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Allozyme variation within and divergence between *Lemna gibba* and *L. disperma*: Systematic and biogeographic implications

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Abstract

Enzyme electrophoresis was employed to assess genetic diversity within and divergence between *Lemna disperma* and *Lemna gibba*, sister species that have often been considered conspecific because of the few technical morphological characters distinguishing them. *L. gibba* is distributed widely except in Australia and New Zealand, where it is replaced by *L. disperma*. Allozyme data were employed to examine: (1) whether species recognition is supported by genetic divergence between accessions assigned to the two taxa, (2) whether the level of diversity in the two species supports the hypothesis that *L. gibba–L. disperma* are related as a progenitor–derivative species pair, and (3) whether estimates of divergence times obtained from allozymes are in general agreement with those from plastid sequences. Accessions of the two species are highly divergent at allozyme loci, with a genetic identity of 0.404, and the putative derivative species (*L. disperma*) has only one-third the diversity of its proposed ancestor, *L. gibba*. Therefore, allozyme data support the continued recognition of the two species and are concordant with the hypothesis that the species are related as progenitor and derivative. The reduced morphology of *L. disperma* and the allozyme data indicate that this species originated via dispersal of *L. gibba* or of a common ancestor of the two species. Estimated divergence times from allozymes and plastid sequences vary widely, but assuming that

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actual divergence was within the broad range of estimates, long distance dispersal is required to explain the present distribution of the two species.

Keywords: Lemnaceae; *Lemna*; Allozymes; Genetic identity; Genetic diversity

1. Introduction

Family Lemnaceae (duckweeds) are aquatic monocotyledons with a nearly cosmopolitan distribution and are the most reduced of all flowering plants, both in reduction of size and loss of organs (Landolt, 1986). The extreme reduction of the duckweed plant body makes taxonomic and phylogenetic studies of the family using anatomical–morphological features challenging because of the few characters and character states available for analysis. Recent studies employing molecular data have provided valuable information for the delimitation of species and for producing a phylogenetic hypothesis for the family (Crawford and Landolt, 1993, 1995; Crawford et al., 1996, 1997, 2001; Les et al., 2002; Kimball et al., 2003).

Lemna is the largest of the five genera in the family, and section *Lemna* is among the most complex and confusing groups within the entire family. The combined analysis of Les et al. (2002) provided robust support for the monophyly of the section, and *Lemna disperma* and *Lemna gibba* received strong support as sister species. Landolt (1986) had suggested a similar relationship between the two species (Landolt, 1986, Fig. 7.5), and he hypothesized that *L. disperma* differentiated from *L. gibba*. However, distinguishing these two species has been somewhat problematic. Landolt (1986) discussed morphological variation in *L. gibba* and provided contrasting features that separated it from *L. disperma*: the former species has fronds mostly without a distinct papule near the tip, and fruits that have two to five seeds and margins that are 0.1–0.2 mm wide; fronds of the latter species have papules near their tips and fruits with one to two seeds and margins that are only 0.05–0.1 mm wide. These are small, technical differences, even by duckweed standards. *L. disperma* has a chromosome number of $2n = 40$, whereas the numbers $2n = 40$, 50, 70, and 80 have been determined for *L. gibba* (Urbanska-Worytkiewicz, 1975, 1980; summaries in Landolt and Urbanska-Worytkiewicz, 1980; Landolt, 1986).

These two very similar species have strongly allopatric distributions. *L. gibba* is nearly cosmopolitan in distribution with the notable exceptions of Australia and New Zealand, precisely the two areas where *L. disperma* is endemic (Fig. 1). The nearest occurrence of *L. gibba* to the distribution of *L. disperma* is nearly 8000 km (Fig. 1), which is the greatest disjunction by far for any sister species of duckweeds. Despite their disjunct distributions, *L. gibba* and *L. disperma* exhibit none of the ecological differences that often characterize closely related duckweed species (Landolt, 1986). The two species occupy similar habitats (Landolt, 1975), and while highly clonal, they have high flowering and fruiting frequencies relative to many duckweeds. Seeds allow both species to survive drought in seasonally dry habitats. The two taxa are unique among sister species in Lemnaceae because they represent a vicariant species pair with little or no ecological divergence (Landolt, 1986).

