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Study of the Evolutionary Relationships among *Limonium* Species (Plumbaginaceae) Using Nuclear and Cytoplasmic Molecular Markers

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The genus *Limonium*, due to the patchiness of the natural habitats of its species as well as the high frequency of hybridization and polyploidy and the possibility of reproduction by apomixis, provides an example of all the principal mechanisms of rapid speciation of plants. As an initial study of evolution in this genus, we have analyzed intra- and interspecific variability in 17 species from section *Limonium*, the largest in the genus, based on RFLPs of cpDNA and nuclear rDNA ITS sequences. In the cpDNA analysis, 21 restriction enzymes were used, resulting in 779 fragments, 490 of which were variable and 339 parsimony informative. *L. furfuraceum* exhibited two relatively divergent cpDNA haplotypes. The relationships found among the species based on cpDNA restriction fragments were coincident using different methods of phylogenetic analysis. Due to the presumed reticulate evolution in the genus *Limonium*, the comparison of these results with data from the nuclear DNA was necessary; ITS sequences were analyzed. The final alignment contained 488 characters, of which 198 were variable and 156 parsimony informative. Two relatively divergent ITS types were present at the intraindividual level in *L. delicatulum*, a triploid species. Each type was related to ITS from different groups of diploid *Limonium* species, one with a base haploid chromosome number $n = 8$ (represented by *L. cossonianum*) and the other with $n = 9$ (represented by *L. minutum*). The different phylogenetic inference methods used for the analysis of ITS sequences rendered very similar topologies. In general, the relationships among the species studied were coincident with those obtained with the chloroplast genome. Both nuclear and cytoplasmic markers support the polyphyly of section *Limonium*, with at least two species, *L. narbonense* and *L. vulgare*, clearly divergent from the rest. Moreover, the remaining subsections into which section *Limonium* is currently divided seem to be artificial.

INTRODUCTION

Taxonomic complexity has frequently been related to the mating system of plants. Hence, taxa having breeding systems favoring selfing or asexual reproduction (apomixis and clonality) are usually prone to taxonomical controversy (Richards, 1986). Apomictic plants circumventing sexuality, obligately or facultatively, defy classical species concepts and make the delimitation of taxa a difficult task (Richards *et al.*, 1996).

Limonium is the most species rich and widespread genus of Plumbaginaceae, although the number of species reported in the genus is rather speculative. A very high percentage of *Limonium* diversity is centered in the Mediterranean basin with nearly 300 taxa currently used in regional floras and checklists (Erben, 1993; Greuter *et al.*, 1989). A significant portion of these taxa belong to section *Limonium*, one of the 12 sections in which the genus has been traditionally split (Boissier, 1848). In turn, section *Limonium* has been divided into six subsections (Boissier, 1848; see also Table 1).

Several of these sections were later grouped into subgeneric ranks. Thus, Pax (1897) included sections *Polyarthrion*, *Myriolepis*, *Siphonantha*, and *Psillyostachys* within subgenus *Siphonantha*. On the other hand, Pignatti (1971) raised section *Pteroclados* to the subgeneric level and excluded section *Myriolepis* from subgenus *Siphonantha* to create a new subgenus (subgenus *Myriolepis*). Other analytical treatments have dealt with some of the sections recognized by Boissier and Pax (section *Circinaria*, section *Schyzimenium*, section *Psillyostachis*, section *Schyzopethalum*, section *Pterolimon*) as separate genera (Linczveski, 1968).

Both sexual (diploid and tetraploid) and apomictic (spanning triploid to hexaploid cytotypes) species have been reported in section *Limonium*. Nevertheless, diploid species are few, and polyploid agamic species constitute the largest portion of the diversity currently known in this section. Several competing hypotheses, based mainly on karyological data, regarding the origin of polyploid *Limonium* species have been postulated (Dolcher and Pignatti, 1971; Erben, 1978, 1979). Dol-

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cher and Pignatti (1971) suggested that triploid taxa arose through hybridization between diploid and tetraploid species, the latter having originated from diploid ancestors. In contrast, Erben (1978, 1979) noted that within section *Limonium*, diploid species show two basic chromosome numbers ($n = 8$ and $n = 9$) and postulated that the complement of the polyploids arose through several combinations, involving reduced and unreduced gametes, of the $n = 8$ and $n = 9$ genomes. Hence, triploid ($2n = 24$, 25, 26, and 27) and tetraploid ($2n = 34$, 35, and 36) taxa combine genomes of the two basic cytotypes. Although conflicting, both hypotheses agree that interspecific hybridization has played a substantial role in the evolution of section *Limonium*. However, this has not been tested by means of a rigorous phylogenetic analysis. Unfortunately, the very similar morphology exhibited by most members of section *Limonium* (with most characters showing continuous variation) has prevented the use of morphological characters in a phylogenetic (cladistic) context. Theoretically, molecular analyses could circumvent this drawback and offer robust hypotheses on the evolution of these species.

