Molecular Phylogeny of Larrea and Its Allies (Zygophyllaceae): Reticulate Evolution and the Probable Time of Creosote Bush Arrival to North America

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Nucleotide sequences of Rubisco Large Subunit (rbcL) and the internal transcribed spacers (ITS) of nrDNA were obtained for the five species of Larrea and one species each of Bulnesia (ITS only) and Plectrocarpa (rbcL only). Parsimony analyses were conducted, including sequences from seven genera of Zygophyllaceae reported by other authors—Kalistratemia, Zygophyllum, Augea, Fagonia, Pintoa, Guaiaicum, and Porlieria. The main conclusions of the present study are (1) the Argentine endemic Plectrocarpa tetracantha belongs to the subfamily Larreoideae (New World Clade); (2) all three phylogenies obtained from rbcL, ITS, and combined data sets show a close relationship between the tetraploid L. cuneifolia (sect. Bifolium) and the diploid multifoliolate pair L. nitida–L. ameghinoi (sect. Larrea), which could result from a possible intersectional hybrid origin of the tetraploid; (3) L. divaricata (sect. Bifolium) and L. tridentata (sect. Bifolium) form a highly supported monophyletic group, which agrees with previous cytogenetic and molecular evidence; and (4) the rate of nucleotide substitution of rbcL was estimated based on geological and fossil records. Under the molecular clock hypothesis, nucleotide sequence divergence between L. divaricata and L. tridentata suggests a Late Neogene (8.4 to 4.2 mybp) time of arrival of the diploid ancestors of L. tridentata to North American deserts.

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INTRODUCTION

The genus Larrea (Zygophyllaceae) is composed of evergreen, woody shrubs of wide geographical distribution in the major warm deserts of the New World, covering large arid and semiarid regions of Argentina, Chile, Bolivia, Peru, Mexico, and the southwestern United States. It comprises five species: four in South America, L. ameghinoi Speg., L. nitida Cav., L. divaricata Cav., and L. cuneifolia Cav. ("jarillas"), and one in North America, L. tridentata (D.C.) Coville ("gobernadora," "creosote bush") (Hunziker et al., 1977, 1978).

Two sections are recognized within the genus (Palacios and Hunziker, 1972). Section Larrea includes diploid species L. ameghinoi and L. nitida (2n = 26) with multifoliolate leaves and rather small flowers. The remaining three species constitute section Bifolium and are distinguished mainly by the presence of bifoliolate leaves and larger flowers, exhibiting different ploidal levels: L. divaricata (2n = 26), L. cuneifolia (2n = 52), and L. tridentata (2n = 26, 52, 78).

Phylogenetic affinities within the genus have been inferred on the basis of morphological, cytogenetic, and biochemical studies (Hunziker et al., 1977, 1978; Yang et al., 1977). Several lines of evidence have given support to the hypothesis of a close relationship between the supposedly more unspecialized representative of the genus, L. nitida (Hunziker et al., 1977), and the chamaephyte L. ameghinoi. Cytogenetic analysis of their interspecific hybrid revealed regular meiosis and bivalent configuration, demonstrating high levels of homology between genomes and the absence of strong postzygotic isolation mechanisms. Different ecological preferences (Hunziker et al., 1978), however, seem to restrict the extent of gene flow between these species, as confirmed by isozyme electrophoresis data (Lia et al., 2000).

Despite their strictly allopatric distributions, L. divaricata and L. tridentata are morphologically and biochemically similar (Yang, 1967; Hunziker et al., 1977; Mabry et al., 1977; Cortés and Hunziker, 1997; Lia et al., 2000). Moreover, the close relationship between these species is also supported by the regular chromosome behavior in the diploid hybrids and the partial gene exchange that is still possible through the semisterile diploid hybrid (Yang et al., 1977).