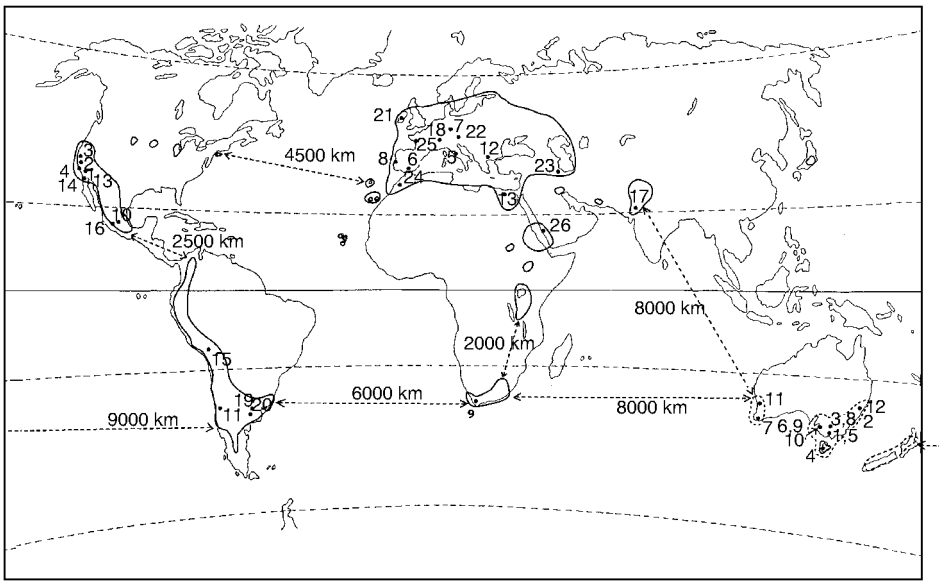

Fig. 1. Geographic distributions of *Lemna gibba* (solid lines) and *L. disperma* (dashed lines). Numbering of accessions is the same as in Table 1; localities from Australia and New Zealand represent *L. disperma* and all others are *L. gibba*. Shortest distances between major distribution areas are shown by dashed lines.

In Lemnaceae, enzyme electrophoresis has proven valuable in assessing genetic variation in species (Vasseur et al., 1991, 1993; Cole and Voskuil, 1996; Crawford et al., 2001). Allozymes also have been valuable in delimiting species and in assessing relationships in the genera *Spirodela* (including *Landoltia*) (Crawford and Landolt, 1993), *Wolffia* (Crawford and Landolt, 1995), *Wolffiella* (Crawford et al., 1997), and in the diverse genus *Lemna* (Vasseur et al., 1991; Crawford et al., 1996, 2001). The results of these studies have demonstrated that, in general, there is high divergence at allozyme loci among congeneric species, even when the taxa are difficult to distinguish using morphological and anatomical characters. The present study of allozyme variation within and divergence between *L. gibba* and *L. disperma* had several objectives. One purpose was to assess genetic divergence at allozyme loci between accessions assigned to the two species to ascertain whether species recognition is warranted. Another objective was to estimate genetic diversity within the two species to ascertain whether the presumed derivative species has the expected lower diversity (Gottlieb, 1977, 2003). A final and somewhat secondary objective was to compare estimated divergence times from allozymes to times estimated from plastid sequences (Les et al., 2003).

