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Evolution

Chloroplast DNA diversity is low in a wild plant, *Lupinus texensis*

(restriction analysis/polymorphism/organelle DNA)

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ABSTRACT **Chloroplast DNA diversity was measured in an annual flowering plant, *Lupinus texensis*. Individual plants were collected from 21 local populations throughout the range of the species in Texas. Chloroplast DNA was isolated separately from each plant and digested with seven restriction enzymes. The most common form of the 150-kilobase-pair genome was cut at 134 sites, so that about 0.5% of the base pairs in the genome were sampled. Of the 100 plants examined, 88 had identical restriction fragment patterns. Three variant forms were found in different local populations. Two, represented in single plants, differed from wild type in the presence or absence of single restriction sites. The third variant was fixed in one of the local populations; it had lost a restriction site and also had a deletion of ≈100 base pairs. The data suggest that chloroplast DNA in this plant is much less polymorphic than mitochondrial DNA from animals and is probably less polymorphic than nuclear genes in the same plant or in animals.**

One of the tasks of experimental population genetics is to measure the levels of genetic variability within species and populations. These data are important not only for understanding evolutionary mechanisms but also for the development of plant and animal breeding programs and of conservation programs aimed at conserving the gene pools of endangered species. For all these purposes, it is important to know the variability of genes in mitochondria and chloroplasts as well as that of nuclear genes. Although the organelle genes are relatively few in number, they play indispensable roles in the key processes of respiration and photosynthesis. Moreover, each genome may contain different levels or kinds of variability. This variability can be measured at the molecular level by using restriction endonucleases. The technique has been used principally for mitochondrial DNA (mtDNA) in animals (reviewed in refs. 1, 2) although it has been applied to a few specific nuclear genes. These studies have shown that (*i*) mtDNA nucleotide diversity within a species is very high and (*ii*) population subdivision (i.e., genotypic differences between local groups) is striking. There is a strong impression that overall genetic diversity and population subdivision may be greater for mitochondrial than for nuclear genes in animals. This is probably due in part to a higher mutation rate (3). The non-Mendelian inheritance of organelle genes (reviewed in refs. 4 and 5) is also expected to influence both the diversity and the subdivision of populations (6–10).

Here we present a large-scale evaluation of genetic variability in the organelle genes of a wild plant. Chloroplast DNA (cpDNA) was studied because it is smaller, easier to isolate, and simpler in structure than mtDNA. The annual plant species, *Lupinus texensis*, was chosen for several reasons: nuclear gene (allozyme) variability has been investigated and is extremely high (11); parameters that may affect the genetic structure of populations, especially gene flow and life history characteristics, have been investigated (12–15); the plant occurs in distinct, well separated local populations; and the plant is easily grown and bred in artificial environments. Our results show cpDNA variability for both point mutations and deletions, but the overall variability is extremely low compared to animal mtDNA and is probably lower than the variability of nuclear genes in plants and animals.

MATERIALS AND METHODS

Lupinus texensis seeds were collected from roadsides and fields at the 21 locations described in Fig. 1. At each locality, seeds were gathered from topsoil along a linear transect approximately 50 m long or shorter, depending on the size of the population. Samples of seeds were grown 4–5 months in greenhouses before leaf tissue was harvested. The isolation of cpDNA from individual plants followed that of Bohnert and Crouse (16) or Palmer (17). cpDNA isolated by the first method could not be digested by some restriction enzymes. This problem was overcome by precipitating cpDNA with spermine tetrahydrochloride according to Hoopes and McClure (18).

cpDNA was digested with restriction enzymes (Bethesda Research Laboratories or New England Biolabs) according to the supplier's instruction. cpDNA (0.5–2.0 μg) from each plant was digested with each enzyme, and the resulting DNA fragments were electrophoretically separated on 0.7–1.0% agarose gels. *Hin*dIII-digested λ DNA or *Hae* III-digested φX174 DNA were included on each gel as molecular weight standards. DNA fragments within the gel were stained with ethidium bromide and visualized by UV illumination.