Molecular approaches have been applied to some genera with a large apomictic element in order to detect species (microspecies) boundaries and to trace the origins of apomictics (Campbell *et al.*, 1997). Molecular data have been rarely applied in Plumbaginaceae (Chase *et al.*, 1993; Williams *et al.*, 1994; Fuertes *et al.*, 1999). Recently, Lledó *et al.* (1998) used *rbcL* sequence data from species of *Limonium, Limoniastrum, Acantholinum, Dictyolimon, Psilliostachys, Armeria, Ceratostigma,* and *Plumbago* to evaluate the monophyly and phylogenetic relationships of Plumbaginaceae. Because the goals of that study were to elucidate relationships at higher taxonomic levels within this family, few conclusions regarding *Limonium* other than its monophyly were attained.

Sequences of the nuclear rDNA internal transcribed spacers (ITS region) have been widely applied to depict evolutionary relationships at lower taxonomic levels, notably at the intrageneric ones (Baldwin *et al.*, 1995). In addition, the ITS region has been a valuable tool for tracing the hybrid origin of diploid (Sang *et al.*, 1995) and polyploid (van Houten *et al.*, 1993; Kim and Jansen, 1994; Wendel *et al.*, 1995; Roelofs *et al.*, 1997) species in flowering plants. Presumed uniparental (maternal) inheritance of the chloroplast genome in *Limonium* (Harris and Ingram, 1991) and the absence of intramolecular recombination prevent the appearance of reticulation in cpDNA phylogenies, as opposed to those based on morphology or nuclear DNA. Therefore, when nuclear phylogenies conflict with molecular markers obtained from the chloroplast genome, hybridization or introgression can be suspected (Doyle, 1992; Rieseberg and Brunsfeld, 1992; Soltis *et al.*, 1992).

In this work, nuclear (ITS sequences) and organellar (RFLP of cpDNA) markers have been obtained from 21 *Limonium* species, mostly from section *Limonium*, inhabiting the western Mediterranean basin. Most of them are endemic to this area, and some are also endangered due to the fragility of the ecosystems that these species inhabit. Species representing the main karyological and reproductive systems present in this section have been included in this work. The main goals of this study were to check the monophyly of section *Limonium*, to test the current division of this section into subsections, and to assess its relationships with other sections of the genus *Limonium*. Also, we have explored whether molecular markers can shed some light on the origins of polyploidy in apomictic species from this section.

MATERIALS AND METHODS

Plant Samples and DNA Isolation

Twenty-six *Limonium* populations corresponding to 17 species from section *Limonium* and 4 species from other sections of the genus were analyzed for cpDNA variation and/or ITS sequence variation (Table 1). This table also shows the number of individuals sampled per population, voucher specimen numbers, locality of origin of the populations, and chromosome numbers and mode of reproduction of each species. The choice of species was dictated mainly by the availability of material. At least 1 species from each of the currently recognized subsections of the section *Limonium* was included, with the only exception being subsection *Sarcophyllae,* for which no material was available. All basic chromosome numbers were included in the sampling by considering diploid ($n = 6$, 8, and 9), triploid ($2n = 25$, 26, and 27), and tetraploid ($2n = 36$) species, with sexual and apomictic taxa among them.

Plant materials were collected as 1–3 g of fresh leaf tissue from the greenhouse or from the field and stored at −80°C until processed. Fresh leaves were not available for *L. lobatum,* and therefore dried leaves from herbarium specimens were used for DNA extraction. When available, leaves from two or more individuals from each population were pooled (Table 1). This approach has been suggested as a strategy to detect intraspecific or intraindividual variation both in cpDNA RFLPs and ITS sequence analyses (Soltis *et al.*, 1989; Baldwin *et al.*, 1995). Total DNA was isolated using the CTAB method (Doyle and Doyle, 1991). One further chloroform–isoamylalcohol (24:1) extraction was done if samples were still turbid after the first organic extraction.

RFLP Analysis of the Chloroplast Genome

Variation among cpDNAs of *Limonium* was detected by DNA digestion with 21 restriction endonuclease enzymes according to suppliers' instructions. We employed 3 four-cutter enzymes (*Cfo*I, *Hae*III, and *Msp*I)

