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An allozyme study of two sibling species of *Lemna* (Lemnaceae) with comments on their morphology, ecology and distribution

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CRAWFORD, D. J. (Department of Plant Biology, The Ohio State University, Columbus, OH 43210), E. LANDOLT (Geobotanisches Institut ETH, Zurichbergstrasse 38, CH-8044, Zurich, Switzerland) AND D. H. LES (Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, CT 06269). 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. 1996.—Enzyme electrophoresis was employed to examine divergence between *Lemna minuta* H.B.&K. and *L. valdiviana* Phil., two nearly indistinguishable species comprising sect. *Uninerves* of *Lemna*. Fifteen presumptive loci were resolved for 25 clones of *Lemna minuta* and 26 of *L. valdiviana*. Genetic identity between the two species is 0.70, which is near the mean value for congeneric species of flowering plants, but is much higher than values reported for other species of Lemnaceae. With the exception of two clones, the taxonomic designations of the strains correlated with monomorphism for alternative alleles at two MDH loci. The two species are highly differentiated at two other loci as well. Although the taxa are morphologically similar, they differ in geographic distribution, flavonoid chemistry, and certain physiological and ecological attributes. The relatively high identity at allozyme loci compared to other Lemnaceae suggests that *L. minuta* and *L. valdiviana* are closely related and recently diverged sister species.

Key words: *Lemna minuta, Lemna valdiviana,* allozymes, genetic identity.

The Lemnaceae (duckweeds) consist of four genera and some thirty-six species (Landolt 1986, 1984). Although a small family in terms of species numbers, duckweeds are morphologically reduced freshwater plants of cosmopolitan distribution. The taxonomic difficulty of duckweeds is due primarily to their highly reduced bodies (referred to as "fronds" or "thalli") which offer few morphological characters for comparison. Also, certain species may have diverged recently, and therefore appear to share many of the same characters.

The two species *Lemna minuta* H.B.&K. and *L. valdiviana* Phil. comprise sect. *Uninerves,* and they are difficult to distinguish morphologically even considering the usual problems associated with identifying duckweeds (Landolt 1986). Also, the two taxa are viewed as the most morphologically reduced members of the genus *Lemna* (Landolt 1986). In his monograph, Hegelmaier (1868) did not distinguish *L. minuta* from *L. valdiviana.* He did, however, characterize and name with a question mark two forms differing from the typical form. These were designated as forma *pellucida* and forma *minima,* and they were recognized primarily on length-width ratios of the fronds. The typical form of *L. valdiviana* has a ratio of 1:1.86–2.6, whereas forma *minima* has a ratio of 1:1.4–1.85 and forma *pellucida* is intermediate. In a later publication, Hegelmaier (1895) elevated one forma to species status (as *L. minima*) and at the same time indicated that there was no distinction between it and some forms of *L. valdiviana.* Daubs (1965) recognized both species and pointed to the fact that in *L. valdiviana* the base is asymmetrical and often 8–10 fronds stay attached whereas in *L. minima* (=*L. minuta*) the base is more symmetrical and more than two fronds rarely remain attached. All the characters, however, are subject to considerable variation under various environmental influences. Landolt (1986) indicated that under certain conditions the two species form fronds that are nearly identical, and that external factors can affect the size and shape of the fronds. The best character for distinguishing the two species is the length of

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the single nerve in the frond relative to the extension of the air spaces. This, however, must be determined from fixed microscopic preparations, and there are other difficulties in evaluating the feature (Landolt 1986). A recent cladistic study of Lemnaceae using 40 morphological and anatomical characters indicates that these two species are sister taxa and form the monophyletic sect. *Uninerves* (Les, Landolt and Crawford, unpubl. data). Results of the cladistic analysis also show this section to be the most highly derived within *Lemna*.

The two species differ somewhat in distribution and ecology. *Lemna minuta* occurred originally in warm temperate zones of North and South America. In the tropics it is restricted to higher elevations. The New World distribution is similar, but slightly larger than *L. gibba*. However, *L. gibba* is also wide-spread in Europe, Asia and Africa. In recent times *L. minuta* has been introduced into Europe and Japan (Landolt 1986). *Lemna valdiviana* tends to occur in the warm temperate to tropical areas of America, primarily in more humid climates as compared to the areas where *L. minuta* is typically found. It does well in stable habitats where it can grow submerged. As *L. minuta* is able to grow in more different types of habitats, it may be transplanted more easily by birds, thus allowing its introduction into Europe and Asia. Both species, as with most members of the Lemnaceae, propagate nearly exclusively vegetatively. Flowers and fruits may occur occasionally, but very rarely and locally. Out of 500 herbarium specimens of *L. valdiviana* and 700 specimens of *L. minuta* investigated, 3% and 5%, respectively, were flowering and half of the flowering specimens showed fruits (Landolt 1986). In culture, flowering occurs only if some chemicals were added, and fruiting was never observed.