For the Southern hybridization experiments, cpDNA was transferred from agarose gels bidirectionally to two layers of nitrocellulose according to Smith and Summers (19). Agarose gel-isolated or cloned cpDNA fragments were nick-translated in the presence of [α-^{32}P]dATP and DNA polymerase I, then used as probes in the Southern hybridization experiments. Prehybridization and hybridization of filters to homologous probes, as well as the washing of filters, followed Maniatis *et al.* (20). After drying, filters were placed in x-ray holders with a Lightning Plus intensifying screen (DuPont) and X-Omat film. Film was exposed 12 hr to 21 days at −70°C. Nitrocellulose filters were reused up to three times after washing the filters in boiling 1.5 mM NaCl, 0.15 mM sodium citrate (pH 7.0), 0.1% $NaDodSO_4$ for two 10-min periods.

Procedures for cloning cpDNA fragments into pBR322 followed those of Bogorad *et al.* (21); the host was *Escherichia coli* strain HB101. Colony hybridization was used to screen large numbers of recombinant *E. coli* colonies for cpDNA inserts homologous to a specific cpDNA probe.

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Abbreviations: cpDNA, chloroplast DNA; mtDNA, mitochondrial DNA; bp, base pair(s).

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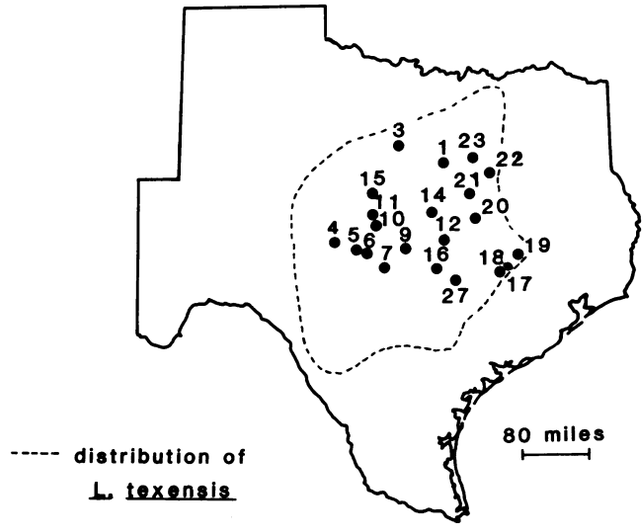

FIG. 1. Distribution of *L. texensis* and location of collection sites from which plants were analyzed. Sites: 1, Cleburn State Park, Johnson County, 6 plants; 3, Strand, Palo Pinto County, 6 plants; 4, Menard, Menard County, 1 plant; 5, Streeter, Mason County, 11 plants; 6, Mason, Mason County, 4 plants; 7, Willow City, Gillespie County, 12 plants; 9, Inks Lake State Park, Burnet County, 2 plants; 10, Richard Springs, San Saba County, 5 plants; 11, Locker, San Saba County, 1 plant; 12, Brownwood, Brown County, 2 plants; 14, Gatesville, Coryell County, 7 plants; 15, Bartlett, Bell County, 3 plants; 16, Austin, Travis County, 2 plants; 17, Brenham, Washington County, 8 plants; 18, Washington, Washington County, 8 plants; 19, Anderson, Grimes County, 6 plants; 20, Marlin, Falls County, 4 plants; 21, Axtell, McLellan County, 11 plants; 22, intersection of FM744 and Tx22, Navarro County, 3 plants; 23, Palmer, Ellis County, 4 plants; 27, Bastrop, Bastrop County, 1 plant.

Colonies were grown on nitrocellulose filters placed on LB plates (20) containing the appropriate antibiotic; the cells were then lysed and the liberated DNA was fixed to the filter according to procedure II of ref. 20.

RESULTS

Wild-Type cpDNA. cpDNA isolated from 100 individual *L. texensis* plants was digested with seven restriction enzymes *Bam*HI, *Bcl* I, *Bgl* II, *Hin*dIII, *Hpa* I, *Sst* I, and *Xho* I. These seven enzymes produced a total of 134 detectable fragments in the most common form of cpDNA. This form, hereafter called wild type, was found in 88 plants. Three variant forms (described below) were identified in the remaining plants by visual comparison of restriction gels.