2. Methods

Enzymes were extracted from plant material grown on agar culture from single fronds isolated from nature, or seeds collected from natural populations were germinated on agar,

Table 1
Accessions of *Lemna* examined for allozymes

	Taxon	Collection number	Origin	Chromosome number
1.	*L. gibba*	6566	California	$2n = 40$
2.	*L. gibba*	6583	California	$2n = 50$
3.	*L. gibba*	6745	California	$2n = 50$
4.	*L. gibba*	6751	California	$2n = 50$
5.	*L. gibba*	6861	Italy	$2n = 40$
6.	*L. gibba*	7021	Spain	$2n = 40$
7.	*L. gibba*	7107	Germany	$2n = 40$
8.	*L. gibba*	7198	Portugal	$2n = 40$
9.	*L. gibba*	7218	South Africa	$2n = 80$
10.	*L. gibba*	7240	Mexico	$2n = 40$
11.	*L. gibba*	7262	Chile	$2n = 40$
12.	*L. gibba*	7263	Greece	$2n = 40$
13.	*L. gibba*	7377	Egypt	$2n = 40$
14.	*L. gibba*	7589	California	$2n = 40$
15.	*L. gibba*	7613	Peru	$2n = 40$
16.	*L. gibba*	7661	Mexico	$2n = 40$
17.	*L. gibba*	7705	India	$2n = 60$
18.	*L. gibba*	7810	France	$2n = 40$
19.	*L. gibba*	7922	Argentina	$2n = 50$
20.	*L. gibba*	7922a	Argentina	$2n = 40$
21.	*L. gibba*	7932	Ireland	$2n = 40$
22.	*L. gibba*	7937	Austria	$2n = 40$
23.	*L. gibba*	8291	Iran	$2n = 40$
24.	*L. gibba*	8384	Morocco	$2n = 40$
25.	*L. gibba*	8405	France	$2n = 40$
26.	*L. gibba*	8682	Saudi Arabia	–
1.	*L. disperma*	7190	Victoria	$2n = 40$
2.	*L. disperma*	7223	Canberra	$2n = 40$
3.	*L. disperma*	7259	Victoria	$2n = 40$
4.	*L. disperma*	7269	Tasmania	$2n = 40$
5.	*L. disperma*	7276	Victoria	$2n = 40$
6.	*L. disperma*	7761	South Australia	$2n = 40$
7.	*L. disperma*	7767	Western Australia	$2n = 40$
8.	*L. disperma*	7777	Victoria	$2n = 40$
9.	*L. disperma*	7816	South Australia	$2n = 40$
10.	*L. disperma*	7842	South Australia	$2n = 40$
11.	*L. disperma*	8717	Western Australia	–
12.	*L. disperma*	8729	New South Wales	–

and the resulting fronds served as sources of enzymes. The collection numbers and geographic origins of the accessions examined are given in Table 1. Twenty-six accessions of *L. gibba* and 12 accessions of *L. disperma* were examined. All plant material was supplied and determined taxonomically by E.L.; voucher specimens are deposited in ETH (ZT).

The extracting buffer was made up in 10% glycerine, and consisted of 0.1 M Tris–HCl, pH 7.5, with 14 mM 2-mercaptoethanol, 1.0 mM EDTA (tetrasodium salt), 10 mM $MgCl_2$, 10 mM KCl, and 5–10 mg polyvinylpolypyrrolidine (PVP-10) per 0.5 mL of buffer

