveals haplotypes in some Lemnaceae species. Three haplotypes were revealed in *L. gibba*. The main haplotype (A_{537}, T_{558}, A_{566}, C_{585}, A_{619}, T_{659}, T_{994}) was detected in *L. gibba* 1.0, *L. gibba* 3.0, and *L. gibba* 4.0 accessions. The haplotype of *L. gibba* 5.0 differs at all seven positions (C_{537}, G_{558}, G_{566}, T_{585}, T_{619}, C_{659}, C_{994}), and the haplotype of *L. gibba* 2.0, at one position (C_{659}).

Two haplotypes were detected in *L. trisulca*: main (A_{688}) and the haplotype specific for *L. trisulca* 9.1 accession (C_{688}).

Based on the *rpS16* intron sequences variability intergeneric and interspeci?c genetic distances (Table 2) have been evaluated and phylogenetic trees (Fig. 2) and PCA plot obtained. (Fig. 3).

For most *Lemna* species, interspecific genetic distances (GD) vary within 0.02–0.04. Unexpectedly, GD values between *L. aequinoctialis* and other *Lemna* species reach 0.09–0.11, that is comparable to intergeneric GD values. For example GD values for the following genera taxa: *Landoltia* vs. *Lemna* (0.06), *Spirodela* vs. *Landoltia* (0.11), and *Landoltia* vs. *Monstera* (0.12) (Table 2).

NJ and MP tree topologies are generally similar, being supported by high bootstrap values. (Fig. 2).

The genus *Lemna* forms a monophyletic and well-supported group (92% bootstrap) both on NJ and MP trees, with *L. aequinoctialis* at the basal position. Of all Lemnaceae, species *Spirodela* occupies the basal positions on the trees, forming a sister clade to *Landoltia*, *Wolffia*, and *Lemna*.

The branch position of the recently recognized genus *Landoltia* is unstable. It forms one cluster with *Wolffia* on the MP tree (100% bootstrap) but falls in the cluster with *Lemna* species on the NJ tree (52% bootstrap). The genetic similarity between *Landoltia* and *Lemna* is also confirmed by PCA. Two-dimensional PCA describes 86% of cumulative variance in Lemnaceae (Fig. 3).

Table 2. Intergeneric and interspecific distances in Lemnaceae and Araceae

Intergeneric genetic distances	
Spirodela–Landoltia	0.11
Spirodela–Wolffia	0.19
Spirodela–Lemna	0.12
Wolffia–Landoltia	0.14
Wolffia–Lemna	0.15
Landoltia–Lemna	0.06
Landoltia–Pistia	0.26
Landoltia–Monstera	0.12
Landoltia–Anthurium	0.15
Spirodela–Pistia	0.28
Spirodela–Monstera	0.15
Spirodela–Anthurium	0.16
Lemna–Pistia	0.27
Lemna- Monstera	0.14
Lemna–Anthurium	0.16
Wolffia–Pistia	0.32
Wolffia–Monstera	0.20
Wolffia–Anthurium	0.23
Pistia–Anthurium	0.23
Pistia–Monstera	0.21
Monstera–Anthurium	0.05
Interspecific genetic distances within *Lemna*	
L. minor–L. gibba	0.04
L. minor–L. turionifera/L. japonica	0.02
L. minor–L. trisulca	0.02
L. minor–L. aequinoctialis	0.09
L. gibba–L. turionifera/L. japonica	0.04
L. gibba–L. trisulca	0.04
L. gibba–L. aequinoctialis	0.11
L. trisulca–L. turionifera/L. japonica	0.02
L. trisulca–L. aequinoctialis	0.09
L. turionifera/L. japonica–L. aequinoctialis	0.09

DISCUSSION

The analysis of the *rpS16* intron polymorphism in 25 Lemnaceae and some Araceae shows applicability of this region for phylogenetic studies in duckweeds both at interspecific and intraspecific levels. According to our results *Lemnoideae* is paraphyletic that is in agreement with data presented by Les et al. [6]. The tree topology is in concordance with the basal placement of *Spirodela* in Lemnaceae and with recognition of *Landoltia* as an independent taxon. However, the *Landoltia* phylogenetic position within Lemnaceae remains uncertain. On the MP tree *Landoltia* and *Wolffia* form one highly supported clade but at the same time on the NJ tree and the PCA plot *Landoltia* show more similarity to *Lemna*. Les et al. [6], considering Lemnaceae phylogeny based on other cpDNA regions (the *matK* gene and the *trnK* intron), noted the ambiguity of *Landoltia* placement with respect to *Lemna* and *Wolffia*. According to the *matK* gene sequence variability, *Landoltia* forms a sister group to *Wolffia* and *Wolffiella*, whereas analysis of the *trnK* intron reveals similarity between *Landoltia* and *Lemna*.