Concerning the origin of the tetraploid L. cuneifolia, analysis of meiotic behavior showed regular formation of 26 bivalents and absence of multivalents, a fact that would suggest allopolyploidy. Evidence of the identity
of one of the parental species was provided by the chromosome behavior of the triploid sterile hybrid L. cuneifolia × L. divaricata, which exhibited 13 bivalents and 13 univalents as the most frequent configuration, indicating that L. divaricata (or a species very closely related to it) may have been involved in its origin (Hunziker et al., 1977, 1978). The biogeographical history of Larrea provides an interesting case for the study of amphitropical disjunctions. There are two general hypotheses concerning the geographical origin of Larrea: (1) Johnston (1940) proposed a South American origin for Larrea, a hypothesis further sustained by Hunziker et al. (1972), Hunziker (1975), and Wells and Hunziker (1977) based on the fact that the genera of Zygophyllaceae most likely to be closely related to Larrea have contemporary distributions that suggest a northern South American origin for most of them, and (2) Turner (1972) proposed that L. tridentata developed as a diploid population in North America millions of years ago and subsequently became established in South America through long-distance dispersal. Porter (1974) also supported this view originally, but later admitted (1979) that it was no longer tenable and concluded that the South American origin “had been proved convincingly in a series of papers by Hunziker and his associates.” Comparative sequencing of chloroplast and nuclear genes has proven to be of great value in reconstructing the phylogenetic history of numerous groups of plants (for a review see Soltis et al., 1998). Chloroplast DNA variation has been useful for elucidating relationships at higher taxonomic levels within the family Zygophyllaceae. On the basis of a cladistic analysis of morphological and anatomical characters, rbcL sequence data, and DNA sequences of the noncoding trnL-F region, five subfamilies were recognized within the Zygophyllaceae s.s. (Sheahan and Chase, 1996, 2000). A monophyletic lineage including Larrea, Bulnesia, Guaiacum, Pintoa, and Porlieria was assigned to subfamily Larreoidae. According to Porter (1974), these New World genera may have separated from the Old World groups by sea floor spreading in the early Cretaceous. Separation between Africa and South America was almost complete about 80–100 mybp (Pitman et al., 1993; Goldblatt, 1993; Hallam, 1994). However, both continents continued to be fairly connected via a series of volcanic islands until approximately the end of the Eocene (~38 mybp), when they were separated by 1400 km and fewer islands remained (Raven and Axelrod, 1974). Leopold and MacGinitie (1972) reported the first known appearance of pollen of Guaiacum in North America by Early Middle Eocene times (~45 mybp). Taking into account the assumptions of Porter (1974), this scenario would place the origin of the New World clade within the time frame delimited by the opening of the Atlantic Ocean and the earliest fossil record belonging to this clade found in America.

The present paper reports the phylogenetic analysis of nucleotide sequences of the rbcL chloroplast gene and internal transcribed spacer regions (ITS-1, ITS-2) of nuclear ribosomal DNA from the five species of Larrea and related genera. The main purposes of this study were to (1) examine the evolutionary relationships within Larrea and among Larrea and other members of the Zygophyllaceae, (2) identify the progenitors of the tetraploid L. cuneifolia, (3) gain further insights into the geographical origin of the genus, and (4) estimate the time elapsed since the origin of the amphitropical disjunction of Larrea, by use of a rbcL rate of nucleotide substitution here obtained for the Zygophyllaceae.

**MATERIALS AND METHODS**

This study includes the five species of Larrea and representatives from nine related genera of the Zygophyllaceae. rbcL sequences were determined for L. ameghinoi, L. cuneifolia, L. divaricata, L. tridentata, and Plectrocarpa tetracantha. Two specimens of the tetraploid, L. cuneifolia, from different geographic areas were included. Kallstroemia maxima, Fagonia indica, Augea capensis, Zygophyllum simplex, Porlieria chilensis, Pintoa chilensis, Guaiacum guatemalense, and Bulnesia arborea rbcL sequences were taken from GenBank (Table 1).

ITS sequences were obtained for all Larrea species and B. arborea. South American species of Larrea and Plectrocarpa tetracantha were collected in the field from natural populations, whereas leaf material corresponding to L. tridentata (2×) and B. arborea was obtained from specimens grown under cultivation at the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires. Voucher specimens were deposited at the herbarium of “Darwinion” Institute (SI).

Species names, GenBank accessions, collection sites, voucher information, and prior publication references are given in Table 1.

DNA extraction, amplification, and sequencing. Total genomic DNA was isolated from 0.3 g of dried leaves following the protocol described by Yang et al. (2000). The rbcL gene and internal transcribed spacers (ITS-1 and ITS-2) were amplified from total genomic DNA by the polymerase chain reaction (PCR). The rbcL gene was amplified with primers Z1 and Z1351R described in Soltis et al. (1992). Two additional internal primers were designed based on the partial sequences obtained for Larrea species (L2F: ATGTTTAATTTCCATTGTGGG; L33R: TGTACTCTCTTTCCCCCTTCA) with the software program Primer3.cgi 0.2c (Rozen and Skaletsky, 1996, 1997). Amplification of ITS-1 and ITS-2 was performed with primer pairs ITS-4/ITS-5 (White et al., 1990) and Q1/Q2 (Samuel et al., 1998). Reaction mixtures contained 80–100 ng of template DNA, 0.5
μM each amplification primer, 200 μM dNTPs, 3 mM MgCl₂, 2 units of Taq DNA Polymerase (Promega), 1× Buffer, and sterile distilled water to a final volume of 50 μl.

The amplified segments were electroforesed in 1% agarose gels and purified with the GeniePrep Kit (Ambion) or QIAQuick gel extraction Kit (QIAGEN Inc.). Nucleotide sequences were obtained with a PE Biosystems automated 377 DNA sequencer.

