

Allozyme studies in *Lemnaceae*: variation and relationships in *Lemna* sections *Alatae* and *Biformes*

Daniel J. Crawford¹, Elias Landolt², Donald H. Les³ & Rebecca T. Kimball⁴

Summary

Crawford, D. J., Landolt, E., Les, D. H. & Kimball, R. T.: Allozyme studies in *Lemnaceae*: variation and relationships in *Lemna* sections *Alatae* and *Biformes*. – Taxon 50: 987–999. 2001. – ISSN 0040-0262.

Allozyme variation was examined within and among three species of *Lemna* (*Lemnaceae*) that have been viewed as closely related based on morphological and DNA sequence data. These include *L. aequinoctialis*, *L. perpusilla*, and *L. tenera*, with the former two species the only members of sect. *Alatae* and the latter the sole species of sect. *Biformes*. *Lemna aequinoctialis* is widely distributed in tropical and subtropical regions whereas the other two species have much narrower distributions. Sampling from one population of *L. aequinoctialis* revealed several multilocus genotypes but a deficiency of heterozygotes, which agrees with observations that the species can self-fertilise and produce seed in addition to reproducing clonally. Two series of localized populations revealed several multilocus genotypes in each group, but no heterozygotes were detected at any of the variable loci. Twenty single clones from much of the geographic range of *L. aequinoctialis* contain the same alleles detected in the local populations; no pattern of large-scale geographic variation was detected. Allozyme data support the recognition of *L. perpusilla* as distinct from *L. aequinoctialis*, but the two species share much higher identities at allozyme loci than either does with any other species of *Lemna*, thus agreeing with morphological and plastid DNA sequence data indicating that the two are closely related yet distinct species. *Lemna tenera* shares very low allozyme identities with *L. aequinoctialis* and *L. perpusilla*. Neither allozymes nor plastid sequences support the hypothesis that *L. perpusilla* is a hybrid between *L. aequinoctialis* and *L. turionifera*. Molecular data indicate that the three species form a strongly supported monophyletic group, with the common ancestor of *Lemna tenera* and *L. aequinoctialis*-*L. perpusilla* having diverged first, much later divergence occurring between the common ancestor of *L. aequinoctialis* and *L. perpusilla*.

Keywords: allozymes, genetic variation and divergence, *Lemna*, *Lemnaceae*, systematic relationships.

Introduction

Assessing genetic variation within and divergence among species of duckweeds (*Lemnaceae*) is challenging because this family of five genera and 38 species of aquatic flowering plants constitutes the smallest and most reduced angiosperms. As a result, duckweeds lack many of the morphological and anatomical features

¹ Department of Ecology and Evolutionary Biology, University of Kansas, Haworth Hall, Lawrence, Kansas 66045, U.S.A. E-mail: dcrawfor@ku.edu (author for correspondence).

² Geobotanisches Institut ETH, Zürichbergstrasse 38, CH-8044 Zürich, Switzerland. E-mail: landolt@geobot.umnw.ethz.ch.

³ Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, Connecticut 06269, U.S.A. E-mail: les@uconnvm.uconn.edu.

⁴ Department of Zoology, University of Florida, Gainesville, Florida 32611, U. S. A. E-mail: rkimball@zoo.ufl.edu.

available for comparative purposes in most angiosperms (Landolt, 1986). Attempts to obtain more refined insights into geographical variation within species, to assess genetic divergence between species, and to infer relationships among species have motivated molecular systematic studies of *Lemnaceae* (e.g., Crawford & Landolt, 1993, 1995; Crawford & al., 1996, 1997; Les & al., in press). The molecular phylogenetic study of Les & al. (in press) using sequences from the plastid genome provides a well-resolved phylogeny for the family, and may serve as a framework within which other data sets are viewed and contrasted. Electrophoretic studies in all species of the genera *Spirodela* (including *Landoltia punctata* described by Les & Crawford, 1999), *Wolffia*, *Wolffiella*, and two species of *Lemna* have shown the following: some widely distributed species exhibit geographical variation at allozyme loci; low genetic identities at allozyme loci may occur between species that are very similar morphologically; allozyme data are often concordant with relationships hypothesised from morphological data; and congeneric species may share no alleles, rendering allozymes of no value for inferring higher level relationships.

Several species of *Lemnaceae* are widely distributed geographically, and among the most widespread of all species is *Lemna aequinoctialis* Welwitsch, which is known from subtropical and tropical regions throughout the world (Fig. 1). This species has been introduced into more northern temperate zones with rice culture (Landolt, 1986). Landolt (1986) discussed morphological variation in *L. aequinoctialis* and presented evidence that several previously described segregate species should not be recognised because of intergradation in features used in their

