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Genetic differentiation studies and phylogenetic inference in the plant genus *Limnanthes* (section Inflexae)

C. I. McNeill and S. K. Jain

Department of Agronomy and Range Science, University of California, Davis, CA 95616, USA

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Summary. An electrophoretic survey of genetic variation in 50 populations representing all ten taxa of section Inflexae of the plant genus *Limnanthes* is reported here with three objectives: 1) to describe genetic differentiation for testing certain phylogenetic hypotheses on the origin of species and infraspecific relationships, 2) to evaluate the concordance of electrophoretic, morphological and hybridization data within the section, and 3) to discuss models of speciation using *Limnanthes* as an example. Species, subspecies, and populations, designated on the basis of morphology and distribution, gave decreasing values of genetic distances that were apparently maintained across a wide ecological and geographical range. Using electrophoretic, morphological and hybrid fertility data, we concluded that *L. montana,* intermediate in range between the two disjunct varieties of *L. gracilis,* is not likely to be a relictual set of populations from what once was a continuously distributed taxon as hypothesized by earlier workers. Neither *L.g. gracilis* nor *L.g. parishii* appear to have been founded by long distance dispersal from one to the other. However, a very close genetic relationship was detected between *L. gracilis* and *L. alba.* This genetic pattern suggested that the two disjunct *L. gracilis* varieties were probably connected by a *L. alba*-like taxon and perhaps originated from that taxon. Evidence based on allozyme variation did not support the thesis that the inbreeder *L. floccosa* is a recent derivative from the outbreeder *L. alba.* Among the remaining five taxa (i.e. two varieties of *L. gracilis,* two varieties of *L. alba,* and *L. montana*), genetic distance and interspecific hybridization data are highly concordant, ($r=-0.92$, $P<0.001$). The agreement of these two approaches with species relationships based on morphological similarity was less certain. *Limnanthes* species appear to exhibit greater interpopulation differentiation than many plant groups, perhaps a reflection of their distinctive island-like distribution pattern. On the other hand, an unusually high crossability is found in *Limnanthes.* Speciation in *Limnanthes* appears largely to follow a model of adaptive geographical divergence but certain other modes cannot yet be ruled out.

Key words: Allozyme variation – Genetic distance – Speciation – Systematics – Population genetic hypotheses

Introduction

Genetic variability within and differentiation among natural populations can be directly estimated by data on electrophoretically detectable alleles at specific enzyme loci. Such variation surveys can be useful in solving certain biosystematic and evolutionary problems when applied in conjunction with the data from natural history and comparative studies of morphology, ecology and cytogenetics and when certain limitations of statistical and adaptive arguments are fully understood (Thorpe 1982; Wake 1981; Lewontin 1974). The electrophoretic morphological and hybridization studies in the genus *Limnanthes* reported here follows 30 years of systematic and experimental work begun by Mason (1952) and pursued by Ornduff (1971), Arroyo (1973), and Brown and Jain (1979), in which several interesting hypotheses regarding the phylogeny of the genus and biosystematic observations on infraspecific variation were presented.

Our purpose in this paper is three-fold: 1) to describe genetic differentiation among all of the ten

taxa comprising the section Inflexae of the genus *Limnanthes* and to test hypotheses about the phylogenetic relationships raised by the earlier work and reviewed below; 2) to evaluate the concordance of electrophoretic, morphological, and hybrid fertility data in determining species relationships, and 3) to discuss models of speciation in relation to *Limnanthes* species.

Biosystematic background: some hypotheses

Limnanthes (Limnanthaceae) is a genus with nine species comprised of 13 infraspecific taxa of spring-flowering, annual, diploid ($n=5$) plants. One species (*L. macounii*) is found in British Columbia; all others are native and restricted to California and southern Oregon. They usually grow in dense colonies in moist habitats such as vernal pools, or along temporary streams and wet meadows. This generates an island-like distribution with highly subdivided population structure (Holland and Jain 1977). A variety of breeding systems is found in different taxa ranging from almost complete selfing (due to cleistogamy) to as high as 100% outcrossing due to protandry and gynodioecy (Jain et al. 1978).

Mason (1952) recognized two sections of the genus, viz. Inflexae and Reflexae, on the basis of a morphological character (petals folding inward versus outward during seed maturation); no attemps to make intersectional crosses were successful. Ten taxa belonging to four species, namely *L. gracilis, L. alba, L. montana* and *L. floccosa,* in the Inflexae section, will be discussed here whereas members of the Reflexae section are treated elsewhere (Kesseli and Jain, in preparation). Figure 1 and Table 1 give the distribution and morphological comparisons respectively, as described by Mason (1952) and Arroyo (1973), and based on our own extensive field, greenhouse, and herbarium studies. Sympatry is rare among taxa within the section; only *L. floccosa* and *L. alba* show neighboring sympatry at five out of nearly 65 known sites. Although all taxa are small and white-flowered,