TABLE 1

***Limonium* Species Used for the Study of cpDNA RFLP Variation and ITS Sequencing**

Species	Populations	Voucher	EMBL Accession no.	Individuals	Chromosome no. (2*n*)	Reproduction mode
Sect. *Limonium*						
Subsections						
Limonium						
L. narbonense Mill.	Almardà (Valencia)*	JAR-96132	AJ222838	2	36	Sexual
L. vulgare Mill.	Cantabria	JAR-96085	AJ222839	1	36	Sexual
Densiflorae Boiss.						
L. dufourii (Girard.) Kuntze	Cullera (Valencia)	JAR-96051	AJ222840	4	27	Apomixis
L. camposanum Erben	Cala Pi (Mallorca)*	JAR-95111	AJ222841	1	27	Apomixis
L. gymnesicum Erben	Sant Pere (Mallorca)*	JAR-94328	AJ222842	1	27	Apomixis
L. interjectum Soler & Rosselló	Cala Blanca (Javea, Alicante)^	JAR-96127	AJ222843	1	Unknown	Apomixis
	El Llano (Javea, Alicante)^	JAR-96124	AJ222844	1		
L. girardianum (Guss.) Fourr.	El Saler (Valencia) ^	JAR-96027	AJ222845	1	26	Apomixis
Dissitiflorae Boiss						
L. delicatulum (Girard) Kuntze	Cala Blanca (Javea, Alicante)*	JAR-96018	AJ222846-51	8	25	Apomixis
L. cavanillesii Erben	Torre Badún (Castellón)	JAR-96217	AJ222852	6	27	Apomixis
L. anguste-bracteatum Erben	Pobla Farnals (Valencia)*	JAR-96127	AJ222853	1	26	Apomixis
L. rigualii MB Crespo & Erben	Cala Blanca (Javea, Alicante)	JAR-96126	AJ222854	2	27	Apomixis
	El Llano (Javea, Alicante)	JAR-96125		5		
L. cossonianum Kuntze	Formentera (Baleares)	JAR-97005	AJ132331	1	16	Sexual
Steirocladae Boiss.						
L. virgatum (Willd.) Fourr.	Cabo Salines (Mallorca) *	JAR-95025	AJ222855	1	27	Apomixis
	Cala Blanca (Javea, Alicante)^	JAR-96141		1		
	El Saler (Valencia)^	JAR-96028		1		
L. furfuraceum (Lag.) Kuntze	Cabo Huertas (Alicante)	JAR-96219	AJ222856	10	18	Sexual
	Sta. Pola (Alicante)	JAR-96218		10		
L. tenuicaule Erben	Artà (Mallorca)*	JAR-95112	AJ222857	1	18	Sexual
L. minutum (L.) Chaz.	Formentera (Baleares)	JAR-97006	AJ132332	1	18	Sexual
Hyalolepidae Boiss.						
L. dichotomum (Cav.) Kuntze	Aranjuez (Madrid)	JAR-96501	AJ222858	1	18	Sexual
Sect. Polyarthrion Boiss.						
L. caesium (Girard) Kuntze	Villena (Valencia)*	JAR-94029	AJ222859	1	18	Sexual
Sect. Pteroclados						
L. lobatum (Lbe.) Chaz.	Terreros (Almería)	VAB-96/4628	AJ132333	1	12	Sexual
L. sinuatum (L) Mill.	C. Gata (Almería)^	JAR-96850	AJ222860	1	16	Sexual
Sect. Schizhymenium Boiss.						
L. echioides (L) Mill.	Cala Blanca (Javea, Alicante) ^	JAR-96129	AJ222861	1	18	Sexual

* Samples from University of Valencia greenhouse facility.

^ Samples used on ITS sequencing study exclusively. The rest have been used on both studies except for the Sta. Pola population sample of *L. furfuraceum*, which was used only in the cpDNA analysis (see text for further details).