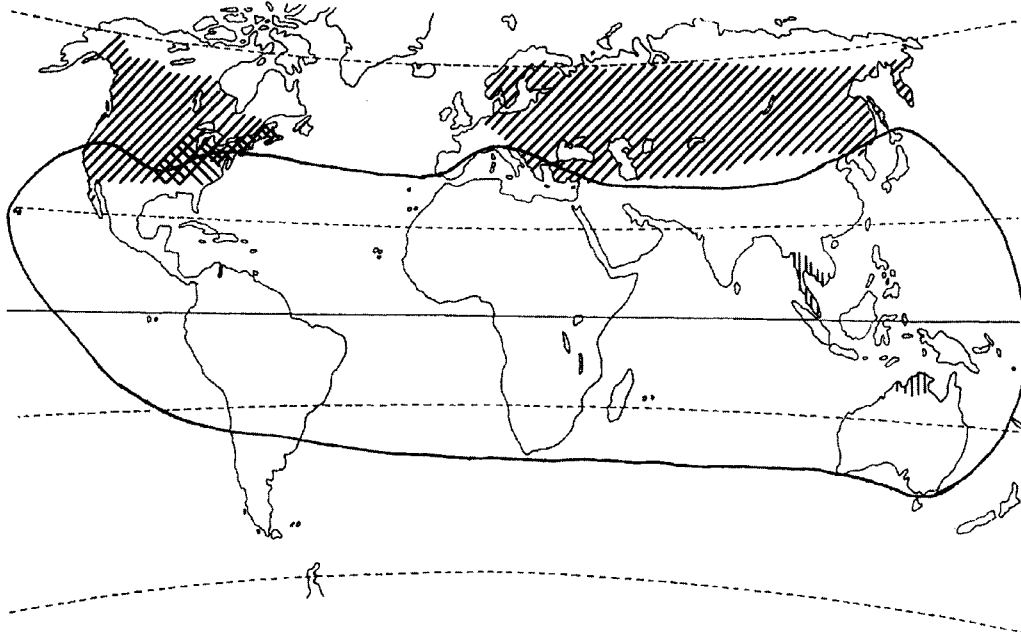


Fig. 1. Distributions of species of *Lemna* studied electrophoretically. Area enclosed by solid line, *L. aequinoctialis*; vertical lines, *L. tenera*; right-slanted lines, *L. turionifera*; left-slanted lines, *L. perpusilla*.

recognition. Kandeler & Huegel (1974) concluded, however, that populations from eastern and central North America (often treated as variants within *L. aequinoctialis* = *L. paucicostata*) should be recognised as the distinct species *L. perpusilla* Torrey (Fig. 1). Landolt (1986) followed Kandeler & Huegel (1974) in recognising *L. perpusilla* and placed it sister to *L. aequinoctialis* in his diagram of relationships, including the two species as the only members of sect. *Alatae*, and raised the question of whether *L. perpusilla* could be of hybrid origin with *L. aequinoctialis* and *L. turionifera* as the parents. The hypothesis of a hybrid origin for *L. perpusilla* was based on a seed coat structure similar to *L. turionifera*. Also, *L. perpusilla* is found where the ranges of the two putative parental species overlap (Fig. 1). A cladistic analysis of morphological and anatomical data in *Lemnaceae* placed *L. aequinoctialis* and *L. perpusilla* together in a moderately supported clade with 70% bootstrap support (Les & al., 1997). The phylogeny from plastid sequences placed *L. aequinoctialis* and *L. perpusilla* together in a clade with 99% bootstrap support (Fig. 2). Sequences of *L. perpusilla* were divergent from each of the putative parental species (Les & al., in press). Thus, sequence data provided no evidence for the putative hybrid origin of *L. perpusilla*.

Landolt (1986) erected sect. *Biformes* with the very rare species *Lemna tenera* Kurz of southeastern Asia as its only member. Later, Landolt (1992) discussed the occurrence and distinctive ecology of *L. tenera* in Australia. *Lemna tenera* is morphologically reduced relative to members of sect. *Alatae*, and Landolt (1986) considered it to be intermediate between sect. *Alatae* and the highly reduced species of sect. *Uninerves*. In the molecular phylogeny of Les & al. (in press), *Lemna tenera* is sister to *L. aequinoctialis* and *L. perpusilla* in a clade with 100% bootstrap support (Fig. 2).

The primary objectives of this allozyme study of *Lemna aequinoctialis*, *L. perpusilla*, and *L. tenera* were to: (1) assess genetic variation at the populational,

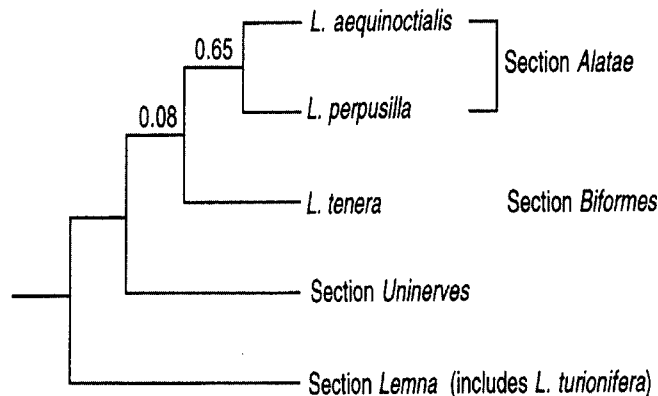


Fig. 2. Phylogenetic relationships in *Lemna* based on plastid DNA sequences (modified and condensed from Les & al., in press). Bootstrap support for all branches shown is 97 percent or higher. Genetic identities at allozyme loci given above branches. Section *Uninerves* shares no alleles with sects. *Alatae* and *Biformes*.

microgeographic and broad-scale geographic levels in *L. aequinoctialis*; (2) determine the level of allozyme divergence between *L. aequinoctialis* and the morphologically very similar *L. perpusilla*; (3) examine the hybrid origin hypothesis of *L. perpusilla*; and (4) assess divergence between *L. tenera* of sect. *Biformes* and the two species of sect. *Alatae*, *L. aequinoctialis* and *L. perpusilla*.

