

LOW LEVELS OF INTRASPECIFIC GENETIC VARIATION AT A RAPIDLY EVOLVING CHLOROPLAST DNA LOCUS IN NORTH AMERICAN DUCKWEEDS (LEMNACEAE)¹

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Although most previous studies on chloroplast (cp) DNA variation in plants have concentrated on systematics and evolution above the species level, intraspecific variation in cpDNA is common and has provided useful insights into population-level evolutionary processes. Polymerase chain reaction methods were used to examine restriction site and sequence variation in the chloroplast *rpL16* gene within and among populations of duckweed species (*Spirodela* and *Lemna*) from the southern and eastern United States. To our knowledge, the *rpL16* region has not previously been used to investigate cpDNA variation in nature. While considerable restriction site and sequence variation were detected among species, no variation was found within populations of either of the two species (*S. punctata* and *L. minor*) selected for sequence analysis, and *S. punctata* showed no interpopulational variation. Two cpDNA haplotypes were identified in *L. minor*, with one haplotype restricted to three sites in Louisiana and the other found in all other populations sampled. This paucity of variation cannot be readily explained as the result of a low mutation rate. In general, group II introns appear to be subject to very little functional constraint, and extensive sequence differences have been found between species in the chloroplast *rpL16* intron in particular. However, factors such as historical range expansions and contractions, founding effects, fluctuations in local population size, and natural selection may play a role in reducing cpDNA sequence variability in these species.

Key words: cpDNA; duckweeds; genetic variation; *Lemna*; *Spirodela*.

Chloroplast (cp) DNA is now used routinely as a tool to investigate evolutionary processes in plants, but due to its relatively low average rate of evolution most previous studies have used cpDNA sequence variation to examine plant systematics and evolution above the species level (Palmer, 1987; Palmer et al., 1988; Soltis, Soltis, and Doyle, 1992 and references therein). However, intraspecific cpDNA variation has been observed in many species (see Harris and Ingram, 1991, and Soltis, Soltis, and Milligan, 1992 for reviews), and has provided useful insights into evolutionary processes within and among populations (e.g., Wagner et al., 1987; Soltis et al., 1989; Rieseberg, Carter, and Zona, 1990; Lavin, Mathews, and Hughes, 1991; Milligan, 1991; Soltis et al., 1991; Kim, Jansen, and Turner, 1992; Hong, Hipkins, and Strauss, 1993; Hooglander, Lumaret, and Bos, 1993; Dong and Wagner, 1994).

A majority of previous studies have used levels of restriction fragment length polymorphism (RFLP) to estimate indirectly levels of cpDNA sequence variation (Soltis, Soltis, and Doyle, 1992), but biases and uncertainties are inherent in these methods (Dowling, Moritz, and

Palmer, 1990). The development of the polymerase chain reaction (PCR) (Mullis et al., 1986) and associated DNA sequencing techniques (Wrischnik et al., 1987; Gyllenstein and Erlich, 1988) has made the amplification and the direct examination of DNA sequences relatively straightforward. Although PCR and direct DNA sequencing methods are commonly used to investigate mitochondrial DNA variation within and among animal populations (Kelley Thomas et al., 1990; Carr and Marshall, 1991; Di Rienzo and Wilson, 1991; Wilkinson and Chapman, 1991; Fajen and Breden, 1992; Volger and DeSalle, 1993; Sang et al., 1994; Thorpe et al., 1994), and have been applied extensively to higher level systematics in plants (Baldwin, 1992; Liston, 1992; Chase et al., 1993; Johnson and Soltis, 1994), they have been used relatively infrequently to examine intraspecific cpDNA variation.

A major advantage of the use of PCR to examine sequence variation is the ability to select specific areas of a genome for analysis. As regions of the chloroplast genome differ in their rate of evolution (Wolfe, Li, and Sharp, 1987; Shimada and Sugiura, 1991; Kim, Turner, and Jansen, 1992; Ogihara, Terachi, and Sasakuma, 1992; Morton and Clegg, 1993), areas showing a level of sequence variation appropriate for the taxonomic level of the evolutionary question addressed may be selected. Areas with a relatively high rate of evolution are more likely to be useful for population-level studies.

Here we report RFLP and DNA sequence variation in the chloroplast *rpL16* gene for species of duckweeds (Lemnaceae). The *rpL16* gene, which codes for a chloroplast ribosomal protein, lies in the large single-copy region of the chloroplast genome and consists of two exons separated by a relatively long intron. The length of the intron is reported by Posno, Van Vliet, and Groot (1986) to be ≈ 1400 base pairs (bp) in *Spirodela oligo-*

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rhiza Hegelmaier (synonymous with *S. punctata* (G.F.W. Meyer) Thompson [Landolt, 1986, p. 440]). This gene appears to be active in protein synthesis (Ellis, 1969; Blair and Ellis, 1973), and, at least in *E. coli*, plays a role in peptidyltransferase activity (Moore et al., 1975; Brosius and Chen, 1976; Gottschalk, 1979 [pp. 69–77]; Neidhardt, 1987 [pp. 627–634]). The *rpL16* intron was chosen for this analysis as a region that could potentially display detectable levels of intraspecific variation while simultaneously being part of a gene that should be functionally stable. Indeed, the *rpL16* intron has been shown to have an exceptionally high rate of sequence change when *Spirodela* is compared with tobacco (Wolfe, Li, and Sharp, 1987).

The duckweeds, which are free-floating aquatic angiosperms, have long been popular models for ecological, physiological, and biochemical studies (Hillman, 1961; Landolt, 1986; Landolt and Kandeler, 1987). The Lemnaceae are in many ways a model system for examination of large- and small-scale geographic patterns of cpDNA variation. Four genera are generally recognized (*Spirodela*, *Lemna*, *Wolffiella*, and *Wolffia*) and collectively they have an almost cosmopolitan distribution (Landolt, 1986). While flowering has been observed in all duckweed species except *Lemna ecuadoriensis* Landolt and *Wolffia elongata* Landolt (Landolt, 1986), it is generally infrequent in nature. Clonal reproduction in this group is vegetative: daughter fronds are produced from meristematic tissue in lateral pouches of the mother frond. By doubling in as little as 1 or 2 d, population growth can be rapid, and densities of up to 200 000 (*Spirodela*)-2 000 000 (*Wolffia*) plants per square metre have been reported (Hicks, 1937). While census population sizes can reach many millions of individuals, seasonal declines in population size are common (Sculthorpe, 1967). Although some duckweed species produce resistant forms (e.g., sinking resting fronds in *L. trisulca* Linné, or turions in *S. polyrrhiza* Schleiden or *L. turionifera* Landolt), the majority have limited resistance to extremes of temperature and pH (Landolt, 1986). Competition between species (Clathworthy and Harper, 1962; Rejmánková, 1975; Keddy, 1976; McIlraith, Robinson, and Shay, 1989; Gopal and Goel, 1993), allelopathy (Gopal and Goel, 1993), changes in nutrient concentrations (Landolt and Kandeler, 1987), and the ephemeral nature of some aquatic habitats may also cause declines in population size.

The aim of this study was to assess the level and distribution of cpDNA diversity in species of duckweed that commonly occur in the southern and eastern United States. Four species were examined for RFLP variation (*Lemna minor* Linné, *L. valdiviana* Phillipi, *Spirodela punctata*, and *S. polyrrhiza*). Two of these (*L. minor* and *S. punctata*) were chosen for more detailed examination of intraspecific differences in DNA sequence. The latter two species, although easily distinguishable, are similar in size and gross morphology; optimal growth conditions differ between the two, but both species display relatively low levels of flowering and neither produces turions (Landolt, 1986).