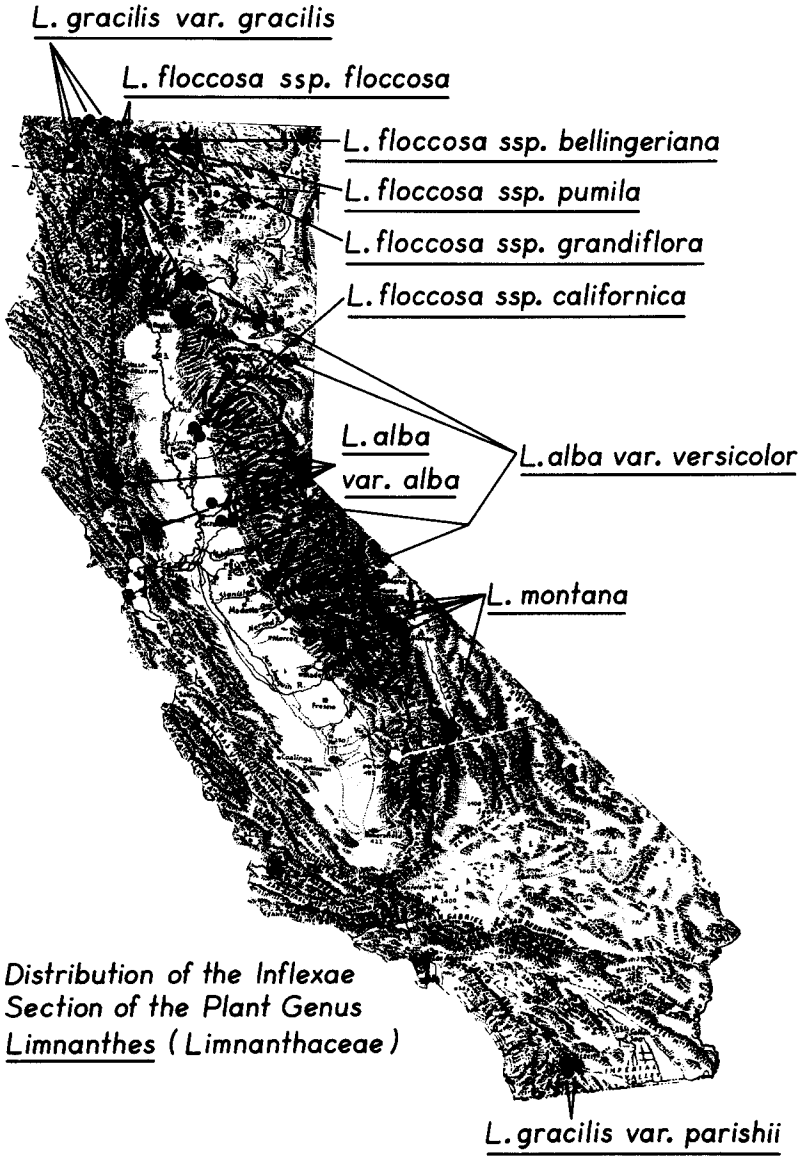

Fig. 1. Map of California and southern Oregon showing the distribution of localities sampled in this study

Table 1. Some morphological features of taxa[a] of the Inflexae section

Character	L.g.g.	L.g.p.	L.a.v.	L.a.a.	L.m.	L.f.f.	L.f.b.	L.f.c.	L.f.g.	L.f.p.
Petal color	white	white	white	white	white	white	white	white	white	white
Petal length (mm)	8.5 – 11.5	8 – 11	9.5 – 12	10.5 – 16	8.5 – 12	5.5 – 8.5	5.5 – 8	8.0 – 9.5	7.5 – 9.5	7.5 – 9
Sepal pubescence	absent	absent	absent to sparse	dense	sparse	dense	absent	dense	sparse to dense	absent
Stem and leaf pubescence	absent	absent	absent to sparse	sparse to dense	sparse	dense	absent	dense	sparse	absent
Pistil length (mm)	3 – 4	2.5 – 3.5	3.5 – 5	4.5 – 6	2.5 – 4	1.5 – 3	2 – 2.5	3.5 – 4	3.5 – 4	3 – 3.5
Stamen length (mm)	3.5 – 4.5	3 – 4	4 – 6	5 – 8	2.5 – 4.5	2 – 4	2 – 3.5	5 – 7	4 – 5	3.5 – 4.5
Flower shape	crateriform to campanulate				funnel-form	urceolate		crateriform		
Color of aging petals	pink	pink	pink	pink	white	pink	white	white		
No. of leaf[b] segments	5 – 9	5 – 9	5 – 9	5 – 9	7 – 11	5 – 9	5 – 9	5 – 9	5 – 9	5 – 9
Nutlet morphology[c]	(6, 6A, 8A) smooth or covered with short or broad conic or sharp tubercules	(6, 6A, 8A)	(6, 6A, 8A, 1)	(6, 6A, 8A, 1)	(8, 8A) abundant small conic tuber-cules	(2, 3) sharply pointed tubercules bearing filamentous apendages	(2) sharply pointed tuber-cular tips	(6A) range of forms from tubercule-less to large conic tubercules	(6A)	(6A)

[a] Names of taxa are abbreviated here (see text for full names)
[b] Leaves are primately dissected with entire, toothed or parted segments (Mason 1952)
[c] see Hauptli et al. (1978) for details of numbers coding various types
Mason's (1952) and Arroyo's (1973a) keys were used to identify almost all of the field populations; however, certain modifications were based on detailed floral and nutlet descriptors (Hauptli et al. 1978; McNeill and Jain, in preparation)

they are distinguishable on the basis of size and shape of leaves, petals and other floral parts as well as by sepal and petal pubescence, and by nutlet morphology.

Mason (1952) described two varieties of *L. gracilis* on the basis of an 800-mile separation. *L. gracilis* var. 'gracilis' occurs in the Klamath Mountain region of southwestern Oregon while *L. gracilis* var. 'parishii' is found only in a few sites in the Cuyamaca Mountains of San Diego County in southern California (Fig. 1). There is no published record of crossing the two *L. gracilis* varieties although we did succeed in obtaining hybrids.

The morphological similarity of the two highly disjunct *L. gracilis* varieties could be due to several factors. First, recurrent gene flow between them might maintain genetic similarity; and second, one variety may be a recent derivation from the other via long distance dispersal. A third hypothesis on their origins was suggested to Mason (1952) by the existence of another related species, *L. montana,* between the two *L. gracilis* varieties along streams through the southern Sierra foothills at elevations of 300 and 1,500 m. He postulated that in the past, *Limnanthes* species were distributed nearly continuously from southern Oregon to southern California and that climatic and geological changes along with extinctions of many *Limnanthes* populations caused subdivisions and subsequent isolation of the various taxa.

Limnanthes alba, a third species in the section, is also quite similar morphologically to *L. gracilis* and *L. montana* and shares a predominantly outcrossing breeding system involving showy white and protandrous flowers which are attractive to insect pollinators. *L. alba* is the most widespread species of the section, occurring in distinct, widely separated populations from the Coast Range east to the Sierra Foothills and from Shasta County south to Tuolumne County (Fig. 1). Mason (1952) described two varieties, namely, *L. alba* var. 'alba' which has large petals, pubescent sepals and tuberculate nutlets and is usually found in vernal pools of the Central Valley, and *L. alba* var. 'versicolor' which has smaller petals, glabrous or slightly pubescent sepals and smooth nutlets, and is found in the Sierra foothills up to an elevation of 1,800 meters. The two varieties can easily be intercrossed and even their distinguishing features show polymorphisms within certain populations. A taxonomic revision is now underway (McNeill et al. in preparation). Their genetic similarity with *L. gracilis* and *L. montana* would be of interest in possibly identifying their phylogenetic relationships.

The fourth species of the section, *L. floccosa,* is a small-flowered species occurring in disjunct populations from Jackson County, in southern Oregon to Butte County, California. Arroyo (1973a) described five subspecies which range in breeding system from essentially full autogamy in *L.f.* ssp. *floccosa* and *L.f.* ssp. *bellingeriana* to partial outcrossing in *L.f.* ssp. *californica, L.f.* ssp. *grandiflora* and *L.f.* ssp. *pumila,* with larger petals and slight protandry. On the basis of morphological similarity, geographical proximity and certain ecological observations, she suggested that *L.f.* ssp. *californica* was a recent derivative of *L. alba,* and that the other subspecies of *L. floccosa* represented progressive evolution toward complete autogamy from *L.f.* ssp. *californica.* This hypothesis is of special interest in relation to the various theoretical ideas on the evolution of inbreeding in plants (Jain 1976).