Our methods may have resulted in a slight underestimate of the amount of variability, for three reasons. First, some of the restriction enzymes generate a number of fragments of less than 1 kbp. These were not included in the analysis because the amount of cpDNA obtained from some of the smaller plants was insufficient for their detection. The sum of these fragments, seen in preparations from larger plants or pooled preparations from several plants, represents less than 8% of the total chloroplast genome per digestion. Second, visual inspection of the gels would not detect very small differences in fragment size. For instance, we were able to detect a deletion of 0.1 kbp in a 3.8-kbp fragment but not in a 7.5-kbp fragment. Third, digests containing apparent variant fragments were run at least twice. Restriction fragments (or sites) were considered variant only if the mobility of a fragment was reproducibly different in side-by-side comparisons of DNAs from different plants. Digests that did not show apparent variants (e.g., those shown in Fig. 2) were not repeated.

Results of restriction site mapping experiments (22) indicate that the *L. texensis* chloroplast genome includes a large inverted repeat containing the *rRNA* genes, as is typical for plant cpDNAs. Consequently, restriction fragments contained entirely within the inverted repeat appear twice as intense as single-copy fragments having the same molecular weight. Without having mapped all sites for all seven restriction enzymes, it is impossible to determine whether a band that appears in nonstoichiometric amounts (i.e., $\geq$2-fold) is due to two or more comigrating-nonhomologous fragments or due to the fragment being repeated in the genome. The latter condition is more likely because the size of the inverted repeat is quite large, 23 kbp. In the many plant species investigated thus far, there have been no reports of the two copies of the repeat having different restriction sites. This, coupled with the observation that induced insertion/deletion mutations within the inverted repeat of *Chlamydomonas* always occur symmetrically in both copies (23), suggests that mutations that occur in one copy of the repeat quickly spread to the other by intra- and intermolecular recombination. For this reason, fragments appearing in 2-fold quantities were scored as single fragments.

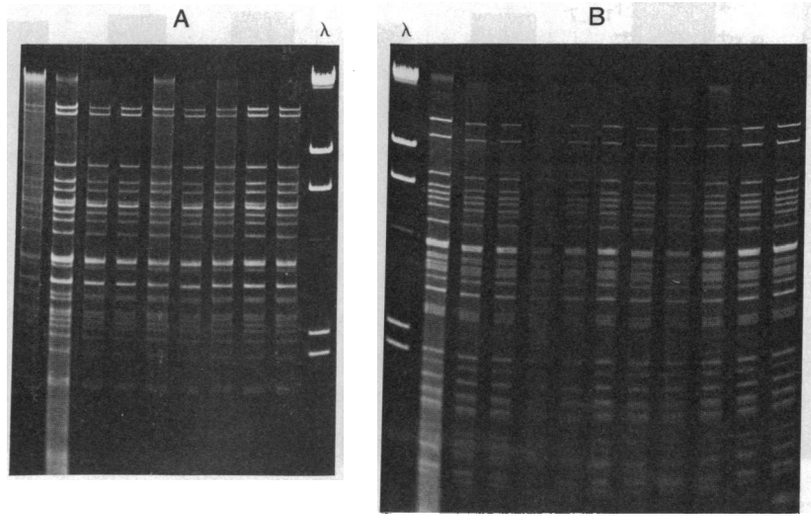

FIG. 2. Samples of wild-type cpDNA digested with *Bgl* II (*A*) and *Bcl* I (*B*). These samples were considered to be identical. Lanes λ: *Hin*dIII-digested λDNA.