Sequence alignment and phylogenetic analysis. Multiple alignment of DNA sequences was accomplished with the CLUSTAL W computer software program (Thompson et al., 1994), followed by minor manual corrections. The rbcL alignment yielded a 1316-character matrix corresponding to positions 34 to 1349 of the *Zea mays* rbcL gene. The boundaries of ITS-1 and ITS-2 were determined by comparison to the published sequence of *Dipteronia sinensis* (Sapindaceae) (GenBank Accession No. AF020386), the most closely related species for which this information was available and complete. Positions 1 to 18 of ITS-1 were excluded from the analyses due to ambiguous base calls in some of the sequences, resulting in a data matrix of 414 characters.

Parsimony analysis of rbcL sequences was performed with the branch and bound search method provided by Swofford’s win-PAUP 4b-4a. The search was repeated with the exact search command in NONA 1.6 (Goloboff, 1993). Selection of outgroup taxa for the analysis of *Larrea* was conducted with regard to the phylogenetic reconstruction previously reported by Sheahan and Chase (1996, 2000). These outgroups included representatives of all genera (except *Metharme*) of subfamily Larreoideae (*G. guatemalense*, *Porlieria chilensis*, *Pintoa chilensis*, *B. arborea*, and *Plectrocarpa tetracantha*), three representatives of the four genera (*F. indica*, *Z. simplex*, and *A. capensis*) of subfamily Zygophylloideae, the sister clade to Larreoideae, and a species from subfamily Tribuloideae (*K. maxima*), which is the sister group to both Larreoideae and Zygophylloideae. All character changes were given equal weight.

The ITS analysis was conducted including a single outgroup represented by *Bulnesia* (B. arborea), a genus considered closely related to *Larrea* (Hunziker et al., 1977). Phylogenies were inferred with the exhaustive search mode provided by the beta test version of win-PAUP 4b-4a under the Fitch criterion. Gaps were treated as fifth base.

Phylogenetic congruence of rbcL and ITS data sets...
was tested by the partition homogeneity test of Farris et al. (1995) with win-PAUP4b-8. One thousand partition replicates were analyzed by maximum-parsimony, with the branch-and-bound search option.

The taxa included in the combined analysis had to be restricted to *B. arborea* (outgroup) and the five species of genus *Larrea*, because no ITS sequence data were available for the remaining species of the *rbcL* phylogeny. Parsimony analysis was performed as described for the ITS data set.

The number of steps required to force monophyly of certain groups was calculated with the branch-and-bound search option with the CONSTRAINTS and ENFORCE commands of win-PAUP4b-8. The two-tailed Wilcoxon signed-ranks test (Templeton, 1983) was used to assess whether the data provided significantly less support for alternative topologies derived from constrained analyses than the most parsimonious topology.

Internal support for the trees generated from all data sets was measured with 1000 replicates of the heuristic search bootstrap option (Felsenstein, 1985) available with PAUP. Starting trees for each bootstrap replicate were obtained via stepwise addition with SIMPLE addition sequence, keeping one tree at each step during the stepwise addition. Branch swapping was performed with the tree bisection-reconnection (TBR) algorithm. Bremer (1988) support values were calculated with NONA 1.6, keeping suboptimal trees up to 20 steps longer than the minimal length tree produced by the exact search.

Based on the principal morphological criteria for the delimitation of section Bifolium, two morphological characters were selected for optimization on the *rbcL* phylogeny to establish their plesiomorphic states in *Larrea*. These characters are (1) number of leaflets (M, multifoliolate leaves; B, bifoliolate leaves), and (2) hairiness of fruits (h, fruit glabrous, verrucose, puberulous, or short haired; H, fruit hirsute, with very long stiff hairs (at least as long as smaller fruit diameter)).

### TABLE 2

<table>
<thead>
<tr>
<th>Species</th>
<th>Character</th>
<th>Species</th>
<th>Character</th>
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<td>M (4–9)</td>
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<td>M (6–18)</td>
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<td><strong>Electrocarpa</strong></td>
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</table>

Note. Number of leaflets: M, multifoliolate leaves; B, bifoliolate leaves; actual number of leaflets is indicated in parentheses. Hairiness of fruits: h, fruit glabrous, verrucose, puberulous, or short haired; H, fruit hirsute, with very long stiff hairs (at least as long as smaller fruit diameter).

Estimation of *rbcL* substitution rate. Pairwise sequence comparisons used for the estimation of the substitution rate of *rbcL* included representatives of all genera of subfamily Larreoideae except *Metharme* (*New World clade*) and *F. indica*, *A. capensis*, and *Z. simplex* as representatives of subfamily Zygophyllae (Old World clade). Despite its almost cosmopolitan distribution, *Fagonia* was considered a member of the Old World clade following the assumptions of Porter (1974), who pointed out that this genus has no close relatives in the New World and invoked long-distance dispersal from east to west during the Early Tertiary to account for its presence in America. The *rbcL* sequence of *K. maxima* was also used for comparison.
Rate constancy across these lineages was tested with Felsenstein’s (1988, 1993) global test as described in the PHYLIP software package. A global test was chosen to avoid the problems that may appear when the statistical conclusions of multiple relative rate tests that share nodes in common are combined.