MATERIALS AND METHODS

Sample collection and species identification—Duckweed samples were collected from sites across the southern and eastern United States

TABLE 1. Details of Lemnaceae samples used in this study.

Species	Sample code	Sample size		Location
		RFLP analysis	DNA sequence	
<i>L. minor</i>	A	17	8	Avery Island, LA
	B		6	Atchafalaya Basin, LA
	C		14	Lafayette, LA
	D		6	Glensboro, KY
	E		5	Seneca Rocks, WV
	F		2	Great Falls, MD
	G		6	Rome, NY
	H		5	Lake Keomeah SP, IA
	I		5	Fraser Exp. Forest, CO
<i>L. valdiviana</i>	C	6		Lafayette, LA
<i>S. punctata</i>	I	22		Avery Island, LA
	II	25	4	Lafayette, LA
	III		6	Tallahassee, FL
	IV		5	Walterboro, SC
	V		5	Wade, NC
	VI		3	Hughes, AR
<i>S. polyrrhiza</i>	II	9		Lafayette, LA

(Table 1, Fig. 1). To avoid sampling from the same population, collection locations were separated by at least 32 km (20 miles) (but usually much greater distances). For samples collected for sequence analysis, no sites were on the same river system. At each site efforts were made to ensure that individual plants were taken from a number of locations throughout the body of water. The species composition of each sample was ascertained using the criteria of Landolt (1986). To examine nerve patterns and air spaces, fronds were boiled in 70% alcohol (2–3 min) and then immersed in a dilute hypochlorite solution (1:5 dilution of household bleach; 2–3 min) (Landolt, 1992). Cleared fronds were then stained with acid aceto-orcein and viewed under magnification using transmitted light. Although taxonomic characters in duckweed can be subtle, differences among species studied in this research were sufficiently robust to provide unambiguous identification. Voucher specimens were deposited in the USL herbarium (LAF).

DNA extraction—DNA was extracted from individual duckweed using a modification of the protocol of Rogers and Bendich (1989). Single plants (one frond and any attached daughter fronds) were transferred to a microfuge tube, dipped into liquid nitrogen, and ground using a disposable plastic pestle. Twenty-five microlitres of 1× CTAB (1% mass/volume cetyltrimethylammonium bromide [CTAB]; 50 mmol/L Tris-HCl pH 8.0; 10 mmol/L EDTA; 0.7 mol/L NaCl) at 65°C were added, and the suspension was incubated at 65°C for at least 5 min. The suspension was then extracted twice using an equal volume of chloroform:isoamyl alcohol (24:1 volume/volume), and the aqueous phase transferred to a fresh microfuge tube on each occasion.

An equal volume of CTAB precipitation buffer (1% mass/volume CTAB; 50 mmol/L Tris-HCl pH 8.0; 10 mmol/L EDTA) was added and the mixture incubated at or below room temperature for 15 min, before being centrifuged at 12 000 g for 2 min at 4°C. The supernatant was discarded and the pellet resuspended in 25 µL high salt TE (10 mmol/L Tris-HCl pH 8.0; 1 mmol/L EDTA; 1 mol/L NaCl). DNA was precipitated for 30 min at –20°C after the addition of 75 µL 100% cold (–20°C) ethanol. Following centrifugation at 12 000 g for 15 min at 4°C, the supernatant was removed and the pellet washed with 75 µL cold 80% ethanol. The purified DNA was vacuum dried and resuspended in 25 µL 0.1× TE (1 mmol/L Tris-HCl pH 8.0; 0.1 mmol/L EDTA). At this scale of extraction DNA concentrations were lower than could be quantified using UV spectroscopic or fluorometric techniques (i.e., <10 ng).

RFLP studies—Oligonucleotide primers were designed to amplify a section of the intron within the chloroplast *rpL16* gene of *S. oligorhiza*



Fig. 1. The locations of sampling sites for plants used in the RFLP and DNA sequence studies. Top: *L. minor* and *L. valdiviana*. Bottom: *S. punctata* and *S. polyrhiza*. Location codes refer to those given in Table 1 and broken lines indicate the approximate distributions of *L. minor* (top) and *S. punctata* (bottom) in the eastern United States according to Landolt (1986).

(Posno, Van Vliet, and Groot, 1986 [in that paper the synonym for *S. punctata*, *S. oligorhiza*, was used; for purposes of distinguishing the Posno, Van Vliet, and Groot findings, *S. oligorhiza* will be used to refer to their results]). Pairs of primers (25-mers) were designed using equations for optimizing annealing, extension, and melting temperatures (Breslauer et al., 1986; Rychlik, Spencer, and Rhoads, 1990). The two regions reported here, PCR1 and PCR2 (Fig. 2), are contiguous. The numbers in the primer nomenclature refer to the 5' position of the primer on the *rpL16* sequence of Posno, Van Vliet, and Groot (1986) for *S. oligorhiza*. PCR1 begins just before the short, 5' exon at position 71 and ends at position 459 in the intron. For PCR1 the primers used were F71:5'-GCTATGCTTAGTGTGTGACTCGTTG-3' and R434:5'-CTCAGGGCTCTTTACCTTTTTGAGT-3'. PCR2 begins at position 435 and ends at position 1686 in the 3' exon. The primers used were F435:5'-CTCAAAAAGGTAAAGAGCCCTGAGT-3' and R1661:5'-CGTACCCATATTTTTCCACCACGAC-3'. Thus, for *S. oligorhiza* (as reported in Posno, Van Vliet, and Groot, 1986), the expected amplification products are 388 bp and 1 251 bp long, respectively. However, in our investigations, amplifications produced products that differed in length and sequence from that reported by Posno, Van Vliet, and Groot (1986), although they were clearly homologous.

For the RFLP studies, standard PCR methods were used to amplify PCR1 and PCR2 (Fig. 2) for >70 individual plants from four species (*L. minor*, *L. valdiviana*, *S. punctata* and *S. polyrhiza*), collected from

the Lafayette and Avery Island (LA) areas (Fig. 1). *Taq* polymerase, nucleotides and reaction buffers (Perkin-Elmer and Boehringer-Mannheim) were used according to suppliers' directions. In general, PCR mixtures in each 25- μ L reaction tube were as follows: template DNA—2.5 μ L (estimated 100 pg—10 ng); *Taq* polymerase—0.5 μ L (1.5 Units); dNTPs—0.5 μ L (200 μ mol/L); primers (each)—1.25 μ L (1 μ mol/L); MgCl₂ (50 mmol/L)—0.1 μ L (200 μ mol); 10 \times reaction buffer—2.5 μ L (1 \times); sterile distilled H₂O—15.1 μ L.

Thermal profiles (on both Perkin-Elmer and Coy thermal cyclers) generally consisted of 3 min at 95°C, followed by five cycles of 94°C (1 min)/52°C (1 min)/65°C (4 min), with a ramp time of 2 min, 15 s between annealing and extension temperatures. This was followed by 35 cycles of 94°C (1 min)/52°C (2 min)/65°C (4 min), again with a ramp time of 2 min, 15 s between annealing and extension. A final 10-min extension was run at 65°C, after which the reaction temperature was lowered to 15°C indefinitely. Eventually, the denaturing temperature was lowered to 92°C and the volume of *Taq* polymerase decreased to 0.3 μ L with equal effectiveness. The extension step of the PCR was performed at 65°C as theoretical predictions of *T_m* and empirical evidence (unpublished data) suggested that the PCR product was denatured at 72°C, the optimum temperature for *Taq* activity.