Materials and methods

Enzymes were extracted from plant material grown on agar culture from single fronds isolated from nature, or from seeds that were collected from natural populations, then germinated and grown on agar. For *Lemna aequinoctialis*, seeds were collected from 16 plants of population 9105 from Okavango Delta in Botswana (Table 1), and the individuals grown from seeds (only one seed produced per frond) were examined to assess diversity within the population. For accession numbers 9084–9108 and 9043–9082 of *L. aequinoctialis*, one individual was isolated from different local populations in Botswana and Zimbabwe, respectively, and used as sources of allozymes (Table 1). For all other accessions of *L. aequinoctialis* and the other three species of *Lemna*, single isolates from populations were used as sources of enzymes (Table 1). More than 15 accessions of *L. turionifera* have been examined and will be treated in a later investigation; in the present study, four accessions (Table 1) were included to test whether *L. perpusilla* is of hybrid origin between the putative parents, *L. turionifera* and *L. aequinoctialis*. All plant material was supplied and identified taxonomically by E. L.

Table 1. Geographic origins and collection numbers of *Lemna* clones and populations used as sources of enzymes for electrophoresis. All collection numbers are those of E. Landolt.

<i>L. aequinoctialis</i>	Single clones: Africa: Malawi, Blantyre: 7382. Angola: Benguela, 7643. China: Nanking, 8340. Cuba: Oriente, 8281. Dominican Republic: Dajabon, 7711. Japan: Kyushu, 7792. U.S.A.: Arizona, 8120; California, 6609, 6612, 6746, 7558; Connecticut, 7001; Florida, 7122; Hawaii, Oahu, 7472; Oklahoma, 8011, 8224; South Carolina, 7006; Texas, 7126, 8110; Virginia, 7594. Population sample (16 progeny examined): Africa: Botswana, Okavango Delta, 9105. Local populations (one sample from each): Africa: Zimbabwe: 9043, 9049, 9050, 9051, 9059, 9060, 9064, 9065, 9068a, 9069, 9075, 9077, 9078, 9082; Botswana, Okavango Delta: 9084, 9085, 9090, 9097, 9098, 9108.
<i>L. perpusilla</i>	U.S.A.: North Carolina, 8473; New Jersey, 8539; Virginia, 8612.
<i>L. tenera</i>	Australia: Northern Territory, Kakadu National Park, 9020, 9021, 9023, 9024.
<i>L. turionifera</i>	U.S.A.: California, 6619; Washington, 6735; Indiana, 7352; Iowa, 7387.

The extracting buffer, which was made up in 10% glycerol, consisted of 0.1 M tris-HCl, pH 7.5, with 14 mM 2-mercaptoethanol, 1.0 mM EDTA (tetrasodium salt), 10 mM MgCl₂, 10 mM KCl, and 5–10 mg PVP-10 per 0.5 ml of buffer (slightly modified from Gottlieb, 1981). One drop of 1.0% bromphenol blue solution was added to the cathodal buffer chamber. 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 12.5% 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 & 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 & Wendel, 1989) were useful for inferring locus number. In addition, in population 9105 the banding patterns from 16 plants grown from single seeds from different plants were compared, and this information was useful for inferring locus number from banding patterns. Allelic frequencies were calculated for the three species, and in *L. aequinoctialis* frequencies were determined for broad geographic regions within species, for different populations within two localised areas, and for a single population. These frequencies were used to calculate genetic diversity statistics (Nei, 1973) and Nei's genetic identity (Nei, 1972); the GeneStat-PC (version 3.3) software (Lewis, 1993) was used to calculate the statistics. In addition, the proportion of polymorphic loci, mean number of alleles per polymorphic locus, and number of multilocus genotypes were calculated (Table 2).

Table 2. Genetic diversity statistics for *Lemna* species. N = number of plants examined; H_{ep} = genetic diversity, A_p = mean number of alleles per polymorphic locus, G = number of multilocus genotypes, and P = proportion polymorphic loci.

Species	Population	N	H_{ep}	A_p	G	P
<i>L. aequinoctialis</i>	9105 (Botswana)	16	0.06	2.00	4	29.0
	9043–9082 (Zimbabwe)	14	0.14	2.11	6	35.7
	9084–9108 (Botswana)	6	0.20	2.33	3	35.7
	6609–8340	20	0.23	2.72	24	50.0*
<i>L. perpusilla</i>		3	0.17	2.00	9	38.5
<i>L. tenera</i>		4	0.00	—	1	0.00

*throughout range

Results

A total of 14 presumptive loci was used to calculate most of the statistics. Due to poor resolution or staining, not all loci were scored in all individuals but all statistics are based on ten or more loci. There was no evidence of duplicated loci for any of the enzymes, that is, the banding patterns could be interpreted as the result of the expression of the conserved minimal number of loci for diploid plants (Gottlieb,

1982; Weeden & Wendel, 1989). Patterns seen for several enzymes, both among progeny from one population and among accessions, did not include complex banding patterns or apparent “fixed” heterozygosity, which would be indicative of duplicated loci (Fig. 3). It is possible, however, that the inability to detect duplicated loci could, in certain instances, be a result of the lack of allelic variation at the loci. Except for *Lemna tenera*, chromosome numbers are known for all accessions used in this study. K. Urbanska (Urbanska-Worytkiewicz, 1980; Landolt & Urbanska-Worytkiewicz, 1980) documented that, with rare exceptions, the accessions have a chromosome number of $2n = 40$, which is the common number in the genus *Lemna* and in *Lemnaceae*.