As previously mentioned, the upper and lower boundaries of the time since divergence of the New World clade were determined based on the fossil record for the group (45–90 My) and an average of the three estimates reported by Goldblatt (1993), Hallam (1994), and Pitman et al. (1993) for the complete separation between Africa and South America (90 My). These boundaries were used as calibration points for the estimation of evolutionary rates (k) based on F84 pairwise distances calculated with win-PAUP 4b-4a (Felsenstein, 1993; Kishino and Hasegawa, 1989). The F 84 model of sequence evolution assumes unequal base frequencies and unequal transition and transversion rates. The averaged F84 distance (D) (between each species of the New World clade and the species from the Old World group, along with the calibration points (t) described above (45–90 My), were used to calculate k following the equation D = 2 kt (Nei, 1987). The resulting k values were utilized to estimate a range of time since divergence between L. tridentata and L. divaricata by division of the corresponding F84 distance by twice the rate of nucleotide substitution.

RESULTS

The new sequences generated in this study are available from GenBank under the accession numbers listed in Table 1. The two specimens of L. cuneifolia included in the sampled taxa yielded identical rbcL sequences and are represented as single entry in all subsequent analyses.

Sequence and Phylogenetic Analyses of rbcL

Of the 1316 aligned characters included in the rbcL data matrix, 1085 (82.4%) characters were invariant, 105 (8.0%) were variable but uninformative with respect to phylogeny, and 126 (9.6%) were phylogenetically informative. Maximum-parsimony analysis yielded a single most parsimonious tree consisting of 355 steps with a consistency index (CI) of 0.7296 (CI: 0.6049 excluding uninformative characters) and a retention index of 0.6811.

The topology of the inferred tree agrees with the subfamilial division proposed by Sheahan and Chase (1996). Moreover, the present analysis confirms that the genus Plectrocarpa should be included within the Larreoideae, a position tentatively proposed by these authors.

The monophyly of the groupings corresponding to subfamilies Larreoideae and Zygophyllidoideae is supported by maximum bootstrap values (100%) and decay values of 19 and higher than 20, respectively (Fig. 1). Within Larreoideae, Pintoa chilensis appears as the sister group to the clade containing all Larrea species, whereas Porlieria chilensis, G. guatemalense, B. arborea, and Plectrocarpa tetracantha formed a separate grouping with Porlieria chilensis as the sister to G. guatemalense and with B. arborea as the sister to Plectrocarpa tetracantha. Intergeneric relationships are weakly supported and do not seem to be concordant with the relationships previously suggested on morphological and cytological grounds (Engler, 1931; Porter, 1974; Poggio, 1978). Moreover, the forcing of sister group relationships between Larrea and the other clades obtained within Larreoideae in the most parsimonious tree—(Larrea, (B. arborea, Plectrocarpa tetracantha)); (Larrea, (Porlieria chilensis, G. guatemalense)); (Larrea, ((B. arborea, Plectrocarpa tetracantha), (Porlieria chilensis, G. guatemalense))—resulted in alternative topologies that were not significantly less parsimonious than the shortest tree (P = 0.5637, 0.7055, 0.1797, respectively).

The species of Larrea formed a clade supported by a bootstrap value of 90% and a decay index of 4. Two major lineages were identified within Larrea (Fig. 1), both of them strongly supported. The first is composed of three species, L. ameghinoi, L. nitida, and L. cuneifolia and the second comprises L. tridentata and L. divaricata. The retrieved groupings do not reflect the infrageneric taxonomic treatment of the genus, because L. cuneifolia (section Bifolium), L. ameghinoi (section Larrea), and L. nitida (section Larrea) are all contained within the same group. In this clade, L. ameghinoi appears as the sister to the clade of L. nitida and L. cuneifolia. The relationship between L. nitida and L. cuneifolia is supported by relatively high bootstrap and decay values. However, only three homoplastic characters support this clade.

The enforced monophyly of Bifolium required the addition of nine extra steps. Evaluation of the resulting topologies with Templeton’s test indicated that they were significantly less parsimonious than the shortest tree (P = 0.029).

Mapping the morphological characters defined as “number of leaflets” and “hairiness of fruits” on the rbcL phylogeny revealed that both the reduction in number of leaflets to two and the presence of very long stiff hairs constitute a derived condition within the genus.

Sequence and Phylogenetic Analysis of ITS-1 and ITS-2

Lengths for the ITS regions sequenced for this study ranged from 212 to 214 and from 213 to 216 for ITS-1 and ITS-2, respectively. These observations are concordant with the range of lengths previously reported for several groups of angiosperms (Baldwin et al., 1995). Sequence alignment revealed four insertion/deletion events within ITS-1 (1 bp each), and two were detected.
within ITS-2 (1 bp each). After removal of ambiguous base calls, a matrix of 414 characters was used for the analyses. Of these characters, 312 (75.4%) were constant and 102 (24.6%) were variable, with 40 (9.7%) of the latter being phylogenetically informative.