Variant Forms of cpDNA. cpDNA from one of the two plants analyzed from population 12 differed from the *Bcl* I wild-type restriction pattern (Fig. 3); cpDNA isolated from this plant is called var12 cpDNA. In it 13.7 kbp *Bcl* I fragment present in wild-type cpDNA was replaced by fragments, 9.9 and 3.7 kbp in length, suggesting that this variant was the result of a base-pair substitution that generated a new *Bcl* I site within the 13.7-kbp fragment. We did not have enough cpDNA from this plant to verify this conclusion by Southern hybridization. A second variant was observed in one of the six plants analyzed from population 1. As shown in Fig. 3, cpDNA from this plant (called var1 cpDNA) lacks two wild-type *Bgl* II fragments, 6.2 and 1.3 kbp, and appears to contain no new fragments. The same *Bgl* II pattern was seen in another variant, var5. Experiments with var5 described below show that wild-type cpDNA has two comigrating fragments at 7.5 kbp and that var1 and var5 have acquired a new site in one of these, producing the new 6.2- and 1.3-kbp fragments.

The third variant (var5) was observed in all of the 11 plants analyzed from population 5. As shown in Fig. 3, var5 cpDNA differs from wild type by (*i*) the loss of the same two *Bgl* II fragments (6.2 and 1.3 kbp) that are absent in var1 cpDNA, (*ii*) the loss of a 1.4-kbp *Bcl* I fragment, (*iii*) the loss of a 3.8-kbp and the gain of a 3.7-kbp *Sst* I fragment, and (*iv*) the loss of a 4.9-kbp and the gain of a 4.8-kbp *Pst* I fragment (data not shown). Var5 cpDNA appears to be identical to the wild type for all other enzymes tested (*Bam*HI, *Hpa* I, *Sal* I, and *Xho* I).

To determine the nature of the *Bgl* II fragment variations in var5 (and var1), the two *Bgl* II fragments present in wild type but absent in the variants were isolated from band in agarose gels, labeled by nick-translation, and used as probes against *E. coli* colonies harboring random wild-type *Hin*dIII fragments cloned into pBR322. The 4.2-kbp-*Hin*dIII fragment 10 was found in all positive clones; this fragment was nick-translated for use as a probe against wild type. Var5 cpDNA was digested with *Bcl* I and *Bgl* II (Fig. 4) and with *Hin*dIII and *Bam*HI (not shown). Although the *Hin*dIII fragment 10 hybridized to the two wild-type *Bgl* II fragments, the same probe hybridized only to a larger (7.5 kbp) *Bgl* II var5 fragment. This 7.5-kbp fragment was not originally detected on stained gels of var5 cpDNA digested with *Bgl* II because it comigrates with another 7.5 kbp *Bgl* II fragment present in both the wild type and var5 *Bgl* II digests. These results indicate that the *Bgl* II variation is due to the loss of a *Bgl* II site in var5 cpDNA relative to wild-type cpDNA. The same site has evidently been lost in var1. Unless we can find an additional plant with the var1 pattern, and sequence it and var5, we cannot tell whether the loss of the sites in var1 and var5 was due to the same mutation. Multiple occurrences of deletions are fairly common in some regions of cpDNA, while parallel base-pair substitutions are extremely rare (24). Alternatively the site could have been lost in a common ancestor that occupied populations 1 and 5 before the deletion was fixed in population 5.

The other variant restriction fragments in var5 cpDNA are evidently due to a single deletion of about 100 bp, as suggested by the results of two hybridization experiments. In the first, the 4.3-kbp *Hin*dIII fragment that is present in wild-type cpDNA (*Hin*dIII fragment 9) but absent in var5 was cloned and shown to hybridize to the following fragments: (*i*) in *Hin*dIII digests, a 4.3-kbp fragment in wild type (i.e., *Hin*dIII fragment 9 to itself) and a 4.2-kbp fragment in var5; (*ii*) *Sst* fragments of 3.8 kbp in wild type and 3.7 kbp in var5 (Fig. 4); (*iii*) *Bcl* I fragments of 1.4 kbp in wild type and 1.3 kbp in var5 (Fig. 4); and (*iv*) *Pst* I fragments of 4.9 kbp in wild type and 4.8 kbp in var5. Second, the 4.9-kbp *Pst* I fragment was in turn cloned and shown to hybridize to the *Sst* I, *Bcl* I, and *Hin*dIII fragments that hybridized to the wild-type *Hin*dIII fragment 9. Thus, all of the variant fragments of var5 (except those due to the loss of the *Bgl* II site) are smaller than the homologous wild-type fragments by approximately 100 bp. Although wild type and var5 cpDNAs are identical in restriction patterns for some enzymes, agarose gel electrophoresis cannot resolve a deletion of 100 bp if it occurs within very large fragments. For example, the wild-type *Hin*dIII fragment 9 that identifies deleted fragments hybridizes to apparently identical var5 and wild-type *Bgl* II fragments, one of which is 16 kbp.