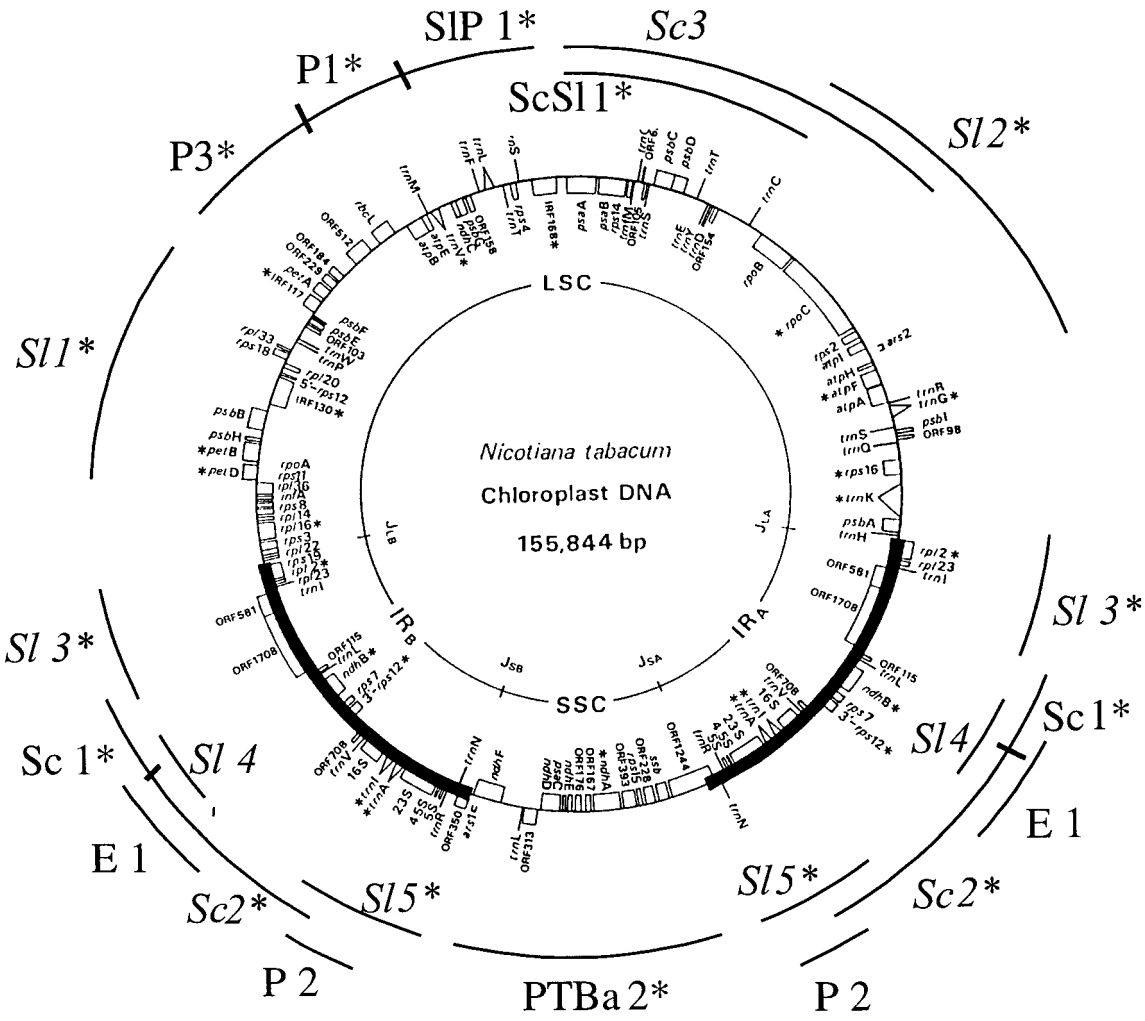

FIG. 1. cpDNA clones from *Limonium narbonense* superimposed on the chloroplast genetic map of tobacco. A genomic library from *L. narbonense* was obtained from an enriched cpDNA extraction (Ko *et al.*, 1984) followed by digestion and cloning of restriction fragments following standard protocols (Maniatis *et al.*, 1982). cpDNA fragments of *L. narbonense* were detected and mapped using cpDNA clones from *Nicotiana tabacum*, kindly provided by M. Sugiura (Sugiura *et al.*, 1986), as probes. *L. narbonense* cpDNA clones are denoted by the restriction enzyme with which they were obtained (Sl, *Sal*I; Sc, *Sac*I; E, *Eco*RV; P, *Pst*I), followed by an arbitrary number. Clones shown in italics are in EMBL20 phage vector; the rest are clones or subclones in pUCBM20 plasmid vector. An asterisk indicates clones used in the study of section *Limonium*.

and 18 six-cutters (*Asp*700, *Ava*I, *Bam*HI, *Bcl*I, *Bfr*I, *Bgl*II, *Cla*I, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Nco*I, *Pst*I, *Sac*I, *Sal*I, *Sca*I, *Xba*I, and *Xho*I). Restriction fragments were separated by electrophoresis on 0.7–1% agarose gels at approximately 2.5 V/cm for 12 h with TBE 0.5× buffer. Nonradioactive hybridization methods were used to detect cpDNA fragments. Transfer of DNA to nylon filters (Hybond-N, Amersham) by Southern blotting, filter prehybridization, hybridization, and detection were performed following manufacturer's instructions (Boehringer Mannheim, B.M.) with slight modifications. Probes were labeled with digoxigenin-11-dUTP using the random priming method. Hybridizations were performed at 65°C overnight, and the filters were washed at 60°C twice for 15 min in a prewarmed 0.1% SDS, 0.5× SSC solution. Probe removal prior to reutilization was performed by washing the membranes in distilled water for 1 min, followed by incubating twice for 15 min at 60°C in 0.4 N NaOH, 0.1% SDS prewarmed solution, and finally rinsing them thoroughly in 2× SSC. Detection of hybridized probes in the first hybridizations was performed by immunochemioluminescence with CSPD substrate (B.M.) followed by autoradiography. Better results were obtained by employing colorimetric reagents (BCIP and NBT; B.M.) to detect the labels in a subsequent rehybridization.

A total of 10 cpDNA restriction fragments obtained from a genomic library constructed from *L. narbonense* (Fig. 1) and a clone from the cpDNA of *Nicotiana tabacum* were used as probes to detect homologous fragments among the different species. Clones were labeled separately and combined in four batches for

filter hybridization, comprising 127 kb in total, which should represent a large fraction of the chloroplast genome (Palmer, 1991).

Fragment observation and reconstruction of the presence–absence character state data matrix were made directly on the autoradiograms from the different hybridizations (Fragment Occurrence Analysis, FOA; Bremer, 1991a). Care was taken not to score a band more than once by overlapping autoradiograms probed with adjacent probe/enzyme combinations. Any band shared in these autoradiograms was scored only once.

Data analysis. Weighted parsimony (Sankoff, 1975) or "generalized parsimony" was used to analyze the presence–absence character state data using the step-matrix option from PAUP 3.1 (Swofford, 1993). We followed the recommendations in Albert *et al.* (1992) but with the following modification. These authors proposed a character state weighting of gains over losses by a factor of 1.3 for analyses at low taxonomic levels, such as species within a genus. This implies a probability of losing a site of 0.565 over 0.435 of gaining it. Because we have screened fragments instead of restriction sites, with the resulting 3:1 ratio character difference among these two types of markers (Bremer and Jansen, 1991b), a fragment gain could result from a gain of a site with probability of ⅔ but also from losing a site with probability of ⅓, with these probabilities being the opposite for losing a fragment. Combining these two considerations, the final cost of fragment gains over losses would be 21:19 (i.e., $1/[(0.565 + 2 \cdot 0.435)/3]:1/[(0.435 + 2 \cdot 0.565)/3]$. Moreover, Albert *et al.* (1992) suggested trying other weighting factors in the range 1.0 to 2.5 for a comparative check. Consequently, we also tried the corresponding weights of these extreme values obtained by applying the previous argument as alternative step-matrices for the FOA approach. In these matrices, gains were weighted as equal to losses for the first extreme value, which is equivalent to Wagner parsimony, and the step-matrix 23:17 was used for the 2.5 factor. A heuristic search ignoring invariant characters and using ACCTRAN optimization was employed for the step-matrix analyses. To avoid problems associated with tree islands (Maddison, 1991), the strategy suggested by Doyle and Doyle (1993) was followed. It consists of conducting searches using 100 random addition sequence option, followed by TBR branch swapping, retaining only a single tree from each run and only the most parsimonious trees (MPT) from all runs. Asymmetric weighting required the definition of ancestral character states, and we chose the option all missing-data for it and forced ingroup to monophyly ["enhanced Wagner" approach (Albert *et al.*, 1992)].