Consider first genetic diversity (H_{ep}) within the one population and among local populations of *Lemna aequinoctialis* (Table 2). The values vary from 0.06 for the 16 progeny for population 9105 from Botswana to 0.20 for populations 9084–9108 (also from Botswana), with populations from Zimbabwe intermediate (Table 2). The proportion of polymorphic loci and mean number of alleles per polymorphic locus show a similar pattern for these different local populations of *L. aequinoctialis*, with diversity lowest in the single population and highest among the local populations from Botswana (Table 2). The mean genetic diversity of 0.23 among the 20 accessions (individuals) that cover much of the geographic range of *L. aequinoctialis* is much higher than the diversity measured in the population 9105 and somewhat higher than was detected among the accessions from the local populations; the proportion of polymorphic loci and the mean number of alleles per polymorphic locus are likewise higher among the 20 accessions (Table 2). Three multilocus genotypes were found among the six individuals from local populations in Botswana, four were detected from the 16 plants from population 9105, six from 14 individuals from local populations in Zimbabwe, and 20 different multilocus genotypes were detected among the 20 plants from the geographic range of the species (Table 2). Three alleles not detected in local population 9105 were found in one or more of the local populations (Botswana and Zimbabwe) and among the 20 accessions collected from much of the geographic range of *L. aequinoctialis*. Genetic diversity and number of multilocus genotypes among the three accessions of *L. perpusilla* and the four accessions of *L. tenera* are also shown in Table 2. No diversity was found in the latter species, but all four accessions were from the same local area in Australia.

Genetic identities among the three groups of local populations range from 0.84 for the two groups from Botswana to 0.98 for population 9105 from Botswana and local populations 9043–9082 from Zimbabwe, with the two groups of local populations from Botswana (9084–9108) and Zimbabwe (9043–9082) intermediate (Table 3). Mean genetic identity among the 20 geographically widespread accessions is 0.91; it is somewhat higher among the 15 New World accessions, and lower among the five Old World accessions (Table 3). The identity between the Old and New World accessions is 0.85. Genetic identity between *Lemna aequinoctialis* (20 accessions) and the three accessions of *L. perpusilla* is 0.65 whereas the 20 accessions have a much reduced identity with the four accessions of *L. tenera*, and