Parsimony analysis generated a single most parsimonious tree consisting of 124 steps (Fig. 2) with a consistency index of 0.8952 (CI: 0.7679 excluding uninformative characters) and a retention index of 0.74. The resulting tree exhibited the same two distinct main lineages that were found within Larrea in the analysis of rbcL sequences (Fig. 2). The only difference between the two arrangements is the placement of L. cuneifolia. In this case, L. cuneifolia appeared as the sister to a highly supported clade containing species of section Larrea, whereas L. tridentata and L. divaricata formed a monophyletic group, as they did in the rbcL analysis.

The alternative placement of L. cuneifolia as sister to L. tridentata and L. divaricata increased the length of the tree by three extra steps. Although evaluation with Templeton's test failed to reject the hypothesis of monophyly of section Bifolium, these results should be interpreted with caution due to the low number of characters that showed variation from one topology to the other.

Combined Analysis of rbcL and ITS Data Sets

Despite the differences in the dynamics of inheritance of the markers employed for this study, relationships among species of Larrea derived from rbcL sequence analysis are generally concordant with those obtained with ITS sequence data. The partition homogeneity test indicated phylogenetic congruence between data sets (P = 1.000), justifying their combination for subsequent analyses. Combined analysis of rbcL and ITS data sets (1729 characters) yielded a single most parsimonious tree consisting of 190 steps with a consistency index of 0.9031 (CI: 0.7625 excluding uninformative characters) and a retention index of 0.7467. The obtained tree was identical to the tree generated by the ITS data matrix, with clades being supported by higher bootstrap values (Fig. 3). The forcing of the monophyly of section Bifolium required the addition of 10 extra steps. This topology was significantly less parsimonious than the shortest tree as evaluated by Templeton's test (P = 0.0124).
rbcL Substitution Rate

Felsenstein's global test did not reveal any significant deviation from rate constancy among the 14 taxa included in the analysis ($\chi^2 = 16.353; \text{df} = 12; P > 0.05$). Pairwise distances calculated under the F84 substitution model and the parameters used for their calculation are depicted in Table 3. With the putative New World dade divergence dates presented under Materials and Methods, rbcL substitution rates for the genera of Zygophyllaceae included in this study were estimated at $4.6 \times 10^{-10}$ substitutions per site per year (90 My) and $9.2 \times 10^{-10}$ substitutions per site per year (45 My). Under these assumptions, and with the nucleotide distance between $L$. divaricata and $L$. tridentata (Table 3) taken into account, these species would have diverged between 8.4 and 4.2 mybp, placing the origin of Larrea in North America within the same time frame, when populations became allopatric.

**DISCUSSION**

Phylogenetic Reconstruction

Engler (1896) conducted a comprehensive study of the distribution and systematics of the Zygophyllaceae. According to his view, Plectrocarpa is so close to Larrea that they can be considered early derivations of a Larrea ancestral type. Phylogenetic analysis of rbcL sequence data shows a sister taxon relationship between Plectrocarpa and Bulnesia (Fig. 1), a genus also considered to be closely related to Larrea (Hunziker et al., 1977). However, it is the Chilean endemic Pintoa chilensis that appears as the sister to Larrea. Pintoa is the only member of subfamily Larreaeae to have large chromosomes and $x = 10$ (Poggio, 1978), whereas the remaining genera show $x = 13$ and have generally smaller chromosomes (Hunziker et al., 1977; Poggio, 1978). Consideration of the constrained analyses and the bootstrap and decay values supporting intergeneric relationships within subfamily Larreaeae suggests that these results should be interpreted with caution until further resolution is achieved.

The monophyly of Larrea is strongly supported in the rbcL tree (Fig. 1). The phylogenies produced from rbcL, ITS, and combined data sets show section Bifolium ($L$. divaricata, $L$. tridentata, and $L$. cuneifolia) as polyphyletic, with the hypothesis of monophyly being significantly less parsimonious than the shortest tree in the rbcL and combined analyses. Delimitation of section Bifolium has been established on the basis of a diversity of vegetative and floral traits, such as number of leaflets, size of flowers, anther shape and length, hairiness of fruits, dehiscence of mericarps at maturity, sclerification of mericarps internal walls, and seed tegument thickness (Palacios and Hunziker, 1972; Hunziker et al., 1972, 1977). Reconstruction of charac-
ter evolution suggests that the similarities in number of leaflets and hairiness of fruits within members of section Bifolium should not be considered retained ancestral features, but shared derived conditions. Biochemical similarities, including seed protein and phe nolic compound profiles, have also been reported (Hunziker et al., 1972, 1977).