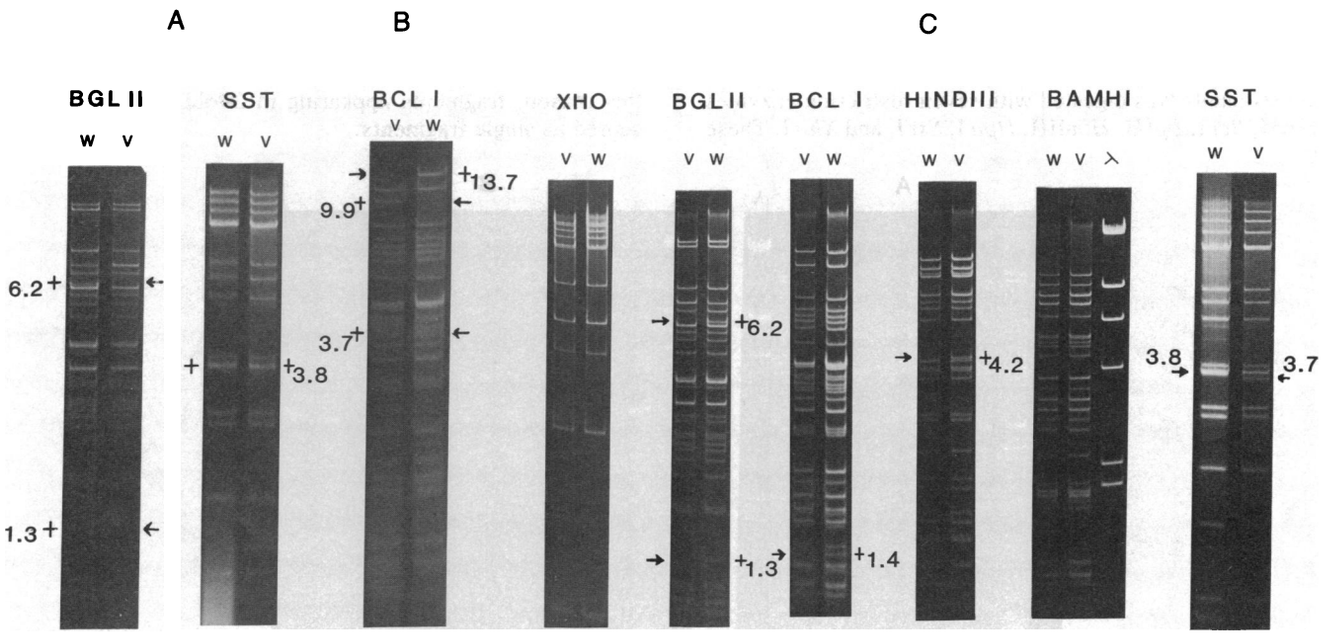

FIG. 3. Comparison of restriction fragment patterns of var1 (*A*), var12 (*B*), and var5 (*C*) cpDNA's to wild-type DNA. The variant DNAs are in lanes labeled v, the wild-type DNAs are in lanes labeled w. The *Sst* digests of var1 and wild-type cpDNA demonstrate that the deletion found in var5 cpDNA is absent in var1 plants, although var1 and var5 appear to share the same *Bgl* II polymorphism. Lane λ: *Hin*dIII-digested λDNA.