In the present study we used enzyme electrophoresis to ascertain whether plants now assigned to *Lemna minuta* and *L. valdiviana* show lowered genetic similarity relative to clones of the same species. Allozymes were employed because they reflect discrete genetic differences and thus represent a potentially useful method for assessing whether one or two gene pools exist within what has been treated as two taxa. Allozymes provided useful data for distinguishing species in *Spirodela* (Crawford and Landolt 1993) and *Wolffia* (Crawford and Landolt, unpubl. data), and thus might be of value for examining the two species of *Lemna*. We also review other data available for these two species and discuss it relative to the allozyme data.

Materials and Methods. Twenty-five clones (strains) of *Lemna minuta* and 26 of *L. valdiviana* were examined electrophoretically. In each case, the strains originated from a single frond from each population, and therefore no assessment of diversity within populations was attempted. Since fruiting never occurred for the two species, the strains should be genetically identical (except for possible somatic mutations) throughout the entire period of cultivation. Clones were selected from throughout the known geographic ranges of the two species, and their origins are given in Table 1. All strains are kept in Zurich in a living collection of 950 clones representing 35 identified species of Lemnaceae. Plants were ground in 0.1 M tris-HCl buffer, pH 7.5, with 14 mM 2-mercaptoethanol, 1 mM EDTA (tetrasodium salt), 10 mM $MgCl_2$, 10 mM KCl, and 5–10 mg polyvinylpolypyrrolidine per 0.5 ml of buffer (Gottlieb 1981).

Polyacrylamide gels employing the methods of Crawford et al. (1987) were used to resolve the following enzymes: glutamate dehydrogenase (GDH, E.C. 1.4.1.2), glyceraldehyde-3-phosphate dehydrogenase (G3PDH NAD-dependent form, E.C. 1.2.1.12), isocitrate dehydrogenase (IDH, NADP form, E.C. 1.1.1.42), phosphogluconate dehydrogenase (PGD, E.C. 1.1.1.44), shikimate dehydrogenase (SKDH, E.C. 1.1.1.25), and superoxide dismutase (SOD, E.C. 1.15.1.1).

Two buffer systems were used to resolve enzymes in 12.5% starch gels. One buffer system used an electrode buffer of 0.5 M tris, 0.65 M boric acid, 0.02 M EDTA at a pH of 8.0, and a 1:9 dilution (with distilled H_2O) of the electrode buffer as the gel buffer. Enzymes resolved with this buffer system were: glucose-6-phosphate isomerase (GPI, E.C. 5.3.1.9) and triose-phosphate isomerase (TPI, E.C. 5.3.1.1). A second system consisted of an electrode buffer of 0.04 M citric acid with N(3-aminopropyl)-morpholine used to adjust the pH to 6.1; a 1:19 dilution (with distilled H_2O) of the electrode buffer served as the gel buffer. Malate dehydrogenase (MDH, E.C. 1.1.1.37) was resolved with this system. The nomenclature of the enzymes and staining protocols were those of Wendel and Weeden (1989).

Several kinds of information were used to infer the genetic basis of the banding patterns ob-

Table 1. Geographic origins of clones of *Lemna* examined for allozymes.