This seemingly contradictory scenario raises the issue of the “gene trees vs species trees” problem. When gene copies are sampled from various species, the gene tree relating these copies might disagree with the species phylogeny. It has been proposed that this discord can arise from horizontal transfer, lineage sorting, and gene duplication and extinction (Doyle, 1997; Maddison, 1997; Slowinsky and Page, 1999). The rbcL and ITS phylogenies may be correct in terms of the history of the molecular sequences, but these histories may or may not reflect that of the species. The incongruence between the infrageneric treatment of the genus and the phylogenetic trees derived from rbcL and ITS sequences can be interpreted as the result of the reticulate allopolyploid origin of L. cuneifolia. Therefore, presentation of the conflict in terms of discord between gene and species trees would not be completely adequate; i.e., rbcL and ITS histories may not be different from the species history, but rather may show just one side of it.

The inference of phylogenetic relationships within Larrea poses the question of how to interpret the bifurcating ancestral–descendant patterns typically obtained from cladistic analysis when species of allopolyploid origin such as L. cuneifolia are considered. In the present study, the evidence provided by nuclear and organellar markers was used in an attempt to obtain a more detailed picture of the origin of this tetraploid. Parsimony analysis of rbcL sequence data suggests that L. nitida (or its ancestor) would have been involved in the original hybridization event that gave rise to L. cuneifolia, acting as the chloroplast donor (Fig. 1). In agreement with this, Yang et al. (2000) have reported that the restriction site patterns obtained for three noncoding regions of cpDNA revealed the existence of two haplotypes among individuals of L. nitida, one of which appears to be identical (or extremely similar) to that of L. cuneifolia. However, the interspecific natural hybrid L. cuneifolia × L. nitida was completely sterile and showed cytomixis at meiosis with more than 20 univalents at Metaphase I. Some of the natural triploid hybrids L. cuneifolia × L. ameghinoi, although also presenting evidence of cytomixis, displayed partial pollen stainability and partial fertility (Hunziker et al., 1978). In this context, it could be proposed that one of the parental species of the tetraploid L. cuneifolia was an ancestor of section Larrea and not any of its extant members. In fact, both the phylogeny obtained from ITS sequence data and the tree derived from the combined analysis support a sister group relationship for L. nitida and L. ameghinoi, with L. cuneifolia as sister to this clade (Figs. 2 and 3).

Recent findings have shown that cpDNA inheritance in interspecific hybrids of Larrea is predominantly paternal, rather than maternal as in most other plants, and does not seem to be affected by parental genotypes (Yang et al., 2000). Hence, the chloroplast phylogeny presented here probably suggests the paternal lineage of L. cuneifolia.

As an alternative explanation of the relationships derived from the molecular analyses, the retrieved groupings could also be regarded a result of a recent hybridization event and cytoplasmic gene transfer between species. Horizontal gene transfer, which in-

### Table 3

<table>
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<th>similarity</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<td>1. Kallstroemia maxima</td>
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<tr>
<td>2. Auga capensis</td>
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<tr>
<td>4. Zypophyllum simplex</td>
<td>0.07295</td>
<td>0.04806</td>
<td>0.05045</td>
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<tr>
<td>5. Porlieria chilensis</td>
<td>0.05809</td>
<td>0.08433*</td>
<td>0.08330*</td>
<td>0.07821*</td>
<td>—</td>
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<tr>
<td>6. Pintoa chilensis</td>
<td>0.06219</td>
<td>0.03853*</td>
<td>0.07908*</td>
<td>0.07548*</td>
<td>0.03028</td>
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<tr>
<td>7. Guaiacum guatemalense</td>
<td>0.06129</td>
<td>0.08861*</td>
<td>0.08403*</td>
<td>0.07801*</td>
<td>0.02795</td>
<td>0.03584</td>
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<tr>
<td>8. Bulnesia arborea</td>
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<td>0.08686*</td>
<td>0.08327*</td>
<td>0.07546*</td>
<td>0.03029</td>
<td>0.03505</td>
<td>0.02949</td>
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<tr>
<td>9. Larrea ameghinoi</td>
<td>0.0569</td>
<td>0.03886*</td>
<td>0.08694*</td>
<td>0.08167*</td>
<td>0.03914</td>
<td>0.03191</td>
<td>0.03916</td>
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<tr>
<td>10. Larrea cuneifolia (4×)</td>
<td>0.06136</td>
<td>0.08517*</td>
<td>0.08159*</td>
<td>0.07638*</td>
<td>0.03186</td>
<td>0.02946</td>
<td>0.03505</td>
<td>0.02717</td>
<td>0.00997</td>
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<td>11. Larrea divaricata</td>
<td>0.05918</td>
<td>0.08637*</td>
<td>0.08446*</td>
<td>0.07924*</td>
<td>0.02885</td>
<td>0.02487</td>
<td>0.03359</td>
<td>0.02724</td>
<td>0.01700</td>
<td>0.01384</td>
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<tr>
<td>12. Larrea nitida</td>
<td>0.06328</td>
<td>0.08718*</td>
<td>0.08358*</td>
<td>0.07834*</td>
<td>0.03196</td>
<td>0.02955</td>
<td>0.03515</td>
<td>0.02806</td>
<td>0.00843</td>
<td>0.00382</td>
<td>0.01543</td>
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<tr>
<td>13. Larrea tridentata (2×)</td>
<td>0.06132</td>
<td>0.08765*</td>
<td>0.08663*</td>
<td>0.07971*</td>
<td>0.02947</td>
<td>0.02551</td>
<td>0.03502</td>
<td>0.03028</td>
<td>0.01773</td>
<td>0.01691</td>
<td>0.00770</td>
<td>0.01618</td>
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<tr>
<td>14. Plectrocarpa tetracantha</td>
<td>0.06978</td>
<td>0.08496*</td>
<td>0.08571*</td>
<td>0.07622*</td>
<td>0.03628</td>
<td>0.03906</td>
<td>0.04472</td>
<td>0.02713</td>
<td>0.03940</td>
<td>0.03272</td>
<td>0.03281</td>
<td>0.03361</td>
<td>0.0327</td>
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</table>