Whole PCR products and digests of whole PCR products produced with *Hinf*I endonuclease (Boehringer-Mannheim) according to supplier's instructions were resolved in 1 \times TBE buffer on 2% agarose gels stained with ethidium bromide and illuminated with ultraviolet light.

DNA sequencing—DNA sequencing was carried out for PCR1 (Fig. 2), the shorter of the two segments of the *rpL16* gene. The F71 and R434 primers were used to prime dideoxy terminal reactions.

Each PCR reaction used 4.0 μ L of purified DNA solution. Amplifications were performed in a final volume of 100 μ L containing 67 mmol/L Tris-HCl pH 8.8, 6.7 mmol/L MgSO₄, 16.6 mmol/L (NH₄)₂SO₄, 10 mM 2-mercaptoethanol, 0.20 μ M each of dATP, dCTP, dTTP, and dGTP, 2.5 units of *Taq* polymerase and 1 μ mol/L of each primer (Kocher et al., 1989). Reaction mixtures were overlaid with 60 μ L of mineral oil to prevent evaporation during thermal cycling. The cycling parameters were 94°C for 1 min, 58°C for 1 min, a 2.5-min ramp to 65°C, and then 65°C for 4 min, (\times 35 cycles) followed by a 10-min step at 65°C.

Eighty microlitres of each PCR reaction were removed, ethanol precipitated, vacuum dried, and resuspended in 20 μ L TE (10 mmol/L Tris-HCl pH 8.0; 1 mmol/L EDTA) before being gel purified in a 1% agarose gel, using 1 \times TBE buffer. The PCR product was retrieved from agarose using Prep-a-gene (BioRad) according to the manufacturer's instructions and eluted in 10 μ L of supplied buffer.

Direct sequencing of the double-stranded PCR product followed the method of Thorpe et al. (1994). This method is modified from that recommended by United States Biochemical for the Sequenase 2.0 polymerase by the use of DMSO as a denaturant (Winship, 1989) and the addition of a chase step (Green et al., 1989). Products of the sequencing reactions were resolved in 1 \times TBE, 8% polyacrylamide gels (19:1 acrylamide:bisacrylamide) containing 7 mol/L urea.

Data analysis—Two measures of intraspecific DNA polymorphism were calculated: the number of segregating sites (*S*) (Watterson, 1975) and the average number of nucleotide differences (*k*) (Tajima, 1983). Under a neutral model of molecular evolution both measures are related to the term $4N\mu$ for nuclear genes (or $2N\mu$ for haploid genomes), where *N* is the effective population size and μ is the mutation rate per DNA sequence per generation (Tajima, 1993). Watterson (1975) showed that in a sample of DNA sequences subject to mutations at a large number of linked sites, the expected number of segregating (variable) sites among them is proportional to the product of the mutation rate (over the entire sequence) and the effective size of the population.

The data were used in a test of the neutral mutation hypothesis for a randomly mating population at equilibrium (Tajima, 1989): the value of

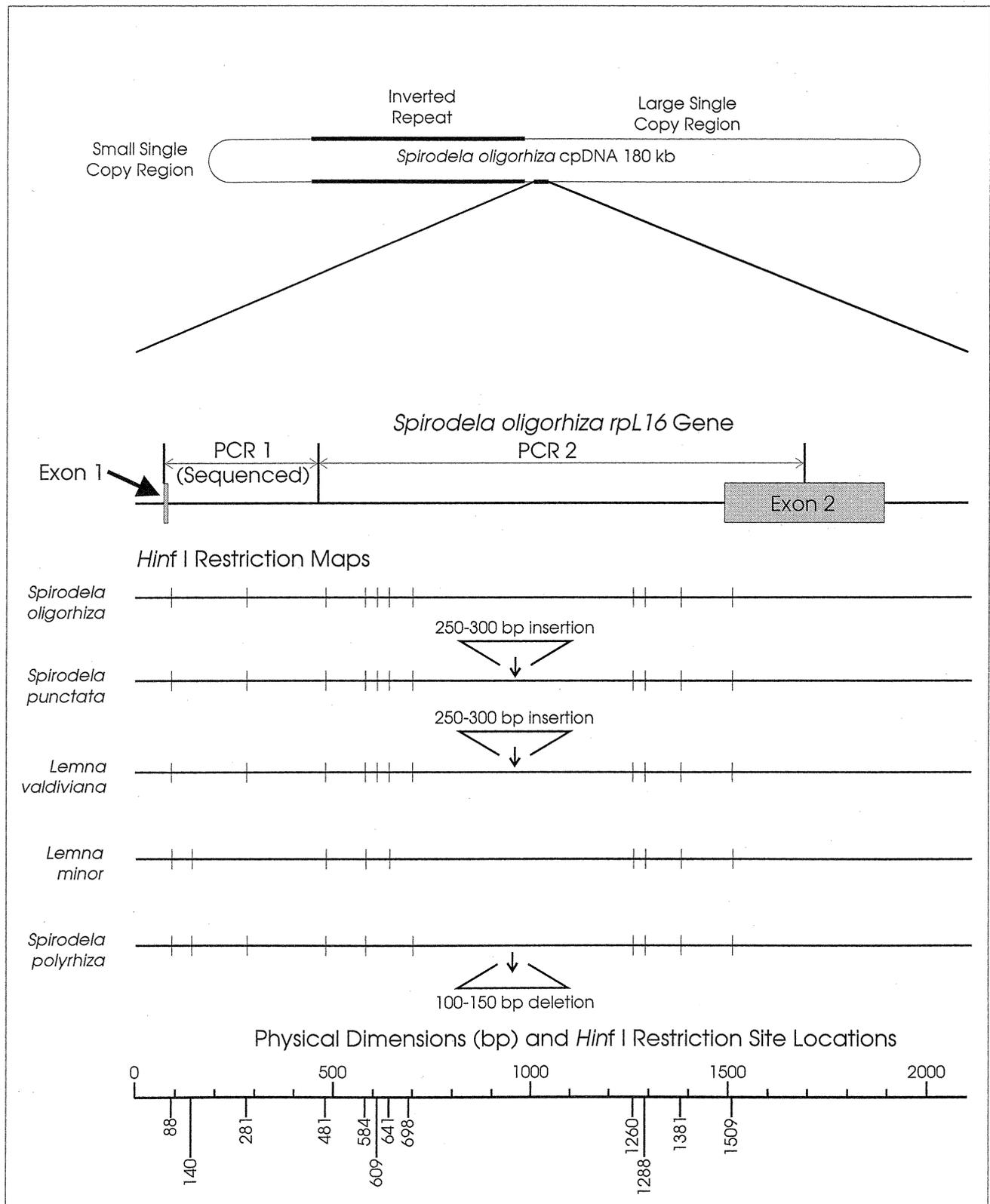


Fig. 2. Physical map of the *Spirodela oligorhiza* chloroplast genome (after Van Ee et al., 1982), indicating the location of the *rpL16* gene (after Posno, Van Vliet, and Groot, 1986), the regions amplified by PCR in this study, and variation observed in *Hinf* I restriction maps among duckweed species.