Materials and methods

Allozyme variation and certain morphological characters were measured in 50 natural populations of a total of ten taxa representing their known geographic range and habitat diversity. (Information on collection localities is on authors' file and is available by request.) Four taxa (viz. *L.f. bellingeriana, L.f. californica, L.f. grandiflora* and *L.f. pumila*) are known to occur in only one to three small natural stands each. Each population stand was comprised of at least several hundred plants and in the case of *L. alba,* usually many thousands, from which seed was randomly sampled in May or June of the years 1975–1980.

For morphological comparisons, 10 to 20 plants from each population were grown under uniform conditions in a greenhouse at U.C. Davis, and scored for the characters listed in Table 1. To carry out electrophoresis, two-week old seedlings, raised in petri dishes, were crushed with one drop of extraction buffer (0.1 M Tris HCl, pH 7.0, with 0.001 ml 0.014 M Mercaptoethanol added per ml buffer). Extract was then absorbed onto two filter paper wicks which were inserted into horizontal starch gels of 12.8% concentration. Gels were cut into as many as eight slices, each of which was assayed for a different enzyme. (Technical details of electrophoretic procedures can be obtained from the senior author.) Variation was observed in 24 zones of enzyme activity; a formal genetic analysis in *L. alba* and *L. gracilis* was based on the progeny tests of certain naturally-occurring heterozygous individuals and of crosses made by hand-pollinations. Bands in the 18 variable zones where shown to be controlled by a total of 18 loci of which ADH, GOT, PGI and MDH are dimeric enzymes while the others are monomeric; intergenic dimers were found only in the case of three MDH loci. Since identical mobility on a gel is not sufficient evidence to establish structural identity due to the possible existence of undetected variation in those proteins (Singh et al. 1975), experiments with various conditions of gel and tray buffer pH and ionic strength as well as gel concentration were carried out to detect more variation. New alleles were found at only one locus but this did not affect our analyses significantly.

Confirmation of the homology of these protein-encoding loci was made possible by the electrophoresis of interspecific hybrid materials; *L. gracilis, L. alba* and *L. montana* have homology for all the loci examined. Failure of hybridization attempts between *L. floccosa* and the other three species precluded use of this test of homology for *L. floccosa.*

Estimates of allelic frequencies for each population and for each taxon, after pooling its populations, provide data with which to estimate genetic similarity using Nei's model (Nei 1972). Mueller's (1979) analytical and numerical results show that an alternative method of estimating Nei's D, the jackknife method (see Miller 1974, for a review) may be superior because it reduces both bias and variance. For each comparison of two populations based on allelic frequency estimates at n loci, the jackknife method calculates D from $(n-1)$ loci (i.e. one locus is dropped out for each calculation of D) and averages n values of D. The jackknife method is more laborious than the delta method used by Nei (1972) but the computations are easily carried out by computer. The 1,225 values of D, based on all pairwise comparisons among the 50 populations, were calculated and populations of each of the taxa were pooled to compute the D values between taxa. The Unweighted Pair Group Method was used for constructing phenograms (Sneath and Sokal 1973).

In order to provide an alternative measure of genetic similarity, interspecific crosses were attempted among *L.g. gracilis, L.g. parishii, L. alba, L. montana, L.f. floccosa,* and *L.f. californica* in the greenhouse during the spring of 1979. At least two populations of each taxon were used in hybridizing each pair of taxa. Seed obtained from various crosses were grown in spring 1980 and a young leaf was electrophoresed to distinguish the hybrids in 82 individual cases from the selfs

since it was not always possible to confirm hybrids on the basis of morphology. Even then certain *L.g. parishii* × *L. alba* hybrids could not be verified due to the lack of diagnostic loci. Hybrids between *L.g. gracilis* × *L.g. parishii*, *L. gracilis* × *L. alba*, and *L. gracilis* × *L. montana* were examined for pollen stainability in a lactophenol blue solution. Approximately 1,000 pollen grains were scored for each hybrid.

All of the 50 populations sampled in this study have been observed by us in the field and most have been grown indoors on the Davis campus. Herbarium specimens were collected and used in verifying most of the morphological descriptors used by Mason (1952) and Arroyo (1973). In addition, a thorough re-examination of the nutlet morphology of populations sampled throughout the range of *L.g. gracilis* (5 populations), *L.g. parishii* (4), *L. alba* (8) and *L. montana* (9) was carried out utilizing the classification scheme, reported by Hauptli et al. (1978), which had included only one population each of *L. gracilis* and *L. montana*.

Results

Distinguishing morphological characters include petal length which separates the smaller flowered *L. floccosa* from the other taxa (Table 1). *L. alba* has the largest flower size in the section. The two characters, flower shape and color of aging petals, delineate the taxa in a like fashion: i.e. *L. gracilis* and *L. alba* varieties differ from *L. montana* while *L.f. floccosa* and *L.f. bellingeriana* differ from *L.f. californica*, *L.f. grandiflora* and *L.f. pumila*. Certain taxa are also identifiable by sepal pubescence, and *L. montana* is distinct from the others on the basis of leaf segment number and flower shape.

Taxa can often be separated out on the basis of nutlet morphology, so a comparison of nutlet types was carried out and results described in Table 1. *L. gracilis* and *L. alba* varieties share most nutlet types while the *L. montana* type is not found in those taxa. *L.f. floccosa* and *L.f. bellingeriana* share nutlet types while *L.f. californica*, *L.f. grandiflora* and *L.f. pumila* have another type in common.

Four parameters were used to quantify genetic variation at allozyme loci: percentage of loci polymorphic in a species (PLS) and per population (PLP); and average number of alleles at a locus per species (K/S) and per population (K/P) (Table 2). Without exception *L. floccosa* subspecies are lower than the three other species in all measures of variation. *L. alba* is the most variable taxon in all categories except PLS. *L. montana* is highly variable when populations are pooled (e.g. PLS, K/S) but lower relative to other taxa when individual populations are considered. *L.g. gracilis* is less variable than *L. alba* and more variable than *L.g. parishii* in all cases. *L.f. floccosa* is extremely homozygous but retains some variability in the form of alternative fixed alleles in different populations at five loci. The single population of *L.f. californica* studied had more polymorphic loci and alleles than the other four *L. floccosa* subspecies. Geographical and ecological aspects of infraspecific variation patterns will be discussed elsewhere.