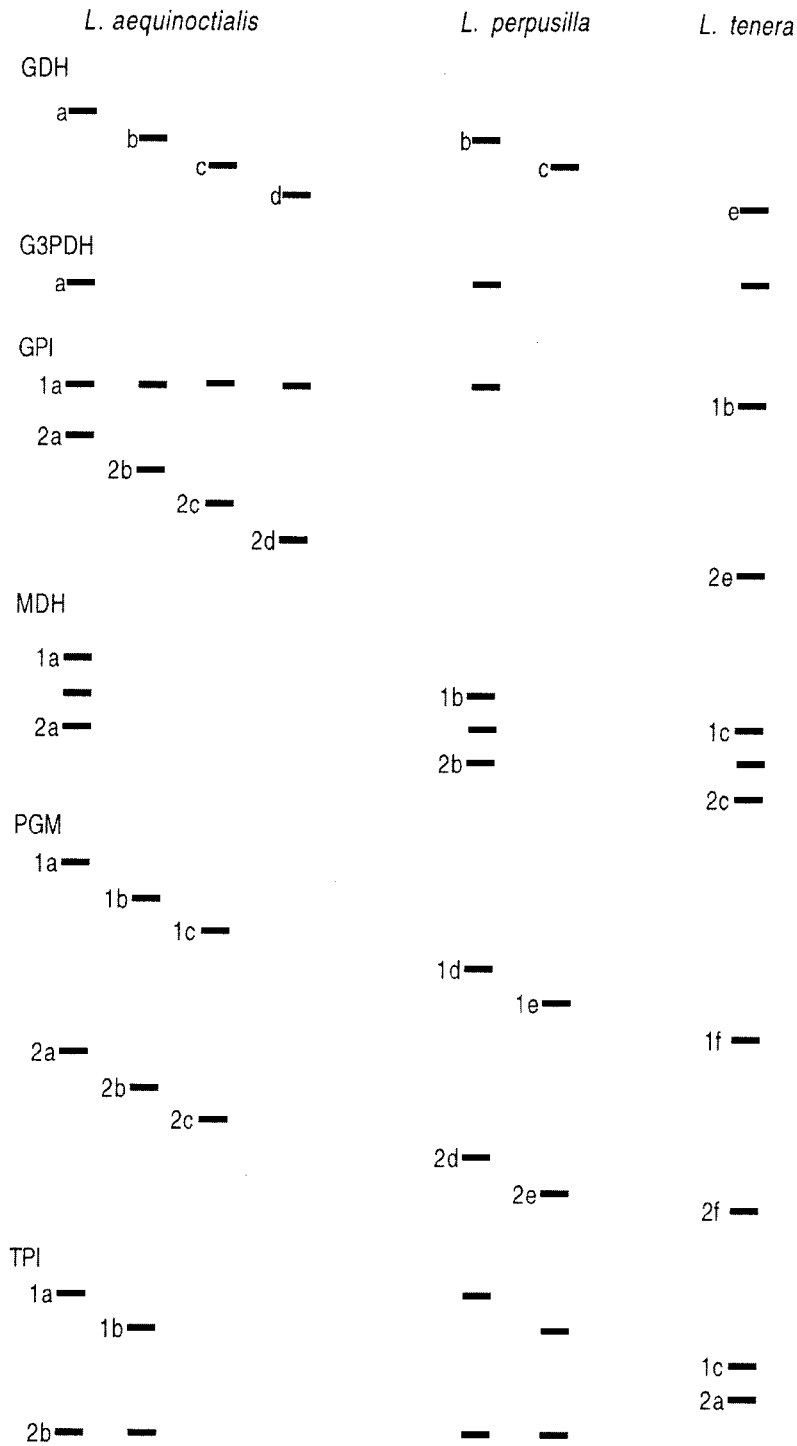


Fig. 3. Drawings of selected banding patterns (multilocus genotypes) for six enzymes and 10 loci illustrating variation within and divergence among three species of *Lemna*. GDH = glutamate dehydrogenase; G3PDH = glyceraldehyde-3-phosphate dehydrogenase; GPI = glucose-6-phosphate isomerase; MDH = malate dehydrogenase; PGM = phosphoglucomutase; TPI = triose-phosphate isomerase. The anode is toward the top; numbers refer to different loci while letters refer to alleles. The intermediate band in the MDH pattern is an interlocus heteromer.

Table 3. Genetic identities among populations and species of *Lemna*.

<i>L. aequinoctialis</i>	
Between population 9105 and local populations 9043-9082	0.98
Between population 9105 and local populations 9084-9108	0.84
Between local populations 9043-9082 and 9084-9108	0.86
Mean for 20 accessions (6609-8340)	0.91
Mean for five Old World accessions	0.82
Mean for 15 New World accessions	0.94
Between Old World and New World accessions	0.85
<i>L. aequinoctialis</i> (6609-8304) and <i>L. perpusilla</i>	0.65
<i>L. aequinoctialis</i> (6609-8304) and <i>L. tenera</i>	0.08
<i>L. perpusilla</i> and <i>L. tenera</i>	0.08

the identity between *L. perpusilla* and *L. tenera* is the same as between *L. aequinoctialis* and *L. tenera* (Table 3).

Discussion

Prior electrophoretic investigations of duckweeds (Crawford & Landolt, 1993, 1995; Crawford & al., 1997) considered local variation within species to a limited extent because the focus was primarily on genetic divergence among congeneric species. In those studies, sampling was from single accessions from throughout the geographic ranges of species. In some cases, different alleles or different multilocus genotypes are largely restricted to different regions of geographically widespread species (Crawford & Landolt, 1993, 1995). Those studies left unresolved the question of whether sampling of single clones from much of the range of a species underestimates species-level diversity (H_{ep}). That is, are additional alleles detected within populations or among samples from localised areas relative to what is found with geographic sampling of single clones? Another question concerns the number of multilocus genotypes (genotypic diversity) within populations or among local populations. The present study provides some preliminary insights into these questions for *Lemna aequinoctialis*, a widely distributed duckweed species.

The diversity among individuals from different local populations in each of the areas is higher than among progeny from individuals within the single population 9105 (Table 2), as might be expected. Also, three alleles were detected among the localised populations in each area that were not found in the single population. Diversity among the 20 individual accessions from throughout the range of *L. aequinoctialis* is much higher than in the single population and only slightly higher than among the localised populations from Botswana (Table 2). The same array of alleles was found in both the African populations and the broader survey of accessions. Thus, at least with regard to *L. aequinoctialis*, it does not appear that sampling single clones from the geographic range of a species materially underestimates allelic diversity within the species. Similarly high genetic identities among localised populations relative to the mean identity among accessions from throughout much of the range of the species likewise indicate that the same high frequency alleles occur at the local level and across broader geographic regions. Whereas patterns of geographic variation were detected in certain other widely distributed species of *Lemnaceae* (Landolt & Crawford, 1993, 1995), none was

found in the present survey. No differences were found, for example, between Old and New World populations, as has been detected in another species of *Lemna* (Crawford & al., unpubl.).