The intermediate *Landoltia* placement between *Spirodela* and other members of Lemnaceae with the following branching order: *Spirodela* > Landoltia >*Wolffia* > *Wolffiella* > *Lemna* have been recently shown by Rothwell et al [15] studied polymorphism in the cpDNA *trnL-trnF* spacer. Thus, in spite of the morphological similarity between *Landoltia* and *Spirodela* [16], molecular data indicate that the *rpS16* intron sequence of *Landoltia* is similar to *Lemna* and *Wolffia*. Interestingly that in concordance with the morphological data RAPD [17] and AFLP analyses (unpublished data by E. Martirosyan) predominantly targeting of random nuclear sequences revealed closer relationship between *Landoltia* and *Spirodela* genomes than between *Landoltia* and *Lemna* or *Wolffia* .

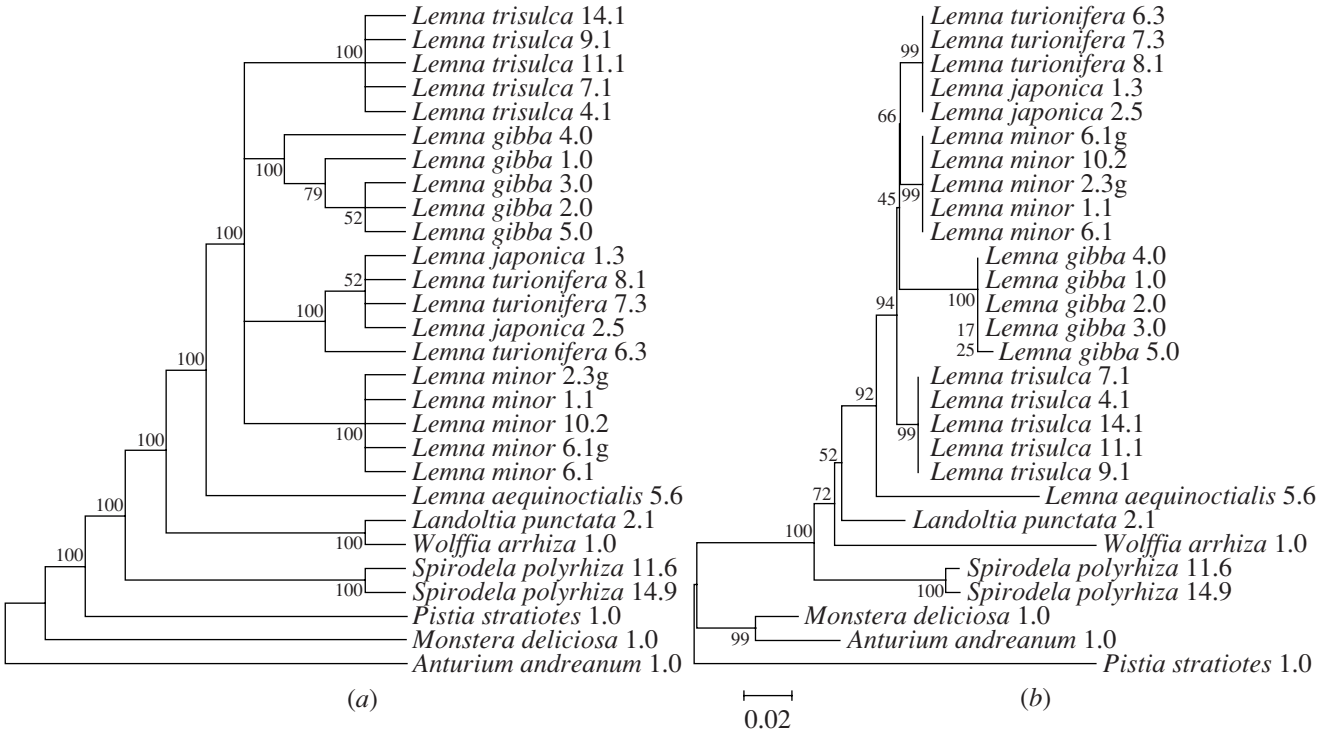

Fig. 2. Phylogenetic trees constructed from analysis of the *rpS16* intron in chlDNA of 25 accessions of Lemnaceae and the related Araceae family: *(a)* MP tree; (*b*) NJ tree. Numerals at branch nodes indicate bootstrap indices (%); the scale bar indicates the number of nucleotide substitutions per site.