Note. Base frequencies and the transition/transversion ratio were estimated from the data (A: 0.28, C: 0.18, G: 0.24, T: 0.30; transition/transversion = 1.6). Values for taxa 1–8 were calculated from sequences given by Sheahan and Chase (1996, 2000).

*Distances included in the calculation of the averaged F84 distance used for estimating rbcL substitution rates (F84 = 0.08263433; SD = 0.00405723).
cludes phenomena such as the transfer of genes between bacteria and hybridization between different species of bisexual eukaryotes, obscures species phylogeny because sequences from one species may introgress into another species (Slowinsky and Page, 1999). If L. cuneifolia had recently captured the cpDNA of L. nitida, one would expect their rbcL sequences to be almost identical, showing no detectable sequence divergence. However, the observed nucleotide distance between L. cuneifolia and L. nitida (Table 3) is not concordant with this assumption. This nucleotide distance would be in agreement with the recent hybridization hypothesis only if we assumed that different and considerably divergent haplotypes coexist within the donor species and that the sequences sampled for each species correspond to two divergent variants.

ITS sequences exhibit different patterns of evolution in several allopolyploid species. In a Krigia polyploid both parental sequences have been maintained (Kim and Janzen, 1994), but in cotton the sequences of allotetraploids have homogenized to that of either parental diploid species by concerted evolution (Wendel et al., 1995). In our case, no additive patterns were observed in the variable positions of L. cuneifolia ITS sequences, suggesting that unidirectional homogenization of ribosomal genes has occurred. However, this assertion should be further confirmed by sequence analysis of recombinant clones.

L. divaricata, or a species closely related to it, has been proposed as one of the progenitors of L. cuneifolia on the basis of anatomical, morphological, and cytological evidence (Hunziker et al., 1977). Furthermore, genomic dot blot hybridization experiments have recently shown that L. cuneifolia has a nuclear genome largely homologous with that of L. divaricata, at least as far as repetitive sequences are concerned (L. Poggio et al., unpublished). This hypothesis may seem to be inconsistent with the results presented here. However, if L. cuneifolia had inherited chloroplast DNA and fixed ITS sequences from a parent from section Larrea and retained much of the ancestral genomic features of L. divaricata, all of these findings could be readily reconciled.

Finally, the molecular data presented here fully agree with previous findings from morphological, biochemical, and cytogenetic studies, which support a close relationship between L. divaricata and L. tridentata. In fact, parsimony analysis showed that these entities constitute a well-supported clade in all topologies.

Geographical Origin of Larrea

The molecular analyses presented in this study argue against Turner’s (1972) hypothesis concerning the geographical origin of Larrea. If South American species of Larrea had derived from ancestral diploid populations of L. tridentata as he suggested, then L. tridentata would be expected to be the sister to the remaining species. However, parsimony analyses of both chloroplast and nuclear regions indicate otherwise (Figs. 1 and 2).

Several lines of evidence have been presented to advocate a South American origin (Hunziker et al., 1977). All taxa of subfamily Larreaeae (New World Clade) have today strictly South American distributions, except for four species of Guaiacum (North America and West Indies). Strictly South American are Bulnesia (nine spp.), Porlieria (three spp.), Plectocarpus (two ssp.), Metharme (one sp.), and Pintoa (one sp.) This subfamily is therefore supposed to have a South American origin with subsequent occasional dispersal to the West Indies and North America. Following from this rationale, Larrea is also assumed to have a South American origin.