Species and collection number[a]	Locality
L. minuta	
6584	U.S.: California: Alameda Co.
6600	U.S.: California: Mono Co.
6747	U.S.: California: Mariposa Co.
6744	U.S.: California: Tuolumne Co.
6752	U.S.: Nebraska: Garden Co.
8065	U.S.: Texas: Brazoria Co.
6737	U.S.: Washington: Asotin Co.
6717	Guatemala: Chimaltenango
7369	Argentina: Tucuman
7921	Argentina: Buenos Aires
8662	Argentina: Corrientes
8807	Argentina: Salta
8808	Argentina: Rio Negro
8904	Bolivia: Potosi
8982	Colombia: Cundinamarca
7612	Peru: Cuzco
7726	Chile: Valparaiso
8686	Chile: Valdivia
8894	Chile: Cocquimbo
8430	England: Cambridge
8406	France: Gironde
8370	Switzerland: Aargau
8951	Greece: Macedonia
8699	Japan: Tokyo
8702	Japan: Kyoto
L. valdiviana	
7514	U.S.: Arkansas: Pope Co.
7546	U.S.: California: San Luis Obispo Co.
7494	U.S.: Florida: Glades Co.
7005	U.S.: Florida: Wakulla Co.
7803	U.S.: Illinois: Chicago
7002	U.S.: Louisiana: Pope Co.
7116	U.S.: North Carolina: Columbus Co.
7161	U.S.: Texas: Travis Co.
7592	U.S.: Virginia: Lancaster Co.
7718	Dominican Republic: Samana
8643	Jamaica: Manchester Par.
7653	Mexico: Tabasco
8411	Panama: Panama City
8821	Argentina: Formosa
7288	Brazil: Sao Paulo
7362	Brazil: Amazonas
8845	Brazil: Rio de Janeiro
8851	Brazil: Rio de Janeiro
8685	Chile: Temuco
7186	Colombia: Bogota
8754	Bolivia: Huatajata
8897	Ecuador: Guayaquil
7614	Peru: Puno
7356	Surinam: Paramaribo
7329	Trinidad: St. Augustine
7408	Venezuela: Carabobo

[a] Collection numbers are those of Landolt.

served in gels. These included the known active subunit composition of the enzymes (Weeden and Wendel 1989), and the number of isozymes of each enzyme normally present in diploid plants (Gottlieb 1982; Weeden and Wendel 1989).

Alleles present in the strains were used to calculate allelic frequencies for each species. These frequencies were then used to compute genetic identities and distances between the two species (Nei 1972) and GENESTAT (Lewis and Whitkus 1989) was employed for the calculations.

Results. A total of fifteen presumptive loci was scored for the two species of *Lemna*, including: GPI (2); GDH (1); G3PDH (1); IDH (2); MDH (2); PGD (2); SKDH (2); SOD (1); and TPI (2). Allelic frequencies for the two species are available from the senior author. The genetic identity for the two species is 0.70 for these 15 loci. Additional loci were expressed for MDH and SOD, but activity and/or resolution were not adequate for interpretation. The number of isozymes detected for most of the enzymes (e.g., GDH, GPI, IDH, PGD, SKDH, TPI) was the same as normally present in diploid plants (Gottlieb 1982; Weeden and Wendel 1989) despite the fact that 31 of the 51 clones used in the present study have been counted and have a tetraploid chromosome number of $2n = 40$ (Landolt 1986; Landolt and Urbanska 1980).

The two species are monomorphic for alternative alleles at *Mdh-1* and *Mdh-2* (but see Discussion section). Also, at *Gpi-1* and *Pgd-1*, each species has a unique allele present in a frequency greater than 0.10. Lastly, *L. valdiviana* has a unique allele at *Gdh* (frequency of 0.55), and both *L. minuta* and *L. valdiviana* each have three unique alleles present in frequencies less than 0.10. Both species are monomorphic for the same alleles at the remaining loci.

Discussion. Allozymes have sometimes proven useful for distinguishing plant species, even when the morphological differences between the taxa are not pronounced (e.g., Crawford and Bayer 1981; Jeffries and Gottlieb 1982). They can serve as useful markers and give some indication of whether the taxa represent distinct gene pools. The fewer the morphological features available for comparison, the more difficult it is to assess relationships between taxa. This is a particular problem in

Lemnaceae because of the extreme reduction of their plant bodies (Landolt 1986). With such extensive reduction it is often difficult, if not impossible, to determine whether species appear similar because of the paucity of characters for distinguishing them and/or because they have diverged recently and few or no distinguishing features have evolved. Within the duckweeds, Landolt (1986) viewed *Lemna minuta* and *L. valdiviana* among the most difficult species to distinguish, and in some instances it becomes nearly impossible to assign a given plant to one of them (Landolt 1986, pers. observ.). Given this morphological similarity, it is somewhat surprising that nearly every clone assigned to a species by one of us (EL) could be grouped with other clones identified as the same species using enzyme electrophoresis. The two diagnostic loci in nearly every case are *Mdh-1* and *Mdh-2*. In only two instances did the MDH profiles not "match" the species assignment. In particular, one clone (*Landolt 7921*) from Argentina and another (*Landolt 8065*) from Texas had been identified as members of *L. minuta* but had the MDH alleles otherwise typical of *L. valdiviana*. Interestingly, these two clones were named with extreme difficulty by one of us (EL), and in fact the name for *8065* was changed three times before *L. minuta* was rather arbitrarily assigned to it. Similar difficulties were encountered in placing a name on *7921*.