Next, consideration will be given to genotypic diversity within a local population of *L. aequinoctialis* and available data on reproduction in *Lemna*. Four different multilocus genotypes were found among the 16 progeny from population 9105 of *L. aequinoctialis*, but no heterozygotes were found at any of the loci variable in the population. Unlike many species of *Lemnaceae*, the flowers of *L. aequinoctialis* are not protogynous, and Landolt (1986) demonstrated that self-pollination and subsequent seed set can occur in the species when in cultivation. This observation helps explain the lack of heterozygotes among the progeny; that is, self-fertilisation would perpetuate the same multilocus genotypes, each of which is homozygous for different alleles at several loci. The two localised series of populations of *L. aequinoctialis* from Botswana (9084–9108) and Zimbabwe (9043–9082) each consist of more than one multilocus genotype, with three found among the six individuals of the former and six among the 14 individuals of the latter. As in population 9105, no heterozygotes were seen in plants from these local populations, but the sampling of single individuals from populations obviously precludes making any assessment of heterozygosity within populations. Vasseur & al. (1993) examined allozyme diversity in eight local populations of *Lemna minor* (sect. *Lemna*) from Ontario, Canada. In that study, sampling of individuals (28–30 individuals/population) was much more extensive than in the present investigation of *L. aequinoctialis*, and a mean of nearly 20 genotypes was found in each population. Vasseur & al. (1993) found large deviations from expected heterozygosities in all populations, with both excesses and deficiencies seen. The two species of *Lemna* differ in that *L. aequinoctialis* flowers frequently and is self-compatible whereas *L. minor* flowers very infrequently, rarely sets fruit when flowering occurs, and is self-incompatible (Landolt, 1986: 175). Vasseur & al. (1993) explained deviations from Hardy-Weinberg equilibrium seen in *L. minor* as the result of the propagation of multilocus genotypes by vegetative reproduction. This same phenomenon has also been observed in the clonal submersed aquatic *Ceratophyllum* (Les, 1991) and in other macrophytes (Triest, 1991). Vasseur & al. (1993) further hypothesised that the presence of genotypic diversity within populations of *L. minor*, which reproduces largely by vegetative propagation, is the result of dispersal of different genotypes into local populations and/or somatic mutations. Additionally, the presumably very rare occurrences of sexual reproduction could generate additional genotypic diversity. In *L. aequinoctialis*, the much more frequent flowering and seed set compared to *L. minor* could, as mentioned above, perpetuate different multilocus genotypes within a population if seed set were the result of self-fertilisation and the different multilocus genotypes were homozygous at all the loci. Under this scenario, the situation would be the same in both *L. aequinoctialis* and *L. minor*, that is, both vegetative reproduction and self-fertilisation would perpetuate multilocus genotypes. By contrast, vegetative propagation would perpetuate multilocus genotypes with at least the presence of heterozygotes at one or more loci, whereas sexual reproduction via self-fertilisation would result in segregation at the heterozygous loci and produce heterozygote

deficiencies, as were seen at three loci in population 9105 in the present study. Outcrossing between different multilocus genotypes in *L. aequinoctialis* during the frequent flowering in the species could generate heterozygotes; however, essentially nothing is known about the reproductive biology of any species of *Lemna* in natural populations. Many aquatic plants appear to possess low levels of genetic variability in comparison to terrestrial species, presumably an indication of selection for genetic uniformity in aquatic habitats (Les, 1988; Philbrick & Les, 1996).

Because of the limited sampling within *L. perpusilla* and *L. tenera*, very little discussion of geographic variation within them is warranted. However, considerable diversity was detected among the three accessions of *L. perpusilla* examined, even when compared to several widely distributed species of duckweeds in which many clones were sampled (Crawford & Landolt, 1993). The causes of the high diversity among these few samples remain unknown. This species is similar to *L. aequinoctialis* in that it flowers and sets fruit with high frequency, it is not protogynous, and it is possible (but has not been demonstrated) that it can self-pollinate (Landolt, 1986).

The distribution and ecology of *L. tenera* were discussed by Landolt (1992). The species is unique among duckweeds in that it grows in forest swamps. It was once thought to be endemic to southeastern Asia, where it is now less common because human activities have destroyed native habitats. It has since been collected in Australia (Landolt, 1992), and more recently in Vietnam. Flowering was observed in culture, but no fruits developed (Landolt, 1992); to our knowledge, no fruits have ever been observed in *L. tenera*. Due to the paucity and poor quality of material from Southeastern Asia, it was not possible to obtain adequate material for allozyme studies. Thus, the allozyme results reported herein are from four accessions from Australia, and no diversity was seen among them. However, plastid sequences from a Vietnam and an Australia accession indicate that the two accessions differ by two substitutions and two gaps in the *rpl16* intron (Les & al., in press). Given the plastid sequence divergence, it is possible that allozyme differences occur between accessions of *L. tenera* from the two geographic localities.