Allelic frequencies for individual populations (not given here) were used to estimate the allele frequencies for each taxon (all populations per taxon pooled) (Table 3). Note that (1) the number of alleles per locus varies from one (fixed in entire section) at loci *Got-2* and *Gdh*, to seven at *Lap-1*, (2) ten of the loci (*Est-2, Got-1, Got-3, Pgm-2, Lap-2, Adh, Mdh-2, Mdh-3, Pgi-1, Pgi-2*) have one of the alleles common across populations; (3) *L.f. floccosa* and *L.f. bellingeriana* are distinguished from *L. gracilis*, *L. alba* and *L. montana* at loci *Est-2, Got-1, Pgm-2* and *Pgi-1* while *L.f. californica*, *L.f. grandiflora* and *L.f. pumila* differ from *L. gracilis*, *L. alba* and *L. montana* at loci *Est-1* and *Pgm-2*, (4) certain alleles at loci *Est-1, Est-2, Got-1, Lap-2* and *Mdh-2* are most common in *L. montana* but absent or rare in *L.g. gracilis*, *L.g. parishii* and *L. alba*. Also note the interesting pattern revealed by a locus-by-locus comparison of the two *L. gracilis* varieties. They share most alleles but at several loci they have quite different or even alternate frequencies (e.g. *Est-2, Pgm-1, Acph, Prx, Lap-1*).

An estimate of genetic similarity is obtained by comparing two taxa for the proportion of loci at which no alleles or all alleles are shared (Table 4). *L.g. gracilis*, *L.g. parishii* and *L.a. alba* share alleles at all loci surveyed while *L. montana* does not share alleles with those taxa at a low proportion (13%) of loci. *L. gracilis*,

Table 2. Genic variation at allozyme loci in *Limnanthes*

Parameter	L.g.g.	L.g.p.	L.a.v.	L.a.a.	L.m.	L.f.f.	L.f.b.[a]	L.f.c.[a]	L.f.g.[a]	L.f.p.
% Polymorphic loci/taxa (PLS)	0.69	0.69	0.81	0.75	0.88	.29	0	.23	0	.06
% Polymorphic loci/popn (PLP)	0.52	0.40	0.58	0.63	0.35	.04	0	.23	0	.03
Avg. no. alleles/ locus/taxon (K/S)	2.33	2.11	2.78	2.56	2.56	1.44	1.00	1.43	1.00	1.11
Avg. no. alleles/ locus/popn (K/P)	1.88	1.82	2.06	2.04	1.54	1.05	1.00	1.43	1.00	1.06

[a] Only one population available for sampling

Table 3. Allelic frequencies at 18 allozyme loci. n = number of individuals assayed

Taxon	No. of populations	n	*Est-1*					*Est-2*							*Acph*				*Got-1*			
			a	b	c	d	e	a	b	c	d	e	f	g	a	b	c	d	a	b	c	d
L.g.g.	4	380	0.01	0.20	0.79					0.15		0.85				0.24	0.14	0.62		1.0		
L.g.p.	7	357		1.0					0.06	0.91		0.03				0.33	0.47	0.20		1.0		
L.a.v.	5	326	0.03	0.91	0.05		0.01	0.01	0.01	0.48		0.50				0.58	0.38	0.04	0.02	0.98		
L.a.a.	6	311	0.16	0.77	0.06		0.01		0.02	0.74		0.23	0.003			0.38	0.51	0.11		0.99		0.01
L.m.	8	321			0.01	0.92	0.07			0.08	0.86	0.02		0.04			0.96	0.04		0.02	0.01	0.97
L.f.f.	15	389		0.50			0.50		1.0							1.0					1.0	
L.f.b.	1	54					1.0		1.0							1.0					1.0	
L.f.c.	1	58		1.0					1.0						1.0						0.16	0.84
L.f.g.	1	61		1.0					1.0						1.0							1.0
L.f.p.	2	109		1.0					1.0						1.0							1.0

Taxon	No. of populations	n	*Got-2*		*Got-3*					*Pgm-1*					*Pgm-2*				*Lap-1*						
			a	b	a	b	c	d	e	a	b	c	d	e	a	b	c	d	a	b	c	d	e	f	g
L.g.g.	4	380	1.0		0.01	0.02	0.01	0.07		0.39	0.54	0.05		0.02	0.96	0.01		0.03			0.81	0.19			
L.g.p.	7	357	1.0			0.87	0.12	0.01		0.01	0.01	0.93	0.02	0.03	0.91	0.08		0.01	0.02	0.94	0.02				0.02
L.a.v.	5	326	1.0		0.04	0.82	0.03	0.11		0.01	0.95	0.04			0.99	0.01					0.86	0.14			0.01
L.a.a.	6	311	1.0		0.04	0.91	0.04	0.01			0.91	0.03		0.06	1.0				0.05	0.12	0.70	0.11	0.01		0.01
L.m.	8	321	1.0		0.05	0.92	0.02	0.006		0.43	0.38	0.18		0.02	0.99	0.01			0.07	0.13	0.54	0.24		0.02	0.01
L.f.f.	15	389	1.0			1.0					1.0					0.93	0.05	0.02			0.12	0.88			
L.f.b.	1	54	1.0			1.0					1.0					1.0						1.0			
L.f.c.	1	58	1.0			0.97	0.03				1.0					1.0					1.0				
L.f.g.	1	61	1.0			1.0				1.0						1.0					1.0				
L.f.p.	2	109	1.0			1.0				1.0						0.58	0.42				1.0				

Taxon	No. of populations	n	*Lap-2*			*Adh*			*Mdh-2*		*Mdh-3*			*Pgi-1*		*Pgi-2*			*Prx*		*Sdh*		*Gdh*	
			a	b	c	a	b	c	a	b	a	b	c	a	b	a	b	c	a	b	a	b	a	b
L.g.g.	4	380	0.92	0.08		0.01	0.96	0.03	1.0		1.0			1.0		0.43	0.57		0.68	0.32	1.0		1.0	
L.g.p.	7	357	1.0				1.0		0.98	0.02	0.84	0.16		1.0		0.09	0.91		0.04	0.96	1.0		1.0	
L.a.v.	5	326	0.90	0.09	0.01	0.03	0.87	0.10	1.0		0.98	0.02		1.0		0.09	0.90	0.01	0.46	0.54	1.0		1.0	
L.a.a.	6	311	0.98	0.02			0.96	0.04	1.0		0.93	0.04	0.03	1.0		0.02	0.84	0.14	0.50	0.50	1.0		1.0	
L.m.	8	321	0.01	0.99	0.01		0.98	0.02	0.99	0.01	0.05	0.95		1.0		0.10	0.86	0.04	0.70	0.30	1.0		1.0	
L.f.f.	15	389		1.0		0.004	0.99	0.004	1.0		0.81	0.13	0.06		1.0		1.0		1.0		1.0		1.0	
L.f.b.	1	54		1.0			1.0		1.0		1.0				1.0		1.0		1.0		1.0		1.0	
L.f.c.	1	58		1.0			1.0		1.0		0.98	0.02		1.0			1.0		1.0		1.0		1.0	
L.f.g.	1	61		1.0			1.0		1.0		1.0			1.0			1.0		1.0		1.0		1.0	
L.f.p.	2	109		1.0			1.0		1.0		1.0			1.0			1.0		1.0		1.0		1.0	