(slightly modified from Gottlieb, 1981). Forms of aspartate aminotransferase (AAT, E.C.2.6.1.1), glutamate dehydrogenase (GDH, E.C.1.4.1.2), glyceraldehyde-3-phosphate dehydrogenase (G3PDH [NADP-dependent form], E.C.1.2.19), and shikimate dehydrogenase (SKDH, E.C.1.1.1.2) were resolved in discontinuous polyacrylamide gels with a 6.0% running gel (0.375 M Tris–HCl, pH 8.9), a 3% spacer gel (0.06 M Tris–HCl, pH 6.7), and an electrode buffer of 0.005 M Tris–0.038 M glycine, pH 8.3 (Davis, 1964). Gels were run until the bromphenol blue tracking dye had migrated ca. 10 cm. The remaining enzymes were resolved in 13.0% starch gels employing two buffer systems. Different forms of malate dehydrogenase (MDH, E.C.1.1.1.37) and phosphogluconate dehydrogenase (PGD, E.C.1.1.1.44) were separated with an electrode buffer of 0.04 M citric acid brought to pH 6.1 with *N*-(3-aminopropyl)-morpholine, and the gel buffer was a 1:19 dilution of the running buffer. A system consisting of an electrode buffer of 0.5 M Tris, 0.65 M boric acid and 0.02 M EDTA, pH 8.0, and a gel buffer that was a 1:9 dilution of the electrode buffer was used to resolve different forms of glucose-6-phosphate isomerase (GPI, E.C.5.3.1.9), phosphoglucomutase (PGM, E.C.5.4.2.2), and triose-phosphate isomerase (TPI, E.C.5.3.1.1). Starch gels were run until the bromphenol blue tracking dye had migrated ca. 15 cm. The staining protocols and the nomenclature of the enzymes followed Wendel and Weeden (1989).

Inferences on the genetic bases of enzyme banding patterns were made from several lines of evidence. The known active subunit composition of the enzymes and the minimal conserved number in diploid plants for each of the enzymes (Gottlieb, 1982; Weeden and Wendel, 1989) were useful for inferring locus number. Resulting allele frequencies were used to calculate genetic diversity statistics (Nei, 1973), proportion of polymorphic loci, and mean number of alleles per polymorphic locus for each species. Genetic identity and distance between the species were calculated using Nei's (1978) unbiased estimates. The program POPGENE, Version 1.31 (Yeh and Boyle, 1997), was used to calculate the statistics.

A neighbor-joining tree (N-J) that summarized the pattern of allozyme variation among the 38 strains studied was constructed from genetic identities using NTSYS (Rohlf, 1998) and PHYLIP's NEIGHBOR (Felsenstein, 1993). The former program was used to determine whether there were ties, i.e., different trees having equal scores (Backeljau et al., 1996), whereas the latter program was used in connection with PAUP* 4.0b10 (Swofford, 2003) to produce an unrooted tree.

Divergence time between the two species was estimated from the allozyme data using Eq. 9.24 from Nei (1987) and the method discussed by Thorpe (1982).

3. Results

A total of 13 loci was scored: AAT (1); GDH (1); G3PDH (1); GPI (1); MDH (3); PGD (2); PGM (1); SKDH (1); TPI (2). Additional zones of activity were present for AAT, GPI, MDH, and PGM but they were not included in the analyses because lack of resolution and staining intensity precluded an unambiguous interpretation. Although polyploidy can influence the interpretation of banding, we observed no differences in banding patterns among the various chromosomal races of *L. gibba* (see Table 1). An average of 10.5 (8–13) and 10 (7–13) loci was scored for strains of *L. gibba* and *L. disperma*, respectively.

Nei's (1973) total genetic diversity in *L. gibba* was 0.126 as compared to 0.041 in *L. disperma*. The proportion of polymorphic loci was 0.539 in *L. gibba* and 0.231 in *L. disperma*. The mean number of alleles per polymorphic locus was 2.43 in *L. gibba* as compared to 2.33 in *L. disperma*, and the mean number of alleles per locus is 1.77 and 1.31, respectively. The three loci polymorphic in *L. disperma* are also polymorphic in *L. gibba*.

Nei's (1973) genetic identity between the two species is 0.404, and the distance is 0.907. A total of 15 unique alleles were detected in *L. gibba* and 9 unique alleles were found in *L. disperma*. The two species are monomorphic for different alleles at five loci (*Mdh-1*, *Mdh-2*, *Mdh-3*, *Tpi-1*, and *Tpi-2*), and they are fixed for the same allele at one locus (*G3pdh*). *L. disperma* had a subset of the alleles present in *L. gibba* (i.e., the former species is monomorphic for an allele occurring at a polymorphic locus in the latter species) at the three loci *Gdh*, *Gpi-2*, and *Pgd-1*; in all instances, the allele in *L. disperma* was the highest frequency allele in *L. gibba*.