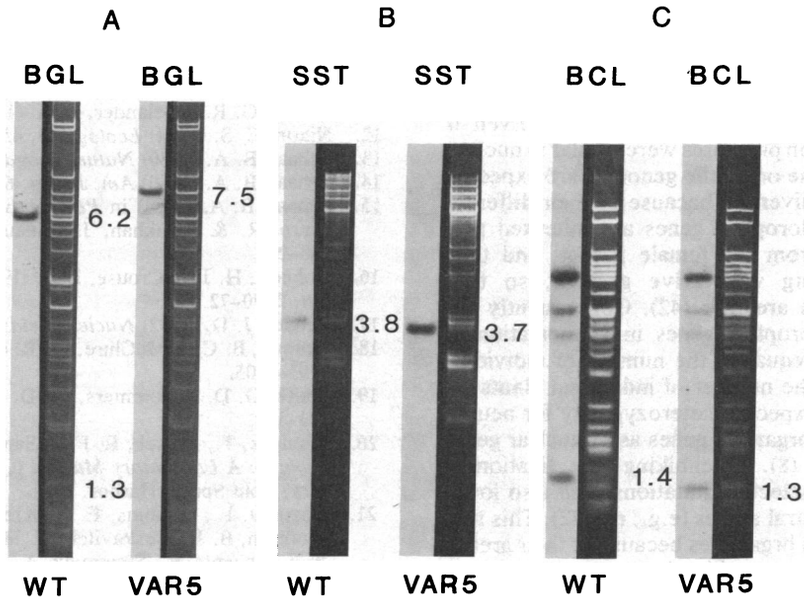

FIG. 4. Southern hybridization experiments demonstrating the basis of the *Bgl* II polymorphism (*A*) and the deletion (*B* and *C*) in var5 plants. (*A*) *Bgl* II digests probed with *Hin*dIII fragment 10. The 1.3-kbp wild-type fragment is seen only on longer exposures of the autoradiogram. (*B* and *C*) *Sst* I and *Bcl* I digests, respectively, probed with *Hin*dIII fragment 9.

The above data locate the var5 deletion in *Pst* I fragment 9, in the large single-copy region of the cpDNA molecule. To locate the *Bgl* II polymorphism on the *Sal* I–*Pst* I restriction map, we used *Hin*dIII fragment 10 as a probe against wild-type cpDNA digested with *Sal* I and *Pst* I. The smallest fragment hybridizing to this probe was *Pst* I fragment 4 (17.4 kbp), which is adjacent to *Pst* I fragment 9. The *Hin*dIII fragment 10 probe, which identifies the *Bgl* II polymorphism, and the *Hin*dIII fragment 9 probe, which identifies the deletion, also hybridize to different *Bcl* I and *Bgl* II fragments. Thus, the loss of the *Bgl* II site in var5 cpDNA is probably independent of the deletion, since they are separated by restriction sites for at least three different enzymes.

DISCUSSION

Restriction fragment differences between the cpDNAs of different populations have been detected in *Zea mays* (25), *Nicotiana debneyi* (26), *Lycopersicon peruvianum* (27), and *Pisum sativum+humile* (24), but not between different races of pearl millet (*Pennisetum americanum*) from around the world (28). Restriction site variation was found within as well as between populations of wild barley (*Hordeum spontaneum*), but significantly fewer differences were found among cultivars and primitive-land races of domesticated barley (*Hordeum vulgare*) (29). In each of these studies the sample sizes were small and the nucleotide diversity was not calculated.