Bootstrap analysis (Felsenstein, 1985) was used to assess the reliability of the phylogenetic reconstructions. One-hundred bootstrap replicates were performed for the three parsimony approaches with heuristic search options as above, except for weighted parsimony in which the stepwise addition was closest instead of random. In this last case, near-MPTs up to 10 steps longer were also examined as alternative phylogenetic hypotheses. Phylogenetic information content of the entire data set was also estimated by the skewness coefficient (g_1; Hillis, 1991) using the random trees option (set to 1000 trees) of PAUP (Swofford, 1993).

Pairwise distances among populations were calculated from the character state data matrix using the nucleotide divergence estimate for restriction fragment data with the iterative method of Nei (1987). Divergence estimates from the 3 four-cutters and the 18 six-cutters were averaged according to Nei and Miller (1990). An unrooted neighbor-joining (NJ) dendrogram (Saitou and Nei, 1987) was obtained from this matrix using the program NEIGHBOR from the PHYLIP package (Felsenstein, 1993).

Sequence Analysis of the ITS Region

Amplification and sequencing strategies. The ITS region comprises ITS-1, ITS-2, and the 5.8S subunit of the nuclear rDNA cistron (Baldwin *et al.*, 1995) and was amplified by PCR using universal eukaryote primers designed by White *et al.* (1990). PCRs were performed in a 25-μL total volume containing 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 μM each primer, approximately 3 ng of template DNA, 2.5 μL of 10× *Taq* buffer, and 1 unit of *Taq* DNA polymerase. The profile for amplification reactions consisted of an initial step at 94°C for 2 min, followed by 30 cycles at 94°C for 1 min, 55°C for 30 s, and 72°C for 45 s; the last cycle was followed by a 9-min extension phase at 72°C; then, samples were held at 4–6°C.

Manual sequencing was performed for all species analyzed in the previous section at least once. PCR products were purified from primers and dNTPs using Ultrafree (Millipore) filters. Cycle-sequencing reactions of these purified products (200 ng, approx) were conducted using the AmpliCycle Sequencing kit (Perkin–Elmer), following the manufacturer's instructions for the [γ^{33}P]ATP end-labeling reaction procedure.

Automated sequencing was performed at least twice for each population using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Inc.) following manufacturer's instructions with some modifications. Purification of PCR products (100 ng, approx) from dNTPs and primers was performed by incubation at 37°C for 15 min with exonuclease and shrimp alkaline phosphatase, followed by 15 min at 80°C. Centri-Sep (Princeton Separations, Inc.) spin columns were used to purify extension products from excess dye terminators after cycle sequencing reactions. Electrophoresis was performed on 4% polyacrylamide at constant voltage (2500 V) on ABI 377 or 373 automated DNA sequencers. No differences

were observed between automated and manually obtained sequences in those species analyzed with both techniques.

Some species showed sequence uncertainties in certain nucleotide positions, which could be due either to insufficient sequence resolution in those positions or to real polymorphisms. They were scored as ambiguities following the IUB code. In one case (*L. delicatulum*), the amount of polymorphism prevented any meaningful interpretation of the nucleotide sequence, and it was necessary to sequence cloned PCR products from single individuals. In this case, ITS PCR products were cloned using the pGEM-T Easy vector system (Promega Corp.), and at least two clones from each individual were sequenced (Bloch, 1991). Recombinant plasmid DNAs were isolated following the modified mini alkaline-lysis/PEG precipitation procedure recommended by ABI (User bulletin No. 18). Cloned fragments were sequenced automatically using T7 and SP6 universal primers.

Sequence alignment. Direct and reverse sequences belonging to the same population or clone were assembled using the program Sequencher (Gene Codes Corp., v. 3.0), and a consensus sequence was obtained. The resulting consensus sequences were aligned using the program PILEUP from the GCG software package (Edelman *et al.,* 1995). Further adjustments of the alignment were done manually to increase similarity, using sequence editors LINEUP (GCG package) and GENEDOC (Nicholas and Nicholas, 1997). A consensus ITS region sequence from the ingroup species was extracted and checked against the Ribosomal Database Project, and it showed the highest similarity with *Arabidopsis thaliana.* The alignment of these two sequences was useful to determine the boundaries of the coding and spacer regions for *Limonium* ITS sequences.

Determination of secondary structure. Secondary structures of ITS-1 and ITS-2 were explored using the minimum free-energy algorithm (Zuker, 1989) with the program MFOLD in the GCG package. The ITS-1 *Limonium* consensus sequence was folded at 37°C. Structures within 2.9 kcal/mol of the optimal structure were recovered using the Squiggles option in PLOTFOLD (GCG). The general model of angiosperm ITS-2 secondary structure proposed by Hershkovitz and Zimmer (1996) was employed to infer a consensus secondary structure model for the ITS-2 region of *Limonium*.