In general, low genetic identities at allozyme loci have been found between congeneric species of duckweeds, even those with few distinguishing characters; genetic identities above 0.50 have rarely been found, and then only between those species which are nearly inseparable morphologically (Crawford & Landolt, 1993, 1995; Crawford & al., 1996, 1997). The characters used to separate *Lemna aequinoctialis* and *L. perpusilla* are not particularly pronounced, even by duckweed standards (reviewed by Landolt, 1986). Vegetative fronds of the two species are very similar but several features of the fruits and seeds are diagnostic, and technical characters such as statistical differences in the widths of the root sheaths are also useful. In contrast to minimal morphological and anatomical differentiation between *L. aequinoctialis* and *L. perpusilla*, there are some rather pronounced ecological differences between the two species. The seeds of *L. aequinoctialis* are released from the fruits when mature and germinate quickly when the temperature is warm. By contrast, in *L. perpusilla* seeds remain within the fruits and sink to the bottom of the water with the dead fronds and require low temperatures for a period of time before germination will occur (Landolt, 1986). Because the fronds of both species

are frost sensitive, they can over-winter only as seeds. *Lemna aequinoctialis* will not form flowers and fruits under cooler temperatures. This means that *L. aequinoctialis* is unable to survive in areas where there are cold winters except in those instances where the seeds fail to germinate quickly during the warm season (with the resulting fronds killed by the cold) because the locality has dried out and remains dry throughout the winter. With the onset of warm weather the next season, the seeds could then germinate (Landolt, 1986). These ecological differences no doubt account for the different distributions of the two species, with *L. aequinoctialis* primarily tropical and subtropical, and *L. perpusilla* temperate in distribution (Fig. 1). The genetic identity of 0.65 between *L. aequinoctialis* and *L. perpusilla* at ten allozyme loci is exceedingly high for congeners of duckweeds, but the lowered identity between the two species relative to accessions of the same species provide strong support for the view of Landolt (1986) that the eastern and central North American populations should be recognised as a separate species and not as variants of *L. aequinoctialis*. The pronounced ecological differences between the two species provide additional support for taxonomic recognition. The hypothesis that *L. aequinoctialis* and *L. perpusilla* are closely related yet distinct species is concordant with plastid sequence data because divergence between the two species in the combined *rpl16*, 5' *trnK*, and 3' *trnK* introns is only 0.79 percent, which is over 30 times lower than the divergence between them and any other species of duckweeds (Les & al., in press). Also, in the phylogeny based on plastid sequences, the two are sister species in a very strongly supported clade (Fig. 3). One plausible evolutionary hypothesis is that *L. perpusilla* originated from peripheral populations of *L. aequinoctialis* (or a common ancestor of the two species) that were better adapted to colder climates. Temperate ecotypes of *L. aequinoctialis* (= *L. paucicostata*) are known (Beppu & Takimoto, 1981a), suggesting that the genetic diversity necessary for differentiation of *L. perpusilla* resides within *L. aequinoctialis*. Beppu & Takimoto (1981b) further discussed cytological and ecological variation in *L. aequinoctialis* in Japan, and Beppu & al. (1985) described a new species, *L. aoukikusa* with two subspecies, to accommodate this variation. Landolt (1986) commented that the features used to distinguish the new species from *L. aequinoctialis* are reliable for Japanese material; however, when variation within *L. aequinoctialis* is considered on a world-wide basis the characters used to segregate the Japanese species fall within the variation found in *L. aequinoctialis*. One subspecies of *L. aoukikusa* has higher chromosome numbers ($2n = \text{ca. } 70$, with counts ranging from 66 to 84) than the $2n = 40$ common in the other subspecies of *L. aoukikusa* and in *L. aequinoctialis* (Urbanska-Worytkiewicz, 1980; Landolt & Urbanska-Worytkiewicz, 1980; Beppu & al., 1985). The one Japanese accession included in the present study is from southern Japan and belongs to *L. aequinoctialis* s.str., thus, whether the Japanese species is distinct at allozyme loci remains unresolved. Indeed, the question of whether variants of *L. aequinoctialis* are worthy of species recognition as *L. aoukikusa* is in need of intensive study including consideration of morphological variation in *L. aequinoctialis* on a global scale.

The hypothesis of Landolt (1986) that *L. perpusilla* is of hybrid origin with *L. aequinoctialis* as one parent and *L. turionifera* as the other is not supported by allozyme data; *L. turionifera* is highly divergent from both *L. aequinoctialis* and *L.*

perpusilla, but none of its unique alleles was found in *L. perpusilla*. As indicated earlier, the plastid sequence data likewise do not support the hybrid origin of *L. perpusilla* because it has unique sequences rather than the sequences of either *L. aequinoctialis* or *L. turionifera*.

Landolt (1986: 490) erected sect. *Biformes* with *Lemna tenera* as the only member, suggesting that *L. tenera* forms a kind of transition between the sections *Alatae* and *Uninerves*. Plastid sequences provide support for this concept because, as mentioned above, *L. tenera* is basal to *L. aequinoctialis* and *L. perpusilla* in a strongly supported clade and this in turn is sister to sect. *Uninerves* in a strongly supported clade (Fig. 2). Genetic identities at allozyme loci between *L. tenera* and both *L. aequinoctialis* and *L. perpusilla* are very low and suggest that the common ancestor of *L. tenera* and *L. aequinoctialis*-*L. perpusilla* diverged much earlier than divergence of the latter two species from a common ancestor. This hypothesis is supported by sequence divergence in the combined *rpl16*, 5' *trnK*, and 3' *trnK* introns because the divergence between *L. tenera* and *L. aequinoctialis* is 3.0 percent and between *L. tenera* and *L. perpusilla* it is 2.6 percent as compared to the aforementioned 0.79 percent divergence between *L. aequinoctialis* and *L. perpusilla* (Les & al., in press). The assignment of *L. tenera* to its own section and the inclusion of the other two species in another section, as was done by Landolt (1986), is fully justified by the molecular data (Fig. 3).