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(i)  TTAGGCTGAA AAAATAGCCT GAATCTTACT ATGAACCAGT AACTATAGAA AGTCATAACT ATAGAAACTA
(ii)  ...A...A... ..T.....

(i)  CTAACCAACT TATGGCTTCG TATTGTGCGAA ATCCCCAAA CAGTCACTAT ATGAGGTATT CATCATATAG
(ii)  .....

(i)  TTGTAGCAAC TGAAAAATGA ATTTAACATA AAAAAATCCC C-GGTTTTGA ATAAAATAAA AAAGAAGGGG
(ii)  ..... ..G..... ---- .A.....

(i)  AGGTGTAGAT AACAGAAGG ATGATAGATA GAAAGAAAAC AAAATATCAA TGATATATTA TTCCAACATA
(ii)  .T.....

(i)  TGTATGGTTT ATGAAAAAAT TTATATAATA AAA
(ii)  .....

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Fig. 3. Sequences from section of chloroplast *rpL16* intron obtained from *L. minor*. Sequence (i) is that for the haplotype found in Louisiana populations, and sequence (ii) is that for the haplotype found at all other sites sampled. A dot (.) indicates that sequences are identical at that position and a dash (-) indicates a gap introduced to improve alignment.

the test statistic D , which measures the deviation between estimates of $4N\mu$ from S and k , is compared to the confidence limits of $D = 0$ for n (number of sequences examined) (Tajima, 1989, table 2). When D differs from zero, then one or more of the assumptions of the neutral model have been violated (Tajima, 1993).

The MEGA software of Kumar, Tamura, and Nei (1993) was used to estimate sequence divergence between species based on the Kimura two-parameter model of base substitution (Kimura, 1980).

RESULTS

RFLP studies—No intraspecific variation was detected in RFLP patterns among the nearly 70 plants sampled. However, interspecific variation in the length of both amplification products (PCR1 and PCR2) was detected. The occurrence of particular restriction sites, as well as the length of amplified regions, varied considerably among species (Fig. 2). For all four species, the length of the PCR1 product uniformly agreed with the 388 bp length predicted from the *S. oligorhiza* sequence. However, the position of *HinfI* sites differed in two of the four species. The length of the PCR2 product varied considerably among species. The sequence published by Posno, Van Vliet, and Groot (1986) indicates that for *S. oligorhiza*, PCR2 is 1 451 bp long; by comparison, *S. polyrhiza* appears to have an ≈ 100 –150 bp deletion. In contrast, *S. punctata* and *L. valdiviana* seem to have an ≈ 200 –250

bp addition in this region. Only *L. minor* reflects the length predicted from the published sequence. Restriction site maps, based on *HinfI* sites, are presented for the four species studied (Fig. 2). While information about variation based on a single enzyme's restriction sites may appear limited, restriction site variation among species was significant enough to justify further analysis of intraspecific variation in DNA sequence.

DNA sequences—Data on up to 320 base pairs of the *rpL16* intron (PCR1) were obtained for individuals from nine populations of *L. minor* (total sample size, $N = 57$) and five populations of *S. punctata* ($N = 23$). No sequence variation was found within populations of either species, and no differences were observed among populations of *S. punctata*.

Two haplotypes were found in *L. minor*, with one restricted to the three populations sampled in Louisiana and the other haplotype found in all other populations sampled (Fig. 3). If insertion/deletion events, inferred from sequence alignments, are treated as single segregating sites regardless of the number of bases involved, the haplotypes found in *L. minor* differ by at least seven mutation steps over 313 sites compared (Fig. 3). On this basis, the average number of nucleotide differences among sequences in *L. minor*, $k = 0.011$, and the number of seg-

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S. punctata  TTAGGATAAA AAAATAGACT TAATCTTACT ATGAACCCAGT AACTATAGAA ACTCATAACT ATAGAAACTA
L. minor      .....C.G.. .....C.. G..... .G.....

S. punctata  CTAACCAACT TATGGCTTCT TATTGTCGAT ATCCCCCAA CAGTCACTCT ATGAGGTATT CATCATATAG
L. minor      .....G .....A .....A. ....

S. punctata  AAGTAGCAAC TGCAAAAT-- -TTTAGATCA AATAACTGGG ATTCGGGTT TGAATAAAAT AAAAA----
L. minor      TT..... ..A....GA A...ACAT. ..A..A.CC- ---...T.. .....AGAA

S. punctata  GGGGATGTAT AGATAAATGG AAAGATGATA GATAGAAAGA AAAAAAAAAA AATTAATGAT ATATTATTC
L. minor      .....G..G. ....CA. ..G..... ...C...T. --.C.....

S. punctata  A--ATATGTAT AATTTATTAA AAATTTTATA TTATAAAA
L. minor      .AC..... GG.....G.. ...A..... .A.....
    
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Fig. 4. Comparison of chloroplast *rpL16* intron sequences from *L. minor* and *S. punctata*. Sequence shown for *L. minor* is the Louisiana haplotype. A dot (.) indicates that sequences are identical at that position and a dash (-) indicates a gap introduced to improve alignment.

regating sites, $S = 7$. Following Tajima (1989) a value of $D = -8.137$ was obtained as a measure of the deviation between estimates of $4Nv$ from S and k : this value of D lies outside the 99.9% confidence limits for $D = 0$. A more conservative analysis, which excluded the data on insertion/deletion events, produced values of $k = 0.008$, $S = 5$, and $D = -2.379$: under this treatment D is still highly significantly different from zero (0.001 K 0.01).

rpL16 sequences from *S. punctata* and *L. minor* show a 11.16-11.77% sequence divergence, depending on the *L. minor* haplotype used in the comparison (Fig. 4). This estimate is conservative in that insertion/deletion events are ignored.

DISCUSSION

Intraspecific cpDNA variation has been observed in the majority of species examined, in many cases even when the low resolution of the techniques used and the sampling design of the study have biased against its detection (Harris and Ingram, 1991; Soltis, Soltis, and Milligan, 1992). However, in the present study cpDNA variation in *L. minor* and *S. punctata* was found to be either low or absent despite the direct examination of DNA sequences, and the number of individuals and extent of the geographic range sampled. This finding was not anticipated, given the extent of restriction site divergence in

the *rpL16* region observed between the species examined. While the low levels of diversity observed may result from a low effective mutation rate at the locus examined, a variety of population genetic processes can also act to reduce genetic variation.

The chloroplast genome has a lower average mutation rate than either plant or animal nuclear DNA, or animal mitochondrial DNA (Wolfe, Li, and Sharp, 1987), but the rate of sequence change is heterogeneous among regions of the cpDNA molecule (Wolfe, Li, and Sharp, 1987; Shimada and Sugiura, 1991; Ogihara, Terachi, and Sasakuma, 1992; Morton and Clegg, 1993). As evolutionary rates have been shown to vary among functionally distinct areas (e.g., coding vs. noncoding regions [Wolfe, Li, and Sharp, 1987]), this heterogeneity appears to be due, at least in part, to differences in the degree of selective constraint for maintenance of function. A number of lines of evidence suggest that the level of functional constraint may be low, and the effective mutation rate relatively high in the *rpL16* intron.