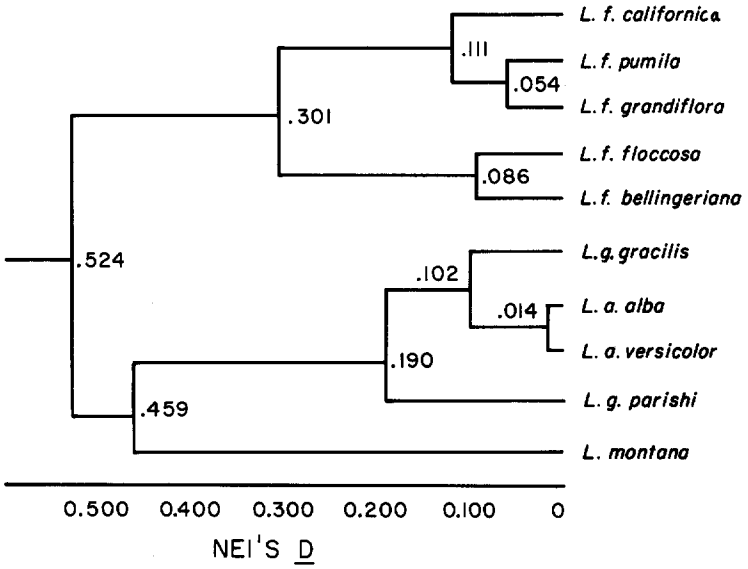

Fig. 2. Phenogram of all ten taxa based on Nei's D values

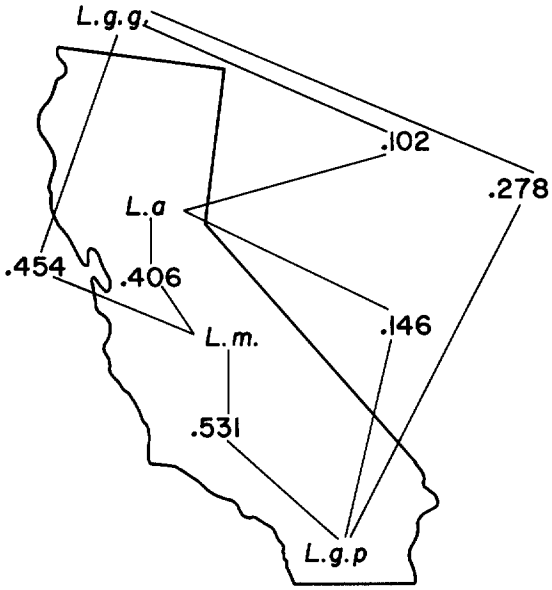

Fig. 3. Map showing D values between *L. alba, L. gracilis* and *L. montana*

Table 4. Shared and unique alleles among *Limnanthes* taxa. Figures *above* the diagonal indicate the proportion of loci at which the specified pair of taxa share no alleles ($P \geqq 0.05$). Figures *below* the diagonal indicate the proportion of loci at which the specified pair of taxa share all alleles ($P \geqq 0.05$)

	L.g.g.	L.g.p.	L.a.	L.m.	L.f.f.	L.f.c., L.f.g., L.f.p.
L.g.g.	–	0	0	0.13	0.14	0.20
L.g.p.	0.44	–	0	0.13	0.14	0.20
L.a.	0.63	0.31	–	0.13	0.14	0.20
L.m.	0.25	0.31	0.25	–	0.14	0.13
L.f.f.	0.21	0.21	0.21	0.07	–	0.07
L.f.c., L.f.g., L.f.p.	0.20	0.27	0.13	0.07	0.50	–

L. alba and *L. montana* share no alleles with *L.f. californica, L.f. grandiflora* and *L.f. pumila* at 13% to 20% loci. High genetic similarity between *L. gracilis* and *L. alba* varieties is apparent from the large proportion of loci at which all alleles are shared (63% between *L.g. gracilis* and *L. alba,* 44% between *L.g. gracilis* and *L.g. parishii* and 31% between *L. alba* and *L.g. parishii*). *L. montana* shares all alleles with *L. gracilis* and *L. alba* at 25 to 31% of the loci as well.

It is also useful to assess genetic relationships between taxa by asking at what proportion of loci will the alleles of one taxon constitute a subset of alleles of another taxon. A recent progenitor-derivative species pair might show all loci of one taxon (the derivative) to be a subset or extraction of another (the progenitor). No such pattern is revealed in the case of *L.g. gracilis* and *L.g. parishii* nor when *L. gracilis, L. alba* or *L. montana* are compared with any of the *L. floccosa* subspecies. However, *L.f. bellingeriana* alleles are all found within *L.f. floccosa* while alleles of *L.f. grandiflora* and *L.f. pumila* at all loci, except for *Pgm-1* and *Pgm-2,* are contained within *L.f. californica* (Table 3).

Estimates of Nei's D between Inflexae taxa are shown above the diagonal in Table 5 while variances, calculated using the jackknife method and appropriate covariance statistics, are shown below the diagonal (Nei and Roychoudhury 1974; Mueller 1979). The D values between *L. gracilis* and *L. alba* are very low (0.099 to 160) revealing genetic similarity between the two species. In fact, the *L. gracilis* varieties are not as similar to each other (D = 0.278) as they are to *L. alba* (D = 0.102, 0.146). The value of D between *L. montana* and *L. gracilis* or *L. alba* varieties is much larger, ranging from 0.429 to 0.531 (Fig. 3), and likewise, all *L. floccosa* subspecies show high D values (0.436 to 0.656) with the other taxa of the section.