Compensatory mutations may be necessary to maintain the seemingly functional ITS secondary structures (Aimi *et al.,* 1992; van der Sande *et al.,* 1992; van Nues *et al.,* 1994). Potential nonindependence of characters due to these compensatory mutations should be considered in phylogenetic analysis. Positional downweighting of the nonindependent positions provides a method to correct for this but the extent of such correlation must be determined empirically (Baldwin *et al.,* 1995). We have performed the χ^2-test proposed by Dixon and Hillis (1993) for character state weighting.

Data analysis. Several phylogenetic and statistical methods were followed to analyze the ITS sequence data. First, Felsenstein's (1981) maximum likelihood model was employed to reconstruct the phylogeny of section *Limonium* using the program FASTDNAML (Olsen *et al.,* 1994). The empirical transition/transversion (ts/tv) ratio calculated as an average over all sequences gave a value of 1.5. Nevertheless, the best tree was searched for using a range of ts/tv ratios from 0.5 to 4.0 as input for the program and using global branch swapping (N1 = 21) and random addition of taxa.

Based on the selection guidelines set forth by Nei (1991), we chose the Jukes and Cantor (JC) one-parameter method (Jukes and Cantor, 1969) to calculate pairwise nucleotide divergence values for all sequences using the program DNADIST from the PHYLIP package (Felsenstein, 1993), as divergence values were in general lower than 0.05 substitutions/site for all ingroup species (Nei, 1991; Kumar *et al.,* 1993). To minimize information loss, gaps and missing data were deleted only on a pairwise basis. A neighbor-joining dendrogram was constructed from the JC distance matrix. Bootstrap values for the different nodes were calculated after 1000 replicates.

Finally, PAUP 3.1 (Swofford, 1993) was employed to conduct parsimony analyses with and without considering compensatory mutations. Ambiguities were considered as polymorphisms or partial uncertainties but the topologies obtained were exactly the same using both alternatives, with the trees differing only in their length, as expected (Swofford, 1993). We report only the results obtained when they were considered as partial uncertainties. The heuristic search option was employed following the same strategy as in the previous section. A 50% majority rule consensus tree was constructed from all most-parsimonious trees. All trees were unrooted and rooted later using *L. sinuatum* as outgroup based on the results from ITS pairwise divergence values.

The previous phylogenetic reconstruction methods did not consider insertion–deletion (indel) mutations. Because some length variation was present in the ITS alignment, the effect of indels on evolutionary change of DNA sequences was also investigated. Gaps were scored as additional presence–absence characters (Brunsfeld *et al.,* 1992; Swofford, 1993; Baldwin *et al.,* 1995) and, by adding this additional set of characters from indel data to the sequence data matrix, parsimony analysis was performed as above.

Neither bootstrap analysis nor the near-MPT method (Doyle and Doyle, 1993) were accomplished in parsimony analyses as computer memory limits were reached before completion. In order to evaluate the nonrandom structure of the data sets, the skewness coefficient, g1,

of Hillis (1991) was used as previously described. One-thousand random trees were generated to establish the significance of the g1 coefficient.

RESULTS

Chloroplast DNA Variation

Intraspecific cpDNA polymorphism was observed only in pooled DNA from the Cabo de las Huertas population of *L. furfuraceum*, a sexual species ($2n = 18$). This variation was diagnosed by two distinct RFLP patterns, of different intensity, with several probe–enzyme combinations. This polymorphism was analyzed in more detail to check whether it existed at the intra- or the interindividual level and whether it was present only in this population or also in other populations of this species. Hence, DNA from 10 individuals of the Cabo de las Huertas population and from a sample of 10 individuals from the Santa Pola population, situated 20 km apart from the previous one, were isolated separately and characterized for those probe–enzyme combinations that showed polymorphism in the Cabo de las Huertas population. Six individuals from the Cabo de las Huertas population presented *L. furfuraceum* cpDNA haplotype A, and the rest had haplotype B, while individuals from the Santa Pola population showed pattern A for all probe–enzyme combinations, except one, which rendered a slightly different pattern and was excluded from the analyses. Consequently, a total of 15 different cpDNA haplotypes, obtained from the 14 *Limonium* species investigated, were subjected to further analyses.

A total of 779 different restriction fragments were scored, of which 490 were variable and 339 were parsimony informative. The numbers of bands shared by each pair of haplotypes are represented in Table 2. The cpDNA data were strongly left-skewed, with g1 of approximately -2.0 ($P \ll 0.001$), indicating a high phylogenetic information content of the entire data set (but see Hillis and Huelsenbeck, 1992).