There appear to be some biochemical and physiological distinctions between the two taxa. McClure (1964) surveyed the Lemnaceae for flavonoids, and several of the strains he examined were the same ones included in the present study. McClure could not distinguish the species morphologically unless they were growing in tap water with very low nutrient content. He could, however, distinguish the two on the basis of flavonoid chemistry. *Lemna minuta* (McClure examined 11 strains under the name of *L. valdiviana*) contained four C-glycosylflavones and three additional unidentified compounds, none of which were glycosides. McClure (1964) also examined five strains of *L. valdiviana* (under the name of *L. minima*) and found three C-glycosylflavones only one of which (vitexin) was shared with *L. minuta*. In addition, *L. valdiviana* had four O-glycosides of apigenin and four unidentified compounds similar to the O-glycosides. Two exceptions were found by McClure; they are referable to *L. valdiviana* on a morphological basis but had the flavonoid chemistry of *L. minuta*. In most cases, it appears that the morphology of the two species is correlated with quite distinctive flavonoid chemistry, but exceptions are found occasionally.

Landolt (1957) carried out experiments in which the two species were grown under various temperature, light and nutrient conditions. Seven strains of *L. minuta* and two strains of *L. valdiviana* were included in the study. Under most conditions, variation between different strains of the same species was as great or greater than between the species. However, growth in the dark was different between six strains of *L. minuta* and the two strains of *L. valdiviana*. *Lemna minuta* was able to grow in the dark with a nutrient solution containing only sugar, whereas *L. valdiviana* required the addition of yeast extract and casamino acids for regular growth. Although sample sizes (particularly for *L. valdiviana*) were very small in these experiments, the results do suggest a difference between the two species. An interesting ecological difference between the two species is the ability of *L. valdiviana* to grow submerged. Whereas *L. minuta* always lives on the surface of the water, *L. valdiviana* develops under the heavy competition of other pleustophytes or under unfavorable nutrient conditions below the surface of the water even if it is densely covered by the leaves of other species. The submerged fronds have the advantage of obtaining newly-released nutrients from the bottom soil. However, their primary uptake is from substances dissolved in the water.

Our results suggest that, although the two species are very similar morphologically, with rare exceptions someone familiar with *Lemna* can sort them into two distinct groups and that these groups correspond with enzyme banding patterns. Previous allozyme studies (Crawford and Landolt 1993; 1995) in Lemnaceae have produced similar results. In nearly all instances, there has also been geographic integrity to the distribution of the enzyme phenotypes, and in certain cases in *Wolffia*, allozymes correlated with morphological characters as well, and additional species were recognized (Landolt 1994). This situation where populations are differentiated at allozyme loci but are distinguishable with difficulty seems to fit into the category of sibling species as defined for plants by Grant (1981). The genetic identity of 0.70 for *Lemna minuta* and *L. valdiviana* is similar to the mean value for congeneric species of flowering plants (Gott-

lieb 1977; Crawford 1989), but within Lemnaceae 0.70 is very high for congeners, and even within what have been viewed as the same species of *Wolffia,* populations from different geographic areas have identities lower than exhibited by *L. minuta* and *L. valdiviana* (Crawford and Landolt, in press).

The very low genetic identities found among species of *Spirodela* and *Wolffia* despite their perceived morphological similarities are viewed as the result of ancient divergence with few characters available for comparison. Given the relatively high allozyme similarity for *L. minuta* and *L. valdiviana* and their morphological similarity (even by duckweed standards), it may be that their divergence has been recent relative to many other congeners in Lemnaceae. The cladistic analysis indicates that these are sister species and the high allozyme similarity likewise suggests that they are more closely related to each other than either is to other species.

The differentiation between *L. valdiviana* and *L. minuta* is primarily ecogeographic in nature. *Lemna valdiviana* grows mainly in the humid parts of the subtropic and tropic zones of South and Central America, and it has radiated northwards along the eastern coast of North America. By contrast, *L. minuta* is a species of drier warm temperate climates in South America and goes northward along the high mountains to Mexico and California. It is possible that the species radiated quickly following their divergence from a common ancestor, because Lemnaceae can colonize new habitats quite rapidly (Landolt 1986). Dispersal is primarily by water birds and it can occur over many kilometers per year. For example, *L. minuta* was first observed in western Europe (Biarritz, France) in 1965, and during the past 20 years it has expanded some 3000 km to southern Russia and Greece (Landolt 1986). In general, Lemnaceae are dependent on relatively high amounts of phosphorus and nitrogen in the water. Under natural conditions, few waters correspond to these needs. Humans have created many new possibilities for successful growth of Lemnaceae with new lakes, water reservoirs, ponds and ditches. The eutrophication of these waters has enabled the Lemnaceae to spread into regions otherwise not accessible to them. In this way, two newly differentiated units could come into contact and hybridize, although presumably this would be rare given how infrequently these two species flower. Still, hybridization may help explain the rare "exceptions" found in which the allozymes and/or flavonoid chemistry do not correspond with the morphology of the plants of *Lemna.*