Table 5. Genetic distance (D) between *Limnanthes* taxa shown *above* the diagonal and variances for each D value, *below* the diagonal. Calculations are based on gene frequencies at 18 allozyme loci

	L.g.g.	L.g.p.	L.a.v.	L.a.a	L.m.	L.f.f.	L.f.b.	L.f.c.	L.f.g.	L.f.p.
L.g.g.	–	0.278	0.099	0.112	0.454	0.582	0.666	0.552	0.598	0.565
L.g.p.	0.0015	–	0.160	0.121	0.531	0.629	0.797	0.704	0.713	0.719
L.a.v.	0.0028	0.0101	–	0.014	0.452	0.436	0.559	0.456	0.592	0.563
L.a.a.	0.0032	0.0070	0.0001	–	0.429	0.470	0.575	0.475	0.609	0.600
L.m.	0.0037	0.0399	0.0355	0.0352	–	0.583	0.717	0.569	0.564	0.525
L.f.f.	0.0051	0.0635	0.0356	0.0424	0.0584	–	0.007	0.271	0.370	0.457
L.f.b.	0.0790	0.1113	0.0651	0.0679	0.0931	0.0065	–	0.325	0.460	0.554
L.f.c.	0.0593	0.0953	0.0495	0.0511	0.0655	0.0270	0.0322	–	0.080	0.141
L.f.g.	0.0678	0.0964	0.0730	0.0750	0.0650	0.0419	0.0506	0.0801	–	0.054
L.f.p.	0.0567	0.0661	0.0727	0.0531	0.0919	0.0471	0.0570	0.0129	0.0032	–

Table 6. Estimates of mean genetic distance, D ± SE, at several taxonomic levels

Taxa (no. of population)		Conspecific populations	Varieties or subspecies	Species
L. gracilis	var. 'gracilis' (4)	0.059 ± 0.012	0.278	0.433 ± 0.065
	var. 'parishii' (7)	0.026 ± 0.003		
L. alba	var. 'versicolor' (5)	0.105 ± 0.019	0.014	
	var. 'alba' (6)	0.026 ± 0.004		
L. floccosa	ssp. *floccosa* (15)	0.109 ± 0.007	0.301	
	ssp. *bellingeriana* (1)			
	ssp. *californica* (1)			
	ssp. *grandiflora* (1)			
	ssp. *pumila* (2)			
L. montana (8)		0.077 ± 0.007	–	

A phenogram (Fig. 2) based on D values confirms the pattern already outlined. *L. floccosa* subspecies are distinctly separated from the other taxa with *L.f. floccosa* and *L.f. bellingeriana* closely related and apart from the cluster of *L.f. californica, L.f. grandiflora* and *L.f. pumila. L.g. gracilis* and *L. alba* are very closely related with *L.g. parishii,* whereas *L. montana* is distinct from both *L. gracilis* and *L. alba.*

Another indicator of genetic relationship relies on the proportion of stainable (therefore, presumably viable) pollen grains in the F_1 hybrids. Figure 4 shows that average pollen stainability values for the hybrids and the estimates of D for the respective interspecific population pairs are significantly, and negatively correlated ($r = -0.92$, $P < 0.001$). This shows that hybrid fertility decreases almost linearly with increasing genetic distance (D) between the populations from which the hybrids derived; for example, hybrids involving *L. montana* produced less viable pollen than those between *L. gracilis* and *L. alba.*

The overall pattern of genetic differentiation in this section can be studied by calculating D at taxonomic levels hierarchically from the population to the species. Average interpopulational D values vary from 0.026 in *L.g. parishii* to 0.109 among *L.f. floccosa* populations

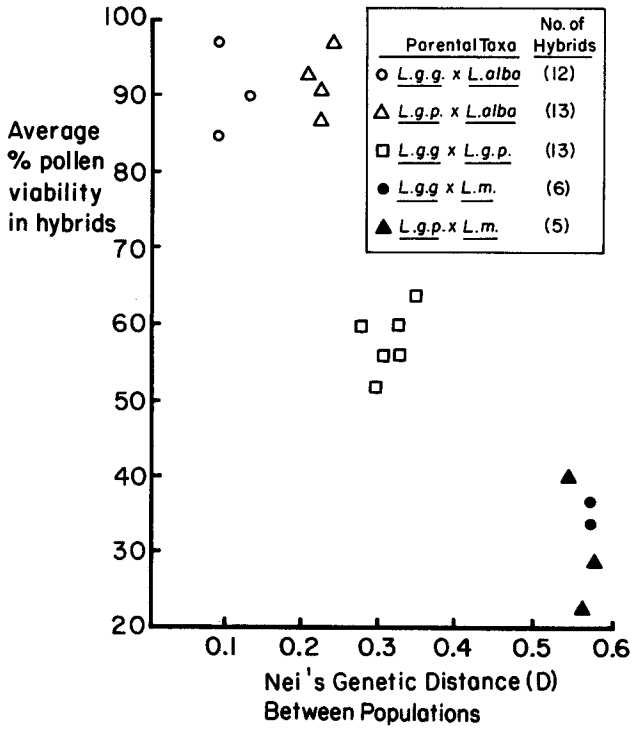

Fig. 4. Correlogram showing relationship of hybrid pollen fertility and genetic distance

(Table 6). Autogamous *L.f. floccosa* appears therefore to have greater interpopulational differentiation than the more outcrossing taxa. Varieties within a species range from 0.014 in *L. alba* to 0.278 in *L. gracilis,* while *L. floccosa* subspecies differ from each other by D=0.301. Average D between species taken pairwise in this section is 0.433.

Discussion

Genetic differentiation within and between species

The distinctive island-like distribution pattern of *Limnanthes* populations raises questions whether interpopulational genetic differentiation is markedly different from that in the other plant groups, presumably with more continuous distribution patterns.

Of the 1,225 D values for pairwise comparisons between the 50 populations, average intraspecific D values ranged from 0.026 to 0.109 with an average of 0.067, while interspecific D values were much higher except for the case of *L. gracilis* and *L. alba* as discussed above.

Comparisons of this value of D (0.067) with D values from 39 other annual plant taxa (Gottlieb 1981) suggests that *Limnanthes* populations generally may be more differentiated than other plants. If these 39 species are representative of short-lived plants in general, this pattern may be due to the restriction of *Limnanthes* species to vernal pools as "islands". Autogamy in *L. floccosa,* however, seemed to account for the classical result of wide divergence among its populations and subspecies.

In terms of hybrid pollen fertility, *Limnanthes* taxa appear less differentiated than many other plant groups at certain taxonomic levels. In a recent review, Levin (1978) reported that hybrids between: 1) remotely related plant species usually have fertilities below 20% (while *L. gracilis* varieties × *L. montana* hybrid fertility averages 33%), 2) closely related species have fertilities which vary around 45% (while *L. gracilis* varieties × *L. alba* hybrid fertility averages 91%) and, 3) races or subspecies have fertilities ranging around a mean of 61% (while *L.g. gracilis* × *L.g. parishii* hybrids have a similar fertility level of 58%).

Recent speciation in Limnanthes?

The genus *Limnanthes* may offer a rare opportunity to observe the process of genetic divergence leading to speciation. Stebbins (1974, 1976) argued on the basis of specialization and other lines of evidence that vernal pool species are the most recently evolved members of the California flora. The development of the Mediterranean climate following the Pliocene provided a major stimulus for the proliferation of species and some genera (Axelrod 1967).