Wagner parsimony analysis identified three most-parsimonious trees with length of 654 steps, consistency index (CI) of 0.765 (with autapomorphies), and retention index (RI) of 0.796. The two other different character state transformational weight methods rendered identical MPT topologies, which were also coincident with the 50% majority-rule consensus Wagner tree, except that *L. caesium* and the *L. narbonense–L. vulgare* clade formed a polytomy in the majority-rule tree. In addition, the topologies of near-MPTs included the three alternative topologies from the Wagner analysis. Figure 2 shows the MPT obtained using the step-matrix 21:19. The basal nodes of these trees are highly resolved. *L. caesium*, chosen as the outgroup species because it belongs to another section of the genus, is in fact situated basal to most species from section *Limonium*. However, *L. narbonense* and *L. vulgare*, from subsection *Genuinae* in section *Limonium*, form a basal monophyletic group, sister to the rest of the species of the section and to *L. caesium*. The mean number of bands shared by these two species with the rest is 259.08 (Table 2), around 100 fewer bands than those shared between pairs of the other ingroup species. Also, the average number of bands shared by *L. caesium* with these species is higher (269.64) than with *L. narbonense* and *L. vulgare*. *L. angustebracteatum* is sister to the clade formed by the remaining ingroup species, which form two relatively well-resolved monophyletic groups. *L. rigualii*, *L. furfuraceum* haplotype A, and *L. cavanillesii* form the first one, and the second comprises the remaining species. Among them, only the

TABLE 2

Number of Bands Shared between Each Pair of *Limonium* Species Generated by Restriction Analysis of cpDNA

	Lnar	*Lvul*	*Lduf*	*Lcam*	*Lgym*	*Ldel*	*Lang*	*Lrig*	*Lvir*	*Lfur B*	*LfurA*	*Lten*	*Ldic*	*Lcae*	*Lcav*
L. narbonense	115/368	114	78	79	79	78	83	81	77	78	80	78	80	88	83
L. vulgare	358	118/367	79	80	80	79	84	82	78	79	81	79	81	80	84
L. dufourii	256	258	119/365	116	116	116	107	109	113	117	105	118	115	91	106
L. camposanum	258	260	358	120/366	119	117	107	108	113	116	105	117	114	90	105
L. gymnesicum	259	261	358	365	120/366	117	107	108	112	116	105	117	114	90	105
L. delicatulum	257	259	353	359	360	120/365	108	109	112	117	105	118	115	89	106
L. angustebract.	259	261	328	330	331	332	118/357	109	103	107	105	108	106	86	107
L. rigualii	258	260	340	344	345	343	337	121/365	105	108	117	109	107	88	118
L. virgatum	258	260	345	352	353	350	328	338	116/362	113	103	114	111	90	102
L. furfuraceumB	260	262	352	359	360	356	330	341	350	120/372	104	119	116	90	105
L. furfuraceumA	257	259	332	336	337	335	331	354	332	349	118/365	105	103	87	114
L. tenuicaule	257	259	354	360	361	358	333	344	354	358	338	120/366	117	91	106
L. dichotomum	256	258	345	351	352	350	330	340	347	351	335	355	120/365	94	108
L. caesisum	264	266	271	271	272	273	272	270	267	271	267	271	267	124/365	91
L. cavanillesii	261	263	327	331	332	331	336	350	328	331	347	334	330	274	121/361

Note. Bands generated with four-cutters are shown in the upper hemimatrix, and those generated by six-cutters in the lower hemimatrix. The main diagonal shows the number of bands (four-cutters/six-cutters) for each species.

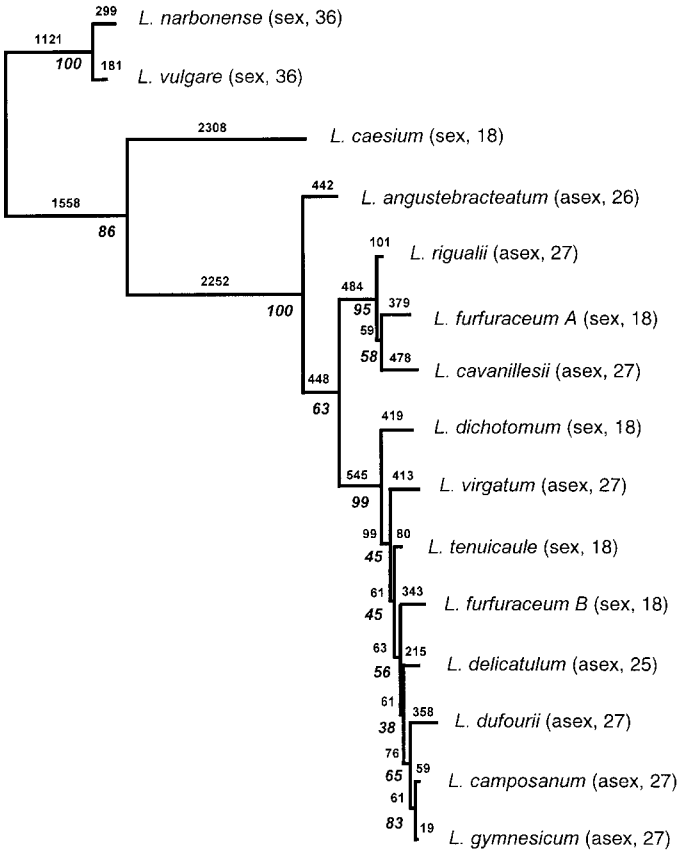

FIG. 2. Maximum parsimony tree derived from "enhanced Wagner" parsimony analysis using the step-matrix 21:19. Numbers along the branches represent mutational steps and those below are bootstrap values for each clade. This topology is identical to that of the NJ tree. Reproduction system (sex, sexual; asex, asexual, apomictic) and number of chromosomes are indicated between parentheses next to each species.

nodes corresponding to *L. dichotomum* and *L. gymnesicum–L. camposanum* have high bootstrap support.