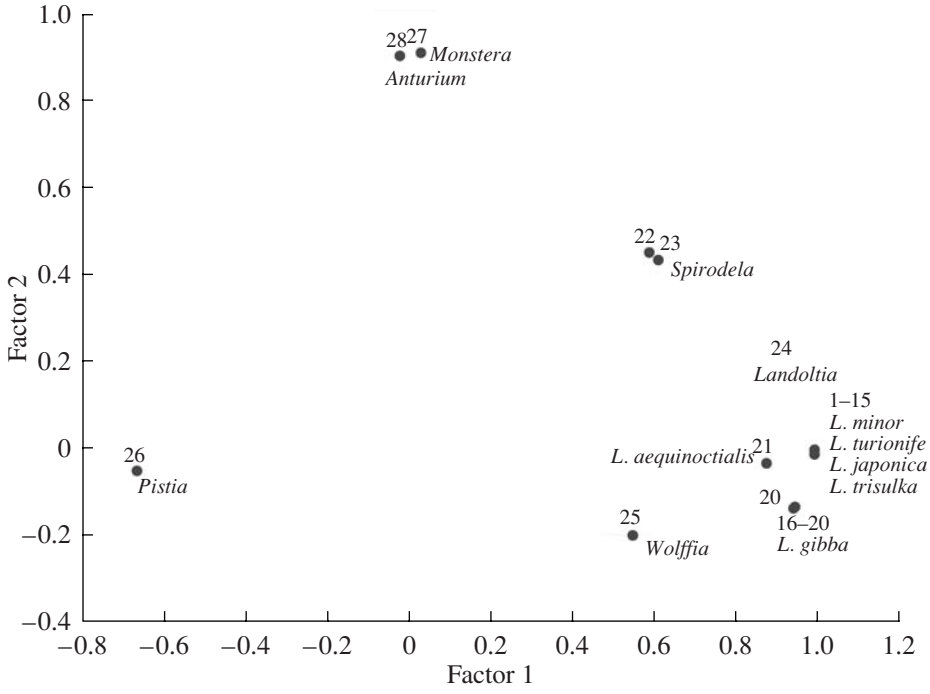

Fig. 3. Principal component analysis (PCA) of the genetic polymorphism of the *rpS16* intron in chlDNA of nine species belonging to four Lemnaceae genera (*Spirodela*, *Landoltia, Lemna*, and *Wolffia*) and in three genera of the related Araceae family (*Pistia, Monstera*, and *Anthurium*).

Numerous studies of nuclei and organelles DNA sequences showed that introgressive hybridization that is a significant factor of evolution of various taxa, occurred more often than it had been thought [18]. The hybrid origin of some Lemnaceae species has been repeatedly supposed [16, 19]. There is an evidence for the hybrid origin of *L. japonica*, whose parental species could be *L. turionifera* and *L. minor* [16]. The supposed hybrid origin of the North American *L. perpusilla* from *L. aequinoctialis* and *L. turionifera* is also under discussion in the literature [6]. The rare flowering of Lemnaceae with predominance of vegetative propagation does not preclude introgressive hybridization among their species [19]. Thus, the disagreement between the positions proposed for *Landoltia* according to nuclear and chloroplast genome data and the overlap of its geographic range with *Spirodela, Lemna*, and *Wolffia* makes the hybrid origin of *Landoltia punctata* discussable.

To sum up, the phylogenetic relationships among *Lemna* species support the notion of the monophyletic state of the genus, as it was concluded by Les et al. [6].

L. aequinoctialis occupies the basal position in the *Lemna* clade. The long branch of this taxon on the NJ tree might reflect a relatively great number of autapomorphic substitutions discriminating this species from other *Lemna* species. The genetic distances between *L. aequinoctialis* and *Lemna* based on the *rpS16* intron polymorphism data exceed the intergeneric distance between *Landoltia* and *Lemna*, being comparable with the intergeneric distance between *Spirodela* and *Landoltia* (Table 2).

Indeed, the current classification of *Lemna* assigns *L. aequinoctialis* to a separate *Alatae* section, whereas *L. turionifera, L. japonica, L. trisulca, L. gibba*, and *L. minor* are placed in another section, *Lemna* [6]. The great number of autapomorphic substitutions and indels and relatively large genetic distance values determined in this study confirm the significant divergence of *L. aequinoctialis*.

It is worth noting that RAPD analysis of Lemnaceae genomes also revealed significant differences between *L. aequinoctialis* accessions and other duckweeds [17]. As reported by Les et al. [6], species of section *Alatae* are a unique divergent *Lemna* group that may be recognized taxonomically as a separate genus.

Morphologically *L. aequinoctialis* is similar to *Lemna* species; for example, *L. minor, L. turionifera* or even *L. gibba*. In contrast, *L. trisulca*, that can be easily identified due to its phenotypic traits and is noticeably different from other *Lemna* species, according to molecular studies of nuclear and chloroplast genomes is much more similar to *L. turionifera, L. japonica, L. gibba*, and *L. minor* than *L. aequinoctialis*.

All above data illustrate the difficulty of correct assessment of phylogenetic relationships by morphology alone in such a worldwide distributed family as Lemnaceae with the extreme vegetative reduction.

As for *L. turionifera* and *L. japonica*, the *rpS16* intron analysis of these morphologically similar species reveals no autapomorphic substitutions or indels. Both species fall into one cluster, forming no species-specific clades. The same was observed in the RAPD analysis [17].

Thus, analysis of the chloroplast *rpS16* intron sequences in 25 Lemnaceae accessions indicates that this cpDNA region is applicable to Lemnaceae phylogenetic studies at different taxonomic levels. According to the polymorphism data *Landoltia* shows more similarity to *Lemna* and *Wolffia* than to *Spirodela*. This supports the recognition of *Landoltia punctata* as a separate genus. Sequence analysis allowed estimating the levels of intergeneric, interspecific, and intraspecific variability of the *rpS16* intron in Lemnaceae and recognition of intraspecific haplotypes for *L. gibba* and *L. trisulca*. The genetic difference between *L. aequinoctialis* and other *Lemna* species is great, comparable with intergeneric differences within Lemnaceae.

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