A critical test of genetic changes involved in speciation, as Gottlieb (1973) pointed out, is to identify a species soon after its origin. Species with a progenitor-derivative relationship are expected to share a high proportion of their alleles shortly after new species formation such that the genome of the derived species is likely to represent a subset of its progenitor's genome (Gottlieb 1973). Also, unless chromosomal rearrangement caused or accompanied the speciation event, pollen viability of hybrids might be relatively high. Examples of rapidly originating species are reported in several genera of diploid annual plants (*Clarkia:* Lewis 1973; Small 1971; *Gaura:* Raven and Gregory 1972; *Stephanomeria:* Gottlieb 1973; *Lycopersicon:* Rick et al. 1976; *Coreopsis:* Crawford and Smith 1982). The genus *Limnanthes,* therefore, would be a logical one in which to look for additional examples of progenitor-derivative species pairs.

Species relationships and origins

Since *L. gracilis, L. alba,* and *L. montana* were found to be quite similar to each other and distant from all of the *L. floccosa* subspecies in most analyses, the two groups of taxa will be discussed separately. First we turn to the disjunct varieties of *L. gracilis* to see if the electrophoretic, morphological and hybrid fertility data can help explain their distribution patterns by distinguishing between the three alternative hypotheses mentioned earlier.

It seems unlikely that the present day gene flow is responsible for the morphological similarity between *L.g. gracilis* and *L.g. parishii* because (a) the most common alleles in one taxon are not always shared in the other, (b) F_1 hybrids between them show only partial fertility (58%), and (c) their 800 mile disjunction would severely restrict gene flow by seed dispersal.

In the absence of historical information and knowledge of the effects of drift and natural selection on gene frequencies, the hypothesis of recent founding of one taxon from the other cannot be rigorously tested. However, a recently founded population is expected to have a subset of genotypes and therefore reduced levels of genetic variability. The fact that at most loci, alleles of neither taxon comprise a subset of those of the other, argues against this hypothesis.

The third hypothesis of similarity due to connection in the past when *Limnanthes* populations had a more continuous distribution appears to be more consistent with the data. An expectation of this hypothesis is that the remnant populations should be more similar to each other than to other populations in the section. This should be true unless selective or random processes have caused divergence among those remnants. We might also expect the geographic extremes of the range to be less similar to each other than they each are to populations intermediate in range. This would be due to gene flow in the past and the expected similarity of selective regimes among proximal populations.

Apparently *L. gracilis* fits the pattern of a large number of northwestern species found in the mountains of San Diego County that do not occur elsewhere in southern California,

being disjunct from their distant northern stands. Raven and Axelrod (1978) argue that these taxa are clearly part of a larger group that ranged southward into the region during the moister, cooler periods of the Quaternary. This is consistent with the current distributional evidence and the fossil record (Munz 1935; Axelrod 1967). Presumably the disjunct northern taxa in southern California disappeared from the intervening areas during the warmer and drier climate of the Xerothermic period.

Mason (1952) suggested that *L. montana,* which is geographically intermediate between the two varieties of *L. gracilis,* is a remnant of this earlier wider range. Ornduff and Crovello (1968) agreed as their numerical taxonomic results supported this idea. Our results show, however, that *L. montana* differs more from *L.g. gracilis* and *L.g. parishii* than the latter two differ from each other based on: 1) percentage of loci at which no or all alleles are shared (Table 4); 2) Nei's D (Table 5, Fig. 3); 3) certain morphological characters such as nutlet type, color of aging petals, flower shape and number of leaf segments per leaflet (Table 1); 4) pollen viability (Fig. 4); 5) Parkers' (1980) work showing that *L. montana* has distinctive petal trichomes which are unique in the genus; and 6) *L. montana's* special germination requirements (unpublished data). These data, indicating that *L. gracilis* varieties are clearly more similar to each other than either is to *L. montana,* are inconsistent with the hypothesis that *L. montana* is a relictual set of populations from what once was a continuous range with the two *L. gracilis* varieties.

On the other hand, the relationship between *L. gracilis* and *L. alba* is surprisingly close and in fact, it fits the pattern predicted by the hypothesis that *L. gracilis* varieties are similar due to the past distributional continuity. Genetic similarity as measured by Nei's D (Table 5) and pollen viability of hybrids (Fig. 4) show that *L. alba* may indeed be more closely related to *L.g. gracilis* of the north and *L.g. parishii* of the south than these two disjunct varieties are to each other. The strong affinity of *L. gracilis* and *L. alba* is further supported by: 1) the high percentage of loci at which all alleles are shared (Table 4); 2) Nei's D (Table 5, Fig. 3); 3) certain morphological characters such as: nutlet type, petal color after pollination, flower shape and number of leaf segments per leaflet (Table 1). In fact, Ornduff (1971) also provides evidence supporting this rearrangement of species relationships by his finding that *L. montana* and *L. gracilis* are "consistently separated by a sterility barrier" while "the relatively dissimilar *L. gracilis* and *L.a. alba* may form relatively fertile hybrids". This is also consistent with Mason's (1952) preliminary hybridization results.

It seems probable that further work on morphology and F_2 progeny may warrant a reclassification of the *L. gracilis* varieties. The present taxonomic designations tend to obscure the evolutionary genetic relationships between these taxa.

Concordance of electrophoretic, morphological and hybridization data

Since the application of biochemical techniques in evolutionary and systematic studies, a critical issue has been to determine whether the study of variation based on various approaches yield concordant results.

The results of our allozyme studies, certain morphological comparisons and pollen viability work, presented above, suggest that *L. gracilis* and *L. alba* have a close relationship while *L. montana* is further removed. Ornduff and Crovello's (1968) "T" analysis (based on a total of 35 vegetative and floral characters) shows *L.g. gracilis, L.g. parishii, L.a. versicolor* and *L. montana* to be clustered and *L.a. alba* slightly separated. Their "F" analysis (using only floral characters) shows these taxa to be spread out fairly continuously while the "V" analysis (based on vegetative characters) indicates that *L.g. gracilis, L.g. parishii* and *L. montana* are associated and distinct from *L.a. versicolor* and *L.a. alba.* It is not clear if the discordance between our allozyme results and the "V" analysis of Ornduff and Crovello (1968) is indeed real since our studies and theirs did not use the same populations. Further, our observations suggest that great morphological variability exists among *L. montana* populations so that use of one or two populations would not adequately represent the species.

In the case of five *L. floccosa* subspecies a striking agreement between the variation patterns for morphology and allozymes is found in which identical schemes of subspecies relationships emerged from the two approaches (Fig. 2, and see Arroyo 1973); *L.f. floccosa* and *L.f. bellingeriana* appeared similar to each other and distinct from the closely related subspecies *L.f. californica, L.f. grandiflora* and *L.f. pumila* in all morphological and electrophoretic analyses.