Acknowledgements

Financial support was provided by NSF grant DEB-9806537 to DJC and DHL. E. Tepe provided technical assistance.

Literature cited

- Beppu, T. & Takimoto, A. 1981a. Growth of various ecotypes of *Lemna paucicostata* in Japan under various temperature conditions, and their wintering forms. *Bot. Mag. Tokyo* 94: 107–114.
- & – 1981b. Geographical distribution and cytological variation of *Lemna paucicostata* Hegelm. in Japan. *Bot. Mag. Tokyo* 94: 11–20.
- , Yanase, D., Nobuchi, T. & Murata, G. 1985. A revision of *Lemna paucicostata* group in Japan. *Acta Phytotax. Geobot.* 36: 45–58. [In Japanese with English summary, tables and figure legends.]
- Crawford, D. J. & Landolt, E. 1993. Allozyme studies in *Spirodela* (*Lemnaceae*): variation among conspecific clones and divergence among the species. *Syst. Bot.* 18: 389–394.
- & – 1995. Allozyme divergence among species of *Wolffia* (*Lemnaceae*). *Pl. Syst. Evol.* 197: 59–70.
- , – & Les, D. H. 1996. An allozyme study of two sibling species of *Lemna* (*Lemnaceae*) with comments on their morphology, ecology and distribution. *Bull. Torrey Bot. Club* 123: 1–6.
- , – , – & Tepe, E. 1997. Allozyme variation and the taxonomy of *Wolffiella* (*Lemnaceae*). *Aquat. Bot.* 58: 43–54.
- Davis, B. J. 1964. Disc-electrophoresis. II. Methods and application to human serum protein. *Ann. New York Acad. Sci.* 121: 1177–1184.
- Gottlieb, L. D. 1981. Gene number of species of *Astereae* that have different chromosome number. *Proc. Natl. Acad. Sci. U.S.A.* 78: 3726–3729.
- 1982. Conservation and duplication of isozymes in plants. *Science* 216: 373–380.
- Kandeler, R. & Huegel, B. 1974. Wiederentdeckung der echten *Lemna perpusilla* Torr. und Vergleich mit *L. paucicostata* Hegelm. *Pl. Syst. Evol.* 123: 83–96.
- Landolt, E. 1986. The family of *Lemnaceae*—a monographic study, vol. 1. *Veröff. Geobot. Inst. ETH Stiftung Rübel, Zürich* 71: 1–566.

- 1992. *Lemna tenera* Kurz, a little known species of *Lemnaceae*. *Geobot. Inst. ETH, Stiftung Rübel, Zürich* 58: 124–131.
- & Urbanska-Worytkiewicz, K. 1980. List of the studied *Lemnaceae* samples: origin and chromosome numbers. *Veröff. Geobot. Inst. ETH, Stiftung Rübel, Zürich* 70: 205–247.
- Les, D. H. 1988. Breeding systems, population structure, and evolution in hydrophilous angiosperms. *Ann. Missouri Bot. Gard.* 75: 819–835.
- 1991. Genetic diversity in the monoecious hydrophile *Ceratophyllum* (*Ceratophyllaceae*). *Amer. J. Bot.* 78: 1070–1082.
- & Crawford, D. J. 1999. *Landoltia* (*Lemnaceae*), a new genus of duckweeds. *Novon* 9: 530–533.
- , – , Landolt, E., Gabel, J. D. & Kimball, R. T. In press. Phylogeny and systematics of *Lemnaceae*, the duckweed family. *Syst. Bot.* 27.
- , Landolt, E. & Crawford, D. J. 1997. Systematics of *Lemnaceae*: inferences from micro-molecular and morphological data. *Pl. Syst. Evol.* 204: 161–177.
- Lewis, P. O. 1993. *GeneStat-PC3.3*. Raleigh, North Carolina.
- Nei, M. 1972. Genetic distance between populations. *Amer. Naturalist* 106: 283–293.
- 1973. Analysis of genetic diversity in subdivided populations. *Proc. Natl. Acad. Sci. U.S.A.* 70: 3321–3323.
- Philbrick, C. T. & Les, D. H. 1996. Evolution of aquatic angiosperm reproductive systems. *BioScience* 46: 813–826.
- Triest, L. 1991. Isozymes in water plants. *Opera Bot. Belg.* 4: 1–264.
- Urbanska-Worytkiewicz, K. 1980. Cytological variation within the subfamily *Lemnaceae*. *Veröff. Geobot. Inst. ETH, Stiftung Rübel, Zürich* 70: 30–101.
- Weeden, N. F. & Wendel, J. F. 1989. Genetics of plant isozymes. Pp. 46–72 in: Soltis, D. E. & Soltis, P. S. (eds.), *Isozymes in biology*. Portland, Oregon.
- Wendel, J. F. & Weeden, N. F. 1989. Visualization and interpretation of Isozymes. Pp. 5–45 in: Soltis, D. E. & Soltis, P. S. (eds.), *Isozymes in biology*. Portland, Oregon.