Group II introns, which include the *rpL16* intron (Posno, Van Vliet, and Groot, 1986), are excised from mRNA transcripts via a series of self-catalyzed reactions (Michel, Umesono, and Ozeki, 1989). Therefore, although generally classed as noncoding DNA, these introns are not without function. A comparison of group II intron sequences, however, reveals extensive variation (Michel,

Umesono, and Ozeki, 1989): the primary sequence appears free to vary, both in composition and length, as long as a limited number of secondary structures and short sequence motifs (e.g., at exon/intron junctions) are conserved (Michel, Umesono, and Ozeki, 1989). Moreover, self-splicing activity *in vitro* has been shown to be maintained despite experimental deletions of substantial sections of a group II intron (Koch et al., 1992).

The chloroplast *rpL16* intron varies considerably in length among species, from 536bp in *Marchantia polymorpha* (Shimada and Sugiura, 1991) to 1 400 bp in *S. punctata* (Posno, Van Vliet, and Groot, 1986). Among the duckweed species examined in this research, large variations (up to 300 bp) in the length of *rpL16* intron were observed (Fig. 2) in an area of the intron outside the sequenced region (PCR1). Together with the comparative and functional studies on group II introns in general, these length variations in the chloroplast *rpL16* intron suggest a low level of functional constraint in this region.

Interspecific comparisons of cpDNA sequences (i.e., *Oryza sativa*, *Zea mays*, or *Nicotiana tabacum* compared to *S. punctata*), show the *rpL16* intron to have such a high rate of evolution that sequences cannot be aligned with confidence at this level of species divergence, in contrast to other coding and noncoding areas of the chloroplast genome (W. C. Jordan, unpublished data; Wolfe, Li, and Sharp, 1987). The difficulties in aligning *rpL16* intron sequences preclude the calculation of measures of sequence divergence and formal comparison of levels and rates of divergence with other chloroplast and nuclear genes. Nonetheless, they do indicate relatively high rates of sequence evolution at the *rpL16* intron.

When the sequence published by Posno, Van Vliet, and Groot (1986) is compared with those of our samples, notable differences emerge (Fig. 2). The sequences for both *S. punctata* and *L. minor* are reasonably similar to those for *S. oligorhiza* in the 20–30 bases immediately downstream of the forward primer (F71). However, the next ≈ 100 bases (positions 125–220) indicate relatively close similarity between *S. punctata* and *L. minor* but a notable lack of similarity between either of these species and *S. oligorhiza*. The divergence between the *S. punctata* sequence in the current study and the *S. oligorhiza* sequence published by Posno, Van Vliet, and Groot (1986), coupled with the lack of observed variation in *S. punctata*, may be explained by putative differences between the *S. oligorhiza* genotype and the limited genotype(s) of *S. punctata* founder(s) in North America. However, these results, coupled with the similarity of *L. minor* and *S. punctata* in positions 125–220, are more difficult to explain on this basis alone. Further systematic analysis with *rpL16* may of course shed light on these patterns; however, one possible explanation is that the founders of North American populations of *L. minor* and *S. punctata* possessed chloroplast genomes closer to that of the common ancestor of *Lemna* and *Spirodela* than the *S. oligorhiza* examined by Posno, Van Vliet, and Groot (1986).

When the sequences of the *L. minor* and *S. punctata* samples obtained in this research are aligned, differences at many sites are apparent, resulting in an 11–12% sequence divergence: a level of divergence reported between some plant orders at the chloroplast *rbcL* locus

(Palmer et al., 1988). Under a neutral model of molecular evolution (Kimura, 1983), this level of divergence implies either a very high mutation rate in this region, or an improbably ancient evolutionary separation of these species.

There is evidence of intraspecific variation within *L. minor*, with the two haplotypes reported here differing by at least seven mutations over the ≈ 300 bp examined. The pattern of polymorphism in *L. minor* does not fit a neutral mutation model (Tajima, 1989, 1993) and suggests that haplotypes, intermediate in state between those observed, may have been lost from the species, or were not detected at this level of sampling. As pointed out by Tajima (1993), a range of possible deviations from the assumptions of the neutral mutation model can cause values of *D* to significantly differ from zero: these include population subdivision, changes in population size, and selection.

Alternatively, the extent of the differences between haplotypes may result from introgression of a chloroplast genome from another species as a result of hybridization and backcrossing. Introgression of this type has been observed in a number of plant taxa (Rieseberg and Brunsfeld, 1992). While some duckweed species are hypothesized to have a hybrid origin (e.g., *L. japonica* Landolt from hybridization between *L. minor* and *L. turionifera*) there are limited data presently available on the ability of duckweed species to hybridize, and on the viability and fertility of any possible hybrids (Landolt, 1986). The extremely low levels of flowering observed in duckweed species reduces, but does not entirely exclude, the possibilities for hybridization in this group (Landolt, 1986).

Alternate explanations of the results for *L. minor* would imply an even lower level of genetic diversity. For example, in the unlikely case that samples from Louisiana may have been misidentified, or may represent morphologically cryptic species, then chloroplast genetic diversity in *L. minor* would in fact be overestimated by this study, and a corrected estimate would take a value of zero for the geographic area sampled, excluding the sites in Louisiana.

Current levels and patterns of cpDNA variation might be expected to reflect the history of *L. minor* and *S. punctata* distributions. For *S. punctata*, which is believed to have been introduced into North America in the 1930s (Saeger, 1934), the absence of cpDNA variation may be due to low levels of genetic diversity in founding populations and to the relatively recent spread and subpopulation partition of the species in the area sampled. On the other hand, *L. minor* is considered endemic to North America (Landolt, 1986). Its range limits within the continent, however, are likely to have been dynamic, and the geographical distribution of cpDNA haplotypes reported here may reflect patterns of range contraction into, and expansion from glacial refugia, most recently in the Pleistocene (Pielou, 1991). By invoking a scenario in which the range of *L. minor* was reduced to two refugia, with loss of genetic diversity and divergence between the isolates due to lineage sorting (Neigel and Avise, 1986), the present-day lack of overall genetic diversity and the seemingly incongruent sequence divergence level of the two haplotypes might be explained simply. However, in

the absence of any fossil data, such an interpretation remains speculative.

Ongoing demographic processes may also affect levels of genetic diversity. Although census population sizes in duckweeds are often of the order of millions of individuals, the predominantly clonal mode of reproduction in these species causes effective population sizes to be much lower (Gliddon, Belhassen, and Gouyon, 1987): numerically large populations may consist of ramets of one or a few founding individuals (Barrett, Eckert, and Husband, 1993). Fluctuations in population size due to seasonal changes in environmental conditions (Landolt, 1986), competition (Clathworthy and Harper, 1962; Keddy, 1976; McIlraith, Robinson, and Shay, 1989; Gopal and Goel, 1993), or allelopathy (Gopal and Goel, 1993) may also act to increase genetic drift and decrease genetic variability in duckweeds. If these declines result in local extinctions in extreme cases, they may have important effects on the distribution of genetic variation in the species, depending on the mode of recolonization (Slatkin, 1977; Wade and McCauley, 1988; Whitlock and McCauley, 1990).