In addition, it has been proposed that reduction of leaflet number would represent an adaptation to aridity and, therefore, a derived condition within the genus (Hunziker et al., 1977). Optimization of the number of leaflets on the rbcL phylogeny also suggests that the multifoliolate condition constitutes the plesiomorphic state in Larrea. Because L. ameghinoi and L. nitida, the multifoliolate and more mesic species of the genus, are presently distributed in Argentina and Chile, a South American origin is again favored. However, this argument does not necessarily mean that the center of origin of Larrea coincides with the present-day range of the multifoliolate species (Hunziker et al., 1977).

In this study, both rbcL and ITS cladograms showed that L. tridentata and L. divaricata form a well-supported monophyletic group. This result is concordant with the relationships proposed by Hunziker et al. (1972, 1977, 1978). According to this hypothesis, present-day diploid populations of both L. divaricata and L. tridentata probably descended from a common South American ancestor and started to diverge allopatrically when migrants reached North America. The dispersal probably occurred through long-distance migratory birds (Wells and Hunziker, 1977).

Time of Arrival of Larrea to North America

A growing number of studies have combined phylogenetic analysis with genetic divergence or fossil data to piece together the biogeographic histories of entire genera or sections of genera (Qu et al., 1995; Sang et al., 1997; Schnabel and Wendel, 1998).

Ultrametricity of the rbcL sequence data included in this study was confirmed by Felsenstein’s (1988, 1993) global tree test. The sequence divergence value of 0.77% between L. divaricata and L. tridentata for rbcL and the range of rbcL substitution rates calculated here indicate that the two species diverged between 4.2 and 8.4 mybp. This divergence time is higher than the estimate of 1.2–0.6 My obtained from allozyme data (Cortés and Hunziker, 1997) and indicates that the
In the present study, the time of divergence between present-day *L. tridentata* and *L. divaricata* is equated to the dispersal of *Larrea* to North America. Alternatively, *L. tridentata* could have evolved in South America and migrated to North America more recently, becoming extinct in South America during the same time. This hypothesis would not only imply the postulation of an extinction event, but would also have to deal with the fact that interspecific hybrids between *L. tridentata* and *L. divaricata* show regular meiosis and are partially fertile. If both populations started to diverge between 4.2 and 8.6 mybp, then a cause should be found to account for the interruption of gene flow. Following William of Occam’s razor principle, we deem that allopatric speciation by long-distance dispersal is the simplest explanation for the present disjunction.

Two different lines of evidence support the estimations of the probable time of arrival of *Larrea* to North America based on rbcL sequence data. First, long-distance dispersal of *Larrea* across the tropics may have occurred through an intermediate series of appropriate “way stations” (Peru, Bolivia) (Raven, 1963), a situation which might have arisen as a result of the expansion of the world’s arid climates in the late tertiary and early Quaternary (10–1.8 mybp) (Axelrod, 1970; Porter, 1974). A detailed discussion on the identity of the putative carriers has been presented by Wells and Hunziker (1977). Second, 30 species in five orders of insects have been found to be associated with *L. tridentata*, many of them being monophagous (Schultz et al., 1977). The diversity and specialization of these herbivores is more likely to have developed during a relatively large time of coevolution, certainly larger than that suggested by the oldest fossil record of *L. tridentata* in North America (18700 ± 1050 years B.P.). Moreover, the uniqueness of *L. tridentata* in North American deserts would argue against biochemical preadaptation of the monophagous species feeding on it.

At present, the arid rain shadow pocket of the Ex-torax–Tula drainage in eastern Querétaro (southern Mexico) harbors an isolated area of landscape dominated by *Larrea*, where the observed cacti endemisms strongly suggest a considerable antiquity for the desert plants confined there (Wells and Hunziker, 1977). Ancestral diploid populations probably established and remained restricted to the proximities of this region, which could have also served as a refugium throughout the Pleistocene glacial advances due to its relatively mild, frost-free climate during this period. Once established in North America, *L. tridentata* underwent a remarkable cytological differentiation within its wide geographic range. This species exists as distinct diploid, tetraploid, and hexaploid cytotypes in the Chihuahuan, Sonoran, and Mohave deserts, respectively (Barbour, 1969; Yang, 1970; Hunziker et al., 1977). Electrophoretic analysis of proteins and chemical analyses of phenolic cuticle resins revealed a uniform chemistry throughout these cytologically differentiated populations, indicating interracial autoploid, and probably recent, origin for the cytogeographic races (Hunziker et al., 1972, 1977; Mabry et al., 1977).

Most of the present areas of the warm deserts in North America were occupied by pluvial woodlands until the recessional phases of the Wisconsin glacial, when a desiccating climate opened a vast and varied desert niche into which *Larrea* populations could have expanded and differentiated explosively (Wells and Hunziker, 1977).

In the light of the evidence presented here, we conclude that, although the present distribution pattern of *Larrea* and its wide dominance in the Sonoran and Mohave deserts would have been achieved in the early Holocene, the amphitropical disjunction of the genus would probably be late Neogene in origin.

**ACKNOWLEDGMENTS**

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Molecular Phylogeny of Larrea and Its Allies