We found variation within as well as between populations; the variation involved apparent point mutations as well as a small deletion. To quantitate this variation, we used the statistics of Nei and Li (30), Nei and Tajima (31), and Engels (32). The detailed calculations are given elsewhere (22). Chloroplast nucleotide diversity (π, the probability that two randomly chosen cpDNA molecules will have different nucleotides at a given nucleotide site; refs. 30 and 31) is 0.00026 (SD = 0.00093). This assumes that the apparent restriction site gains or losses are single base-pair substitutions and gives the proposed var5 deletion the same weight as a substitution; π = 0.00007 (SD = 0.00072) if the deletion is excluded from the calculation. These numbers can be compared to values of π calculated by Nei (2) for nuclear and mitochondrial DNA sequences in animals. cpDNA diversity is significantly lower than animal nuclear DNA diversity, which ranges from 0.002 for the human β-globin gene family (33) and the human albumin locus (34) to 0.006 for the *Drosophila* alcohol dehydrogenase region (35). It is also lower than values for animal mtDNA, which range from 0.004 in humans (36) to 0.013 in chimpanzees (37). Nei and Tajima (31) defined a nucleon as any segment of DNA; here, it is the entire cpDNA molecule. The calculation of nucleon diversity (h, the probability that two randomly chosen cpDNA molecules will differ in at least one nucleotide site) includes both point mutations and deletions (31). In *L. texensis* cpDNA, h = 0.2331 (SD = 0.0370). This can be compared to the nucleon diversity of animal mtDNA, calculated from the data of Shah and Langley (35) by Nei and Tajima (31) to be 0.7100 in *Drosophila melanogaster*. A third measure of genetic diversity is the probability (P) that a given nucleotide position will be polymorphic in a population of molecules (32). From our data, P = 0.00124 (counting the var5 deletion as a single base-pair substitution to provide an upper estimate). This estimate is much lower than ones for human mtDNA (0.0264) or globin genes (0.0048) (32).

It is clear that by any measure, cpDNA diversity in *L. texensis* is much lower than the diversity of mtDNA, and it is probably lower than the diversity of nuclear DNA, in animals. There are no comparable data for mitochondrial or nuclear gene diversity at the DNA level in wild plants. However, the average expected heterozygosity of nuclear genes in *L. texensis* calculated from enzyme electrophoresis data is 0.4138. This is substantially higher than that for most animals (38) and is higher than that for most other plants (39). Since the animal nuclear genes for which nucleotide diversities can be calculated are more diverse than the cpDNA of *L. texensis*, it is very likely that cpDNA is substantially less diverse than nuclear DNA in this plant.

One possible explanation for low genetic variability is a low mutation rate. The rate of synonymous base pair substitutions in plant chloroplast genes appears to be much lower than the rate for animal mitochondria and may be somewhat lower than the synonymous and pseudogene substitution rates in animal nuclear genes (40, 41), which suggests a lower mutation rate. Unfortunately, there are no comparable data

for plant nuclear or mitochondrial genes. Low genetic variability could also be due to stringent selection against new mutations (i.e., a high porportion of new mutations would be detrimental as opposed to neutral or nearly neutral). There are no data with which to test this possibility. Even if mutation rates and selection pressures were similar in nuclear and organelle genomes, the organelle genomes are expected to have a lower genetic diversity because of their different mode of inheritance. Chloroplast genes are inherited predominantly or entirely from the female parent, and they segregate rapidly during vegetative growth, so that heteroplasmic individuals are rare (42). Consequently the effective number of chloroplast genes in a population of hermaphroditic plants is equal to the number of individual plants, while it is twice the number of individual plants for nuclear genes; thus the expected heterozygosity for neutral alleles is half as great in organelle genes as in nuclear genes in the same population (8). Hitchhiking (the fixation of neutral alleles linked to selected mutations) will also lower the heterozygosity of neutral alleles (e.g., ref. 42). This may be especially important in organelles because of their greatly reduced opportunities for recombination.

Moreover, if the plant population is subdivided, organelle genes will have lower effective migration rates than nuclear genes because the pollen will transmit them with reduced probability or not at all. In the lupines, pollen dispersal is greater than seed dispersal (13); it is not known whether these plants transmit chloroplast genes via the pollen, but pollen transmission is greatly reduced or absent in all angiosperms where it has been studied (43). Lower migration rates for chloroplast genes relative to nuclear genes are expected to increase genetic diversity between demes and in the species as a whole, while decreasing diversity within local populations (ref. 10; unpublished results). Our sample sizes from individual populations are too small, and the total genetic diversity is too low to permit the accurate calculation of measures of population subdivision. However, it is worth noting that all 11 individuals sampled from population 5 had the same variant cpDNA type, while the individuals taken from the closest local populations we sampled (sites 4, 6, and 10) were all wild type; other populations with large samples (sites 7 and 21, with 12 and 11 individuals respectively) were also homogeneous for the wild-type pattern.

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