A further consequence of clonal reproduction for levels of genetic diversity is the complete linkage disequilibrium established between nuclear and cytoplasmic genomes. Nuclear–cytoplasmic disequilibria can arise in sexually reproducing species, but they decay over generations in all mating systems except complete selfing (Schnabel and Asmussen, 1989). Complete linkage among chloroplast, mitochondrial, and nuclear genes increases the potential for “hitchhiking” effects, where selection at one locus can reduce genetic diversity at linked loci (Maynard Smith and Haigh, 1974; Kaplan, Hudson, and Langley, 1989; Maruyama and Birky, 1991). It has also been suggested that selection may act on clonal organisms to produce a single general-purpose genotype that is competitively superior across a range of environmental conditions (Baker, 1965; Lynch, 1984).

The relationship between levels and patterns of organellar and nuclear genetic variation in sexually reproducing species is complex, and depends on factors such as mutation rates, mode of inheritance of organellar DNA, sex ratio in populations and among migrants, and selection (Schnabel and Asmussen, 1989; Birky, Fuerst, and Maruyama, 1989; Maruyama and Birky, 1991). For clonal organisms, where all genomes are transmitted together, levels and patterns of variation in nuclear and organellar genes might be expected to be more similar than in sexually reproducing species, even if the types of selection discussed above are not involved. This seems to be the case for *S. punctata*. In the geographical range covered in this study, where a single cpDNA clone was found, only one multilocus allozyme genotype was detected (Crawford and Landolt, 1993; D. J. Crawford, personal communication, Ohio State University), although one other allozyme genotype was found at a site north of the area sampled here (i.e., Massachusetts).

The pattern of chloroplast DNA variation in *L. minor*, however, contrasts strikingly with the relatively high levels of inter- and intrapopulation variation reported for allozyme loci in this species (Vasseur, Aarssen, and Bennett, 1993). Vasseur, Aarssen, and Bennett (1993) found 157 multilocus genotypes in samples of *L. minor* along

a 12-km transect in Ontario, Canada, with an average of 19.6 genotypes per population. It is possible that the estimates of nuclear genetic diversity are inflated by environmental and ontogenetic variation observed in allozyme expression in *L. minor* (Vasseur, Aarssen, and Lefebvre, 1991). However, as not all the loci reported as polymorphic display nonMendelian variation it would be unlikely to account for all of the difference in genetic diversity between the two genomes. Higher mutation rate and larger effective population size for the nuclear genome could act to increase variation over that of the chloroplast genome, but sexual reproduction may also play a role. Although generally low rates of flowering have been reported for duckweeds, increased levels have been found under adverse environmental conditions (e.g., drought [Hicks, 1932] and nitrogen deficiency [Tanaka et al., 1991]). Under such conditions, if outcrossing is successful, the linkage of nuclear and chloroplast genes would be uncoupled (Schnabel and Asmussen, 1989), creating the potential for different patterns of diversity in the two genomes.

While duckweeds have been the focus of laboratory studies for many years, relatively little information is available on the biology of natural populations. Fluctuations in the size of natural populations are known to occur, and single- and mixed-culture experiments have provided a great deal of insight into factors affecting such fluctuations, but quantitative data on their extent are missing from the literature. Similarly, a variety of factors are known to influence flowering, and therefore mating system in duckweeds, but there are few data available on mating systems in natural populations. Data of this type are essential for understanding the relative importance of factors influencing levels of cpDNA variability and the relationship between cytoplasmic and nuclear genetic diversity. Because of their ecological vagility, facultatively sexual reproduction, ample biotic potential, wide geographic distribution, and relative ease of laboratory maintenance and manipulation, duckweeds potentially present a highly suitable model with which to examine this relationship, as well as a wide range of evolutionarily interesting questions.

LITERATURE CITED

- BAKER, H. G. 1965. Characteristics and modes of origin of weeds. In H.G. Baker and G.L. Stebbins [eds.], *The genetics of colonizing species*, 147–172. Academic Press, New York, NY.
- BALDWIN, B. G. 1992. Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: an example from the Compositae. *Molecular Phylogenetics and Evolution* 1: 3–16.
- BARRETT, S. C. H., C. G. ECKERT, AND B. C. HUSBAND. 1993. Evolutionary processes in aquatic plant populations. *Aquatic Botany* 44: 105–145.
- BIRKY, C. W., JR., P. FUERST, AND T. MARUYAMA. 1989. Organelle gene diversity under migration, mutation and drift: equilibrium expectations, approach to equilibrium, effects of heteroplasmic cells, and comparisons to nuclear genes. *Genetics* 121: 613–627.
- BLAIR, G. E., AND R. J. ELLIS. 1973. Protein synthesis in chloroplasts. I: Light-driven synthesis of the large subunit of fraction I protein by isolated pea chloroplasts. *Biochimica et Biophysica Acta* 319: 223–234.
- BRESLAUER, K. J., R. FRANK, H. BLOCKER, AND L. A. MARKY. 1986. Predicting DNA duplex stability from the base sequence. *Proceedings of the National Academy of Sciences, USA* 83: 3746–3750.
- BROSIUS, J., AND R. CHEN. 1976. The primary structure of protein L16