The NJ tree derived from the pairwise genetic distance matrix of restriction fragment data (Table 3) renders a topology identical to that of the weighted parsimony analysis (Fig. 2). Divergence values ranged from 0.0003 to 0.0229. The extreme values of this range are due, on the lower side, to *L. camposanum* and *L. gymnesicum*, two highly related species from Mallorca included in subsection *Densiflorae*, which always form a monophyletic group. The upper value corresponds to the typical level of divergence of the species from subsection *Genuinae*, *L. narbonense* and *L. vulgare*, with respect to the other species (mean 0.0219), including *L. caesium*. However, *L. caesium* had levels of pairwise divergence with all the other ingroup species in the range 0.0176–0.0196, despite being the outgroup. Levels of divergence among the other ingroup species ranged from 0.0010 to 0.0066. It is remarkable that the pairwise divergence between *L. furfuraceum* A and B haplotypes is in the middle of this range.

Based on these cpDNA RFLP results, the current classification of section *Limonium* may be questioned, as divergence levels of *L. narbonense* and *L. vulgare* with other species of the same section were similar to those obtained with the outgroup species. Consequently, we decided to include as possible outgroups for the analysis of the nuclear ITS region three species that are classified under other sections of the genus, *L. sinuatum*, *L. lobatum*, and *L. echioides* (Table 1). Other species included in the ITS study, and for which cpDNA was not studied, correspond to a separate study on *L. interjectum*, a presumed hybrid species, and to *L. cossonianum* ($2n = 16$) and *L. minutum* ($2n = 18$), two diploid, sexually reproducing species with differing basic chromosome numbers.

TABLE 3

Distance Matrix Obtained Using Nei and Miller (1990) Procedure to Combine Four- and Six-Cutter Restriction Enzyme Fragment Analysis of cpDNAs from *Limonium* Species

	Lnar	*Lvul*	*Lduf*	*Lcam*	*Lgym*	*Ldel*	*Lang*	*Lrig*	*Lvir*	*LfurB*	*LfurA*	*Lten*	*Ldic*	*Lcae*	*Lcav*
L. narbonense	0.0000														
L. vulgare	0.0015	0.0000													
L. dufourii	0.0229	0.0225	0.0000												
L. camposanum	0.0225	0.0221	0.0014	0.0000											
L. gymnesicum	0.0223	0.0219	0.0014	0.0002	0.000 0										
L. delicatulum	0.0228	0.0224	0.0020	0.0012	0.0011	0.0000									
L. angustebract.	0.0209	0.0205	0.0059	0.0058	0.0056	0.0053	0.0000								
L. rigualii	0.0222	0.0217	0.0047	0.0044	0.0043	0.0043	0.0045	0.0000							
L. virgatum	0.0223	0.0219	0.0030	0.0022	0.0022	0.0025	0.0061	0.0051	0.0000						
L. furfuraceum B	0.0227	0.0222	0.0024	0.0018	0.0016	0.0020	0.0062	0.0052	0.0028	0.0000					
L. furfuraceum A	0.0223	0.0219	0.0062	0.0057	0.0056	0.0058	0.0057	0.0017	0.0060	0.0044	0.0000				
L. tenuicaule	0.0229	0.0224	0.0016	0.0011	0.0010	0.0012	0.0052	0.0043	0.0018	0.0015	0.0054	0.0000			
L. dichotomum	0.0227	0.0222	0.0032	0.0026	0.0025	0.0026	0.0059	0.0050	0.0030	0.0027	0.0061	0.0017	0.0000		
L. caesium	0.0201	0.0195	0.0183	0.0186	0.0185	0.0184	0.0184	0.0191	0.0188	0.0190	0.0197	0.0185	0.0186	0.0000	
L. cavanillesii	0.0210	0.0206	0.0067	0.0064	0.0062	0.0062	0.0047	0.0020	0.0067	0.0067	0.0028	0.0058	0.0060	0.0177	0.0000

ITS Region Variation

Aligned sequences of ITS-1 and ITS-2 were obtained for all species classified under section *Limonium* and the outgroup species with 488 characters in all (Fig. 3). The length of the ITS-1 and ITS-2 regions varied from 192 to 214 bp and from 233 to 250 bp, respectively. Most of the length variation encountered is due to small insertion–deletion events of only 1 or 2 bp. Only the outgroup species, especially *L. sinuatum* and *L. lobatum*, have larger indels (of up to 8 bp in ITS-2).

Among the 26 populations from the 21 *Limonium* species studied, 26 different ITS sequence types were identified. Although some level of uncertainty/polymorphism was detected in several species, this prevented the identification of a unique sequence only in *L. delicatulum*, a triploid species ($2n = 25$; Table 1). Hence, DNA from three individuals of this species was amplified, cloned, and sequenced. Six different ITS sequences, two from each individual studied, were obtained from eight clones. The two sequences derived from each individual differed in several indels, which prevented the direct sequencing of PCR products. Most of the ITS-2 sequence of *L. cossonianum* is missing due to problems in obtaining material. A single ITS type