Fig. 2. Unrooted neighbor-joining tree for accessions of *Lemna gibba* and *L. disperma*. Numbering of accessions is the same as given in Table 1. The geographic origin of each accession is indicated.

There were six tied N-J trees, one of which is shown in Fig. 2. The six trees were very similar, with strains of each species forming a distinct group in all trees. However, strains of *L. gibba* did not group entirely by their geographic origin (Fig. 2). Two examples illustrating the lack of geographic cohesion in *L. gibba* include strains 10 and 16 from Mexico that associate with plants originating in Iran and Peru, respectively, and 19 and 20 from Argentina, which ally with European and South African accessions (Fig. 2).

The estimated divergence time for the two species using Eq. 9.24 of Nei (1987) is approximately 13 million years before present (mybp). The values using the method of Thorpe (1982), depending on whether one uses 15 or 20 million years for a genetic distance of 1.00, are 13.6 or 18.1 mybp.

4. Discussion

Sequences from plastid DNA, combined with morphology and secondary chemistry, produced a completely resolved and strongly supported phylogeny for Lemnaceae (Les et al., 2002). *L. gibba* and *L. disperma* receive strong support as sister taxa in this phylogeny as well as support from separate analysis of three of the four plastid regions sequenced (the other was not informative). The plastid sequences also support the recognition of the two taxa because, although they are more similar to each other than to any other species, they exhibit sequence divergence in all four plastid regions (Les et al., 2003). Landolt (1975) discussed the morphological similarities and differences between the two species while reserving judgment as to whether they should be maintained as distinct; later, he recognized them as separate species (Landolt, 1986).

Landolt (1986) hypothesized that the two are related as progenitor–derivative species, with *L. disperma* a derivative of *L. gibba*. He suggested that differentiation in several small technical morphological features occurred following dispersal of ancestral colonists to Australia. Reduction in features such as frond size, number of nerves, and number of ovules in *L. disperma*, together with its narrower geographic distribution, was used by Landolt (1986) to support the hypothesis that it was derived from *L. gibba* (or from a common ancestor of the two species).

Allozyme data address two major questions about *L. gibba* and *L. disperma*: whether they represent distinct species and whether they are related as progenitor and derivative. Nei's (1978) genetic identity of 0.404 for the two species and the clear separation of accessions on the N-J tree (Fig. 2) provide compelling evidence that *L. gibba* and *L. disperma* represent distinct, isolated genetic entities. Numerous allozyme studies of congeneric species of flowering plants have revealed a grand mean in the range of 0.650–0.700 (Gottlieb, 1977; Crawford, 1989, 1990). Prior electrophoretic studies of duckweeds have shown identity values ranging from 0.00 to 0.94 (Crawford and Landolt, 1993, 1995; Crawford et al., 1996, 1997, 2001). The genetic identity of 0.404 for *L. gibba* and *L. disperma* may be placed in perspective relative to other duckweed congeners by noting that of the 95 pairwise comparisons, only 14 are equal to or higher than the values obtained in the present study. Thus, *L. gibba* and *L. disperma* are less divergent at allozyme loci than duckweed species in general. However, when *L. gibba–L. disperma* are compared to other duckweed sister species, four species pairs exhibit higher genetic identities (Crawford

et al., 1996, 1997, 2001); two values are essentially the same, and one is considerably lower (Crawford and Landolt, 1993, 1995). Thus, *L. gibba* and *L. disperma* are more divergent at allozyme loci than the majority of other sister species of duckweeds examined. These two *Lemna* species contrast with other sister species of duckweeds in two respects: they have highly disjunct distributions and are very similar ecologically (Landolt, 1986, 1987).