Literature Cited

CRAWFORD, D. J. 1989. Enzyme electrophoresis and plant systematics, pp. 146–164. *In* D. E. Soltis and P. S. Soltis [eds.], Isozymes in plant biology. Dioscorides Press, Portland.

——— AND R. J. BAYER. 1981. Allozyme divergence in *Coreopsis cyclocarpa* (Compositae). Syst. Bot. 6: 373–386.

———, T. F. STUESSY AND M. SILVA O. 1987. Allozyme divergence and the evolution of *Dendroseris* (Compositae: Lactuceae) on the Juan Fernandez Islands. Syst. Bot. 12: 436–443.

——— AND E. LANDOLT. 1993. Allozyme studies in *Spirodela* (Lemnaceae): variation among conspecific clones and divergence among species. Syst. Bot. 18: 389–394.

——— AND ———. 1995. Allozyme divergence among species of *Wolffia.* Plant Syst. Evol. 197: 59–69.

DAUBS, E. H. 1965. A monograph of Lemnaceae. Illinois Biolog. Mon. 34. 118 pp.

GOTTLIEB, L. D. 1977. Electrophoretic evidence and plant systematics. Ann. Missouri Bot. Gard. 64: 161–180.

———. 1981. Gene number in species of Asteraceae that have different chromosome number. Proc. Natl. Acad. Sci. USA 78: 3726–3729.

———. 1982. Conservation and duplication of isozymes in plants. Science 216: 373–380.

GRANT, V. 1981. Plant speciation. 2nd Ed. Columbia Univ. Press, New York. 388 pp.

HEGELMAIER, F. 1868. Die Lemnaceen. Eine monographische Bearbeitung. Verlag W. Engelmann, Leipzig. 169 pp.

———. 1895. Systematische Ubersicht der Lemnaceen. Botanische Jahrbucher 21: 268–305.

JEFFRIES, R. L. AND L. D. GOTTLIEB. 1982. Genetic differentiation of the microspecies *Salicornia europaea* L. (sensu stricto) and *S. ramosissima,* J. Woods. New Phytol. 92: 123–129.

LANDOLT, E. 1957. Physiologische und okologische Untersuchungen an Lemnaceen. Ber. Schweiz. Bot. Ges. 67: 271–410.

———. 1986. The family Lemnaceae—A monographic study 1. Veroff. Geobot. Inst. ETH, Stiftung Rubel, Zurich. 71. 566 p.

———. 1994. Taxonomy and ecology of the section *Wolffia* of the genus *Wolffia* (Lemnaceae). Ber. Geobot. Inst. ETH, Stiftung Rubel, Zurich. 60: 137–151.

——— AND K. URBANSKA. 1980. List of the studied Lemnaceae samples: Origin and chromosome numbers. Veroff. Geobot. Inst. ETH, Stiftung Rubel, Zurich. 70: 205–247.

LEWIS, P. AND R. WHITKUS. 1989. GENESTAT for microcomputers. Amer. Soc. Pl. Tax. Newslet. 2: 15–16.

MCCLURE, J. 1964. Taxonomic significance of the flavonoid chemistry and the morphology of Lemnaceae in axenic culture. Unpubl. Ph.D. Diss. Univ. Texas, Austin, 255 p.

NEI, M. 1972. Genetic distance between populations. Am. Nat. 106: 283–293.

WEEDEN, N. F. AND J. F. WENDEL. 1989. Genetics of plant isozymes, pp. 46–72. *In* D. E. Soltis and P. S. Soltis [eds.], Isozymes in plant biology. Dioscorides Press, Portland.

WENDEL, J. F. AND N. F. WEEDEN. 1989. Visualization and interpretation of plant isozymes, pp. 5–45. *In* D. E. Soltis and P. S. Soltis [eds.], Isozymes in plant biology. Dioscorides Press, Portland.