- located at the peptidyltransferase center of *Escherichia coli* ribosomes. *FEBS Letters* 68: 105–109.
- CARR, S. M., AND H. D. MARSHALL. 1991. Detection of intraspecific DNA sequence variation in the mitochondrial cytochrome *b* gene of Atlantic cod (*Gadus morhua*) by the polymerase chain reaction. *Canadian Journal of Fisheries and Aquatic Sciences* 48: 48–52.
- CHASE, M. W., ET AL. 1993. Phylogenetics of seed plants: an analysis of nucleotide sequences from the plastid gene *rbcL*. *Annals of the Missouri Botanical Garden* 80: 528–580.
- CLATHWORTHY, J. N., AND J. L. HARPER. 1962. The comparative biology of closely related species living in the same area. V. Inter- and intraspecific interference within cultures of *Lemna* spp. and *Salvinia natans*. *Journal of Experimental Botany* 13: 307–324.
- CRAWFORD, D. J., AND E. LANDOLT. 1993. Allozyme studies in *Spirodela* (Lemnaceae): variation among conspecific clones and divergence among the species. *Systematic Botany* 18: 389–394.
- DI RIENZO, A., AND A. C. WILSON. 1991. Branching pattern in the evolutionary tree for human mitochondrial DNA. *Proceedings of the National Academy of Sciences, USA* 88: 1597–1601.
- DONG, J., AND D. B. WAGNER. 1994. Paternally inherited chloroplast polymorphism in *Pinus*: estimation of diversity and population subdivision, and tests of disequilibrium with a maternally inherited mitochondrial polymorphism. *Genetics* 136: 1187–1194.
- DOWLING, T. E., C. MORITZ, AND J. D. PALMER. 1990. Nucleic acids II: restriction site analysis. In D.M. Hillis and C. Moritz [eds.], *Molecular systematics*, 250–317. Sinauer, Sunderland, MA.
- ELLIS, R. J. 1969. Chloroplast ribosomes: stereospecificity of inhibition by chloramphenicol. *Science* 163: 477–478.
- FAJEN, A., AND F. BREDEEN. 1992. Mitochondrial DNA sequence variation among natural populations of the Trinidad guppy, *Poecilia reticulata*. *Evolution* 46: 1457–1465.
- GLIDDON, C., E. BELHASSEN, AND P-H. GOUYON. 1987. Genetic neighbourhoods in plants with diverse systems of mating and different patterns of growth. *Heredity* 59: 29–32.
- GOPAL, B., AND U. GOEL. 1993. Competition and allelopathy in aquatic plant communities. *Botanical Review* 59: 155–210.
- GOTTSCHALK, G. 1979. Bacterial metabolism. Springer-Verlag, New York, NY.
- GREEN, P. M., D. R. BENTLEY, R. S. MIBASHAN, I.M. NILSSON, AND F. GIANNELLI. 1989. The molecular pathology of haemophilia B. *EMBO Journal* 8: 1067–1072.
- GYLLENSTEN, U. B., AND H. A. ERLICH. 1988. Generation of single-stranded DNA by the polymerase chain reaction and its application to direct sequencing of the *HLA-DQA* locus. *Proceedings of the National Academy of Sciences, USA* 85: 7652–7656.
- HARRIS, S. A., AND R. INGRAM. 1991. Chloroplast DNA and biosystematics: the effects of intraspecific diversity and plastid transmission. *Taxon* 40: 393–412.
- HICKS, L. E. 1932. Flower production in the Lemnaceae. *Ohio Journal of Science* 32: 115–131.
- . 1937. The Lemnaceae of Indiana. *American Midland Naturalist* 18: 774–789.
- HILLMAN, W. S. 1961. The Lemnaceae, or duckweeds: a review of the descriptive and experimental literature. *Biological Review* 27: 221–287.
- HONG, Y-P., V. D. HIPKINS, AND S. STRAUSS. 1993. Chloroplast DNA diversity among trees, populations and species in the California closed-cone pines (*Pinus radiata*, *Pinus muricata*, and *Pinus attenuata*). *Genetics* 135: 1187–1196.
- HOGLANDER, N., R. LUMARET, AND M. BOS. 1993. Inter-intraspecific variation of chloroplast DNA of European *Plantago* spp. *Heredity* 70: 322–334.
- JOHNSON, L. A., AND D. E. SOLTIS. 1994. *matK* DNA sequences and phylogenetic reconstruction in Saxifragaceae. s. str. *Systematic Botany* 19: 143–156.
- KAPLAN, N. L., R. R. HUDSON, AND C. H. LANGLEY. 1989. The “hitchhiking effect” revisited. *Genetics* 123: 887–899.
- KEDDY, P. A. 1976. Lakes as islands: the distributional ecology of two aquatic plants, *Lemna minor* L. and *L. trisulca* L. *Ecology* 57: 353–359.
- KELLEY THOMAS W., S. PÄÄBO, F. X. VILLABLANCA, AND A. C. WILSON. 1990. Spatial and temporal continuity of kangaroo rat populations shown by sequencing mitochondrial DNA from museum specimens. *Journal of Molecular Evolution* 31: 101–112.
- KIM, K-J., R. K. JANSEN, AND B. L. TURNER. 1992. Evolutionary implications of intraspecific chloroplast DNA variation in dwarf dandelions (*Krigia*: Asteraceae). *American Journal of Botany* 79: 708–715.
- , B. L. TURNER, AND R. K. JANSEN. 1992. Phylogenetic and evolutionary implications of interspecific chloroplast DNA variation in *Krigia* (Asteraceae-Lactuceae). *Systematic Botany* 17: 449–469.
- KIMURA, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16: 11–20.
- . 1983. The neutral theory of molecular evolution. Cambridge University Press, Cambridge.
- KOCH, J. L., S. C. BOULANGER, S. D. DIB-HAJI, S. K. HEBBAR, AND P. S. PERLMAN. 1992. Group II introns deleted for multiple substructures retain self-splicing activity. *Molecular Cell Biology* 12: 1950–1958.
- KOCHER, T. D. W. K. THOMAS, A. MEYER, S. V. EDWARDS, S. PÄÄBO F. X. VILLABLANCA, AND A. C. WILSON. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences, USA* 86: 6196–6200.
- KUMAR, S., K. TAMURA, AND M. NEI. 1993. MEGA: Molecular Evolutionary Genetics Analysis software for microcomputers. *Computer Applications in the Biosciences* 10: 189–191.
- LANDOLT, E. 1986. The family Lemnaceae—a monographic study. 1. Veröffentlichungen der Geobotanischen Institutes der ETH, Stiftung Rübél, Zurich 71.
- . 1992. Lemnaceae. *Journal of the Arizona-Nevada Academy of Science* 26: 10–14.
- , AND R. KANDELER. 1987. The family Lemnaceae—a monographic study. 2. Veröffentlichungen der Geobotanischen Institutes der ETH, Stiftung Rübél, Zurich 71.
- LAVIN, M., S. MATHEWS, AND C. HUGHES. 1991. Chloroplast DNA variation in *Gliricida sepium* (Leguminosae): intraspecific phylogeny and tokogeny. *American Journal of Botany* 78: 1576–1585.
- LISTON, A. 1992. Variation in the chloroplast genes *rpoC1* and *rpoC2* of the genus *Astragalus* (Fabaceae): evidence from restriction site mapping of a PCR-amplified fragment. *American Journal of Botany* 79: 953–961.
- LYNCH, M. 1984. Destabilizing hybridization, general-purpose genotypes and geographic parthenogenesis. *Quarterly Review of Biology* 59: 257–290.
- MAYNARD SMITH, J., AND J. HAIGH. 1974. The hitchhiking effect of a favorable gene. *Genetical Research* 23: 23–35.
- MARUYAMA, T., AND C. W. BIRKY, JR. 1991. Effects of periodic selection on gene diversity in organelle genomes and other systems without recombination. *Genetics* 127: 449–451.
- MCILRAITH, A. L., G. G. C. ROBINSON, AND J. M. SHAY. 1989. A field study of competition and interaction between *Lemna minor* and *Lemna trisulca*. *Canadian Journal of Botany* 67: 2904–2911.
- MICHEL, F., K. UMESONO, AND H. OZEKI. 1989. Comparative and functional anatomy of group II catalytic introns—a review. *Gene* 82: 5–30.
- MILLIGAN, B. G. 1991. Chloroplast DNA diversity within and among populations of *Trifolium pratense*. *Current Genetics* 19: 411–416.
- MOORE, V. G., R. E. ATCHISON, G. THOMAS, M. MORAN, AND H. F. NOLLER. 1975. Identification of a ribosomal protein essential for peptidyl transferase activity. *Proceedings of the National Academy of Sciences, USA* 72: 844–848.
- MORTON, B. T., AND M. T. CLEGG. 1993. A chloroplast DNA mutational hotspot and gene conversion in a noncoding region near *rbcL* in the grass family. *Current Genetics* 24: 357–365.
- MULLIS, K. B., F. A. FALOONA, S. J. SCHARF, G. T. HORN, AND H. A. ERLICH. 1986. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harbor Symposium on Quantitative Biology* 51: 263–273.
- NEIDHARDT, F. C. 1987. *Escherichia coli* and *Salmonella typhimurium*. American Society for Microbiology, Washington, DC.
- NEIGEL, J. E., AND J. C. AVISE. 1986. Phylogenetic relationships of mitochondrial DNA under various demographic models of specia-