The allozyme data are concordant with the hypothesis that *L. disperma* is derived from *L. gibba* (or from a common ancestor). Derivative species typically have a subset of the allelic variation detected in their progenitors, often have very few or no unique alleles, and exhibit high genetic identities with their progenitor species (above 0.85) (Gottlieb, 1977; Crawford, 1989, 1990). These observations indicate that divergence has been recent; indeed, progenitor–derivative species pairs were often identified initially by their morphological similarities (e.g., Gottlieb, 1973; Gottlieb and Pilz, 1976; Crawford and Smith, 1982). The two *Lemna* species fit the progenitor–derivative model in certain respects. *L. gibba* exhibits 15 alleles not detected in *L. disperma*, and the latter species is monomorphic for high frequency alleles at three polymorphic loci in *L. gibba*. The lower total genetic diversity in *L. disperma* than in *L. gibba* also is characteristic of a progenitor–derivative situation. The two species differ from more typical progenitor–derivative cases by their lower genetic identity (higher divergence) and the relatively high number (nine) of unique alleles detected in the putative derivative. These differences are ostensibly the result of the greater divergence time for the duckweeds compared to other progenitor–derivative species pairs.

The estimated divergence time from allozyme data (13–18.5 mybp, depending on the method employed) is higher than estimates derived from plastid sequences. Les et al. (2003) estimated divergence times ranging from 2.4 to 10.3 mybp, depending on which of the four plastid sequences was used in the calculations. The mean value from plastid sequences was 5.5 mybp, but with a large deviation of 3.5 mybp. The range of divergence times estimated from the nuclear allozyme loci and the different plastid sequences indicate the difficulty of placing an accurate divergence time for the two species. Despite these difficulties, it seems reasonable to conclude that they diverged between 5 and 15 mybp. Within this range of times, there was little difference in the distances between Australia and New Zealand and possible source areas, and their present positions (see Fig. 3 in Les et al., 2003). Thus, even with the limitations in timing the divergence, it seems clear that the present disjunct distribution of the two species is the result of long distance dispersal of *L. gibba*, or a common ancestor of *L. gibba* and *L. disperma*, to Australia–New Zealand with subsequent molecular and morphological differentiation.

Given the relatively high seed set in these species compared to many Lemnaceae, seeds may have been the dispersal agents. Also, the seeds of *L. gibba* and *L. disperma* are more drought resistant than most other species of duckweeds (Landolt, 1986), which could facilitate the survival of desiccation during long distance dispersal by birds. The migration of birds is directed primarily North and South, with East–West migration quite rare (Berthold, 2001). Thus, migration between continents is rare except from Eastern Asia to Australia. However, *L. gibba* does not occur East of Kashmir, except for its recent introduction into Japan by humans (Fig. 1). The absence of *L. gibba* in Eastern Asia could be the reason why this species has not been introduced by birds to Australia during the past several million years. The initial dispersal to Australia likely occurred as a unique incident

of extremely low probability, i.e., it provides a prime example of a ''one bird in a million'' event as Hutchinson (1975) previously had conjectured (Les et al., 2003). Interestingly, *L. gibba* shows no allozyme differentiation among geographic areas, even though the distance between the two major areas, the Americas and Africa–Eurasia, is 6000 km (Fig. 1). Within these two major areas of distribution, distances between known populations do not exceed 2500 km, and bird migration between areas must be frequent. Bird migration from the Northeastern parts of North America to Western Africa are known, but *L. gibba* presently does not occur in these Northeastern regions. However, if we assume that the distribution of *L. gibba* reached Southeastern Canada and the Northeastern United States during past warmer periods, the distance from North America to the island of Madeira, where *L. gibba* now occurs, is reduced to 4500 km (Fig. 1). It is then a short distance from Madeira to Europe and more plausible that occasional transport of diaspores had occurred along this route (Fig. 1).

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