L. alba and L. floccosa: recent evolution of autogamy?

Several examples of an outcrossing species giving rise to a selfer have been suggested and documented (*Clarkia:* Moore and Lewis 1965; *Leavenworthia:* Lloyd 1965; *Stephanomeria:* Gottlieb 1973; *Lycopersicon:* Rick et al. 1976). *L. alba* and *L.f. floccosa* have outbreeding and high selfing systems respectively (Arroyo 1973a; Brown and Jain 1979), and their morphological similarity and overlapping ranges make them a likely pair in which to look for this kind of progenitor-derivative relationship.

However, the high genetic divergence between *L. alba* and *L. floccosa* argues against the postulate that *L. floccosa* was recently derived from *L. alba.* The two species share no alleles at 14–20% of the loci surveyed and at 36–40% of the loci alleles of *L. floccosa* do not constitute a subset of *L. alba* alleles. The high D between them further indicates substantial

divergence. None of the other taxa in this section is any more closely related to *L. floccosa* than *L. alba,* thus, unlike the two sibling *Lycopersicon* species (Rick et al. 1976) which show evidence for the evolution of autogamous species through the fixation of a subset of ancestral genotypes, *L. floccosa* might have either originated from some unknown taxon or diverged rapidly in its genome organization. Hybridization and cytogenetic studies are now being initiated to verify the latter thesis.

Arroyo (1973 b) hypothesized that *L.f. californica* gave rise to *L.f. grandiflora* and *L.f. pumila* to form a closely knit group of subspecies on the one hand and to *L.f. floccosa* and *L.f. bellingeriana* on the other. A comparison of allelic composition of the two sets of subspecies provides no indication as to which set may have given rise to the other. Within those sets of subspecies, *L.f. bellingeriana* alleles are clearly a subset of *L.f. floccosa* while *L.f. californica* contains most alleles found in *L.f. grandiflora* and *L.f. pumila.* It seems likely then that Arroyo (1973) was correct that *L.f. bellingeriana* derived from *L.f. floccosa* and perhaps *L.f. grandiflora* and *L.f. pumila* evolved from *L.f. californica.*

Speciation in Limnanthes

Evidence needed to determine the modes of speciation cover the whole field of evolutionary biology and include observations from morphology, genetics of species hybrids, karyotype, genetic distance, specific gene frequencies, mating system, dispersal rates and gene flow, time since divergence, and sequence and nature of habitat change through time. Lacking data on many of these categories as most studies do, can present day patterns of divergence tell us much about the modes and outcomes of speciation events? The two basic approaches to the genetics of species differences, i.e. hybridization experiments and comparisons of gene products at loci such as those encoding enzymes when considered in a population genetic context, can be used to make certain inferences about the likelihood of certain modes of speciation.

Ayala et al. (1974) found progressively greater differentiation in *Drosophila* at various levels of taxonomic distinction from local populations to morphologically distinguishable species. In several different groups of organisms one finds congruence of patterns of differentiation with the successive "taxonomic" stages of speciation. Evolutionists have often discussed the geographic mode of speciation in terms of such gradual increases in genetic distances accompanied by the origin of reproductive barriers. In contrast, the so-called "genetic transilience" mode (Templeton 1981) assumes certain abrupt genetic shifts due to such factors as inbreeding, random drift or chromosomal translocations which have been widely recognized by the botanists (Raven 1976, for a review). The reliability of a measure of genetic divergence such as Nei's D to reflect the stage of speciation depends therefore on the mode of speciation.

The comparison of molecular data and hybrid fertility data in the study of species relationships can indicate whether genetic and chromosomal differentiation work in parallel or not and thus whether genetic or chromosomal transilience has occurred. Low D values and high hybrid sterility would suggest significant chromosomal repatterning while a high D and low hybrid sterility would support a "genetic transilience" – like phenomenon. Concordant differentiation, on the other hand, as observed here for *L. alba, L. gracilis* and *L. montana,* is consistent with more gradual adaptive genetic change affecting several aspects of the genetic system simultaneously (i.e. karyotype, enzyme coding loci, etc.).

The geographical distance between *L. alba* and *L. gracilis* populations casts doubt on a clinal model of speciation but Axelrod (personal communication) believes that in the past it is likely that *Limnanthes* was continuously distributed throughout California. More support for a clinal divergence mode comes from gene frequency patterns at several loci (e.g. *Est-2, Pgm-1, Acph, Prx, Lap-1*) in which the northern and southern *L. gracilis* populations have alternate alleles while *L. alba* has intermediate frequencies (Table 3).

A careful study of differences between *L. alba* populations may further allow us to distinguish between the clinal and adaptive divergence modes (Templeton 1981). If *L. alba* populations show a clinal pattern at electrophoretic or morphological loci (Ritland and Jain, in press) then we could hypothesize the extinction of certain intermediate populations which lead to *L. gracilis* populations at the north and south extremes of an *alba*-like taxon.

Certain aspects of the population biology of *Limnanthes* make it a likely candidate for several other modes of speciation. Lack of chromosomal rearrangement between species (Mason 1952; Ornduff 1971) and fairly high hybrid fertility between certain taxa do not support a "chromosomal transilience" mode; however, failure to cross species of the two sections of the genus or the inbreeder *L. floccosa* with other Inflexae members, do not allow us to exclude this mode entirely.

Conditions for "genetic transilience" are quite restrictive (Templeton 1981) and yet it may be common under certain circumstances such as the evolution of selfing populations from outcrossers. *L. floccosa* subspecies could be candidates for this mode, and note that *L. gracilis* populations have higher levels of selfing than *L. alba,* suggesting a progressive stepwise change toward higher autogamy (McNeill and Jain, in preparation). Highly subdivided population structure of most *Limnanthes* taxa, and rather dramatic fluctuations in population sizes would provide an optimal fit to the Wright's model of "shifting phase" evolution (Wright 1970).

Although Grant (1971), Lewis (1973), and others emphasized the importance of interspecific hybridiza-

tion in plant speciation, our electrophoretic data revealed no distinct examples of natural hybrids. In comparing levels of crossability and hybrid fertility among plants, Grant (1971) pointed out sharp differences between annuals, herbaceous perennials, shrubs and trees. Annuals tend to have low crossability and low hybrid fertility, whereas the opposite pattern is found among other life forms. This pattern implicates chromosomal rearrangements in speciation. *Limnanthes,* an annual group seems to provide a rather striking exception to this pattern when *L. alba, L. gracilis, L. montana* and certain species clusters of the Reflexae section (Kesseli and Jain, in preparation) are considered. As a final perspective of our work to elucidate species relationships, we tend to agree with Maynard Smith (1982) in arguing that numerical taxonomic techniques should aim at a classification to reflect phylogeny, and echo Thorpe's (1982) view: "studies of evolution and systematics are so intimately linked that there should not be any clearcut border between them." Any "natural" scheme of classification must provide the basis of some phylogenetic inference howsoever it warrants verification from different areas of evolutionary biology.

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