- tion. In E. Nevo and S. Karlin [eds.], *Evolutionary processes and theory*, 515–534. Academic Press, London.
- OGIHARA, Y., T. TERACHI, AND T. SASAKUMA. 1992. Structural analysis of length mutations in a hot-spot region of wheat chloroplast DNAs. *Current Genetics* 22: 251–258.
- PALMER, J. D. 1987. Chloroplast DNA evolution and biosystematics uses of chloroplast DNA variation. *American Naturalist* 130: S6–S29.
- , R. K. JANSEN, H. J. MICHAELS, M. W. CHASE, AND J. R. MANHART. 1988. Chloroplast DNA variation and plant phylogeny. *Annals of the Missouri Botanical Garden* 75: 1180–1206.
- PIELOU, E. C. 1991. *After the Ice Age*. University of Chicago Press, Chicago, IL.
- POSNO, M., A. VAN VLIET, AND G. S. P. GROOT. 1986. The gene for *Spirodela oligorhiza* chloroplast ribosomal protein homologous to *E. coli* ribosomal protein L16 is split by a large intron near its 5' end: structure and expression. *Nucleic Acids Research* 14: 3181–3195.
- REIMÁNKOVÁ, E. 1975. Comparison of *Lemna gibba* and *Lemna minor* from the production ecological viewpoint. *Aquatic Botany* 1: 423–427.
- RIESEBERG, L. H., AND S. J. BRUNSFELD. 1992. Molecular evidence and plant introgression. In P. S. Soltis, D. E. Soltis, and J. J. Doyle [eds.], *Molecular systematics of plants*, 151–176. Chapman and Hall, New York, NY.
- , R. CARTER, AND S. ZONA. 1990. Molecular tests of the hypothesized hybrid origin of two diploid *Helianthus* species (Asteraceae). *Evolution* 44: 1498–1511.
- ROGERS, S. O., AND A. J. BENDICH. 1989. Extraction of DNA from plant tissue. In S. B. Gelvin, R. A. Schilperoot, and D. P. S. Verma [eds.], *Plant molecular biology manual: Update 1*, A6/1–A6/11. Kluwer Academic, Dordrecht.
- RYCHLIK, W., W. J. SPENCER, AND R. E. RHOADS. 1990. Optimization of the annealing temperature for DNA amplification in vitro. *Nucleic Acids Research* 18: 6409–6412.
- SAEGER, A. 1934. *Spirodela oligorrhiza* collected in Missouri. *Bulletin of the Torrey Botanical Club* 61: 233–236.
- SANG, T.-K., H.-Y. CHANG, C.-T. CHEN, AND C.-F. HUI. 1994. Population structure of the Japanese eel. *Molecular Biology and Evolution* 11: 250–260.
- SCHNABEL, A., AND M. A. ASMUSSEN. 1989. Definition and properties of disequilibria within nuclear-mitochondrial-chloroplast and other nuclear-dicytoplasmic systems. *Genetics* 123: 199–215.
- SCULTHORPE, C. D. 1967. *The biology of aquatic vascular plants*. Edward Arnold, London.
- SHIMADA, H., AND M. SUGIURA. 1991. Fine structural features of the chloroplast genome: comparison of the sequenced chloroplast genomes. *Nucleic Acids Research* 19: 983–995.
- SLATKIN, M. 1977. Gene flow and genetic drift in a species subject to frequent local extinctions. *Theoretical Population Biology* 12: 253–262.
- SOLTIS, D. E., M. S. MAYER, P. E. SOLTIS, AND M. EDGERTON. 1991. Chloroplast-DNA variation in *Tellima grandiflora* (Saxifragaceae). *American Journal of Botany* 78: 1379–1390.
- , P. E. SOLTIS, AND B. G. MILLIGAN. 1992. Intraspecific chloroplast DNA variation: systematic and phylogenetic implications. In P. S. Soltis, D. E. Soltis, and J. J. Doyle [eds.], *Molecular systematics of plants*, 117–150. Chapman and Hall, New York, NY.
- , ———, AND J. J. DOYLE. 1992. *Molecular systematics of plants*. Chapman and Hall, New York, NY.
- , ———, T. A. RANKER, AND B. D. NESS. 1989. Chloroplast DNA variation in a wild plant, *Tolmiea menziesii*. *Genetics* 121: 819–826.
- TAJIMA, F. 1983. Evolutionary relationship of DNA sequences in finite populations. *Genetics* 105: 437–460.
- . 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* 123: 585–595.
- . 1993. Measurement of DNA polymorphism. In N. Takahata and A. G. Clark [eds.], *Mechanisms of molecular evolution*, 37–59. Sinauer, Sunderland, MA.
- TANAKA, O., T. YAMAMOTO, Y. NAKAYAMA, T. OZAKI, AND G. TAKEBA. 1991. Flowering induced by nitrogen deficiency in *Lemna paucicostata* 151. *Plant Cell Physiology* 32: 1173–1177.
- THORPE, R. S., D. P. MCGREGOR, A. M. CUMMING, AND W. C. JORDAN. 1994. DNA evolution and colonization sequence of island lizards in relation to geological history: mtDNA RFLP, cytochrome b, cytochrome oxidase, 12s rRNA sequence and nuclear RAPD analysis. *Evolution* 48: 230–240.
- VAN EE, J. H., Y. J. VOS, H. J. BOHNERT, AND R. J. PLANTA. 1982. Mapping of genes on the chloroplast of *Spirodela oligorhiza*. *Plant Molecular Biology* 1: 117–131.
- VASSEUR, L., L. W. AARSSSEN, AND T. BENNETT. 1993. Allozymic variation in local apomictic populations of *Lemna minor* (Lemnaceae). *American Journal of Botany* 80: 974–979.
- , ———, AND D. D. LEFEBVRE. 1991. Allozymic and morphometric variation in *Lemna minor* (Lemnaceae). *Plant Systematics and Evolution* 177: 139–148.
- VOLGER, A. P., AND R. DESALLE. 1993. Phylogeographic patterns in coastal North American Tiger Beetles (*Cicindela dorsalis* Say) inferred from mitochondrial DNA sequences. *Evolution* 47: 1192–1202.
- WADE, M. J., AND D. E. MCCAULEY. 1988. Extinction and recolonization: their effects on the genetic differentiation of local populations. *Evolution* 42: 995–1005.
- WAGNER, D. B., G. R. FURNIER, M. A. SAGHAI-MAROOF, S. M. WILLIAMS, B. P. DANCİK, AND R. W. ALLARD. 1987. Chloroplast DNA polymorphisms in lodgepole and jack pines and their hybrids. *Proceedings of the National Academy of Sciences, USA* 84: 2097–2100.
- WATTERSON, G. A. 1975. On the number of segregating sites in genetical models without recombination. *Theoretical Population Biology* 7: 256–276.
- WHITLOCK, M. C., AND D. E. MCCAULEY. 1990. Some population genetic consequences of colony formation and extinction: genetic correlations within founding groups. *Evolution* 44: 1717–1724.
- WILKINSON, G. S., AND A. M. CHAPMAN. 1991. Length and sequence variation in evening bat D-loop mtDNA. *Genetics* 128: 607–617.
- WINSHIP, P. R. 1989. An improved method for directly sequencing PCR amplified material using dimethyl sulphoxide. *Nucleic Acids Research* 17: 1266.
- WOLFE, K. H., W. H. LI, AND P. M. SHARP. 1987. Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. *Proceedings of the National Academy of Sciences, USA* 84: 9054–9058.
- WRISCHNIK, L. A., R. G. HIGUCHI, M. STONEKING, H. A. ERLICH, N. ARNHEIM, AND A. C. WILSON. 1987. Length mutations in human mitochondrial DNA: direct sequencing of enzymatically amplified DNA. *Nucleic Acids Research* 15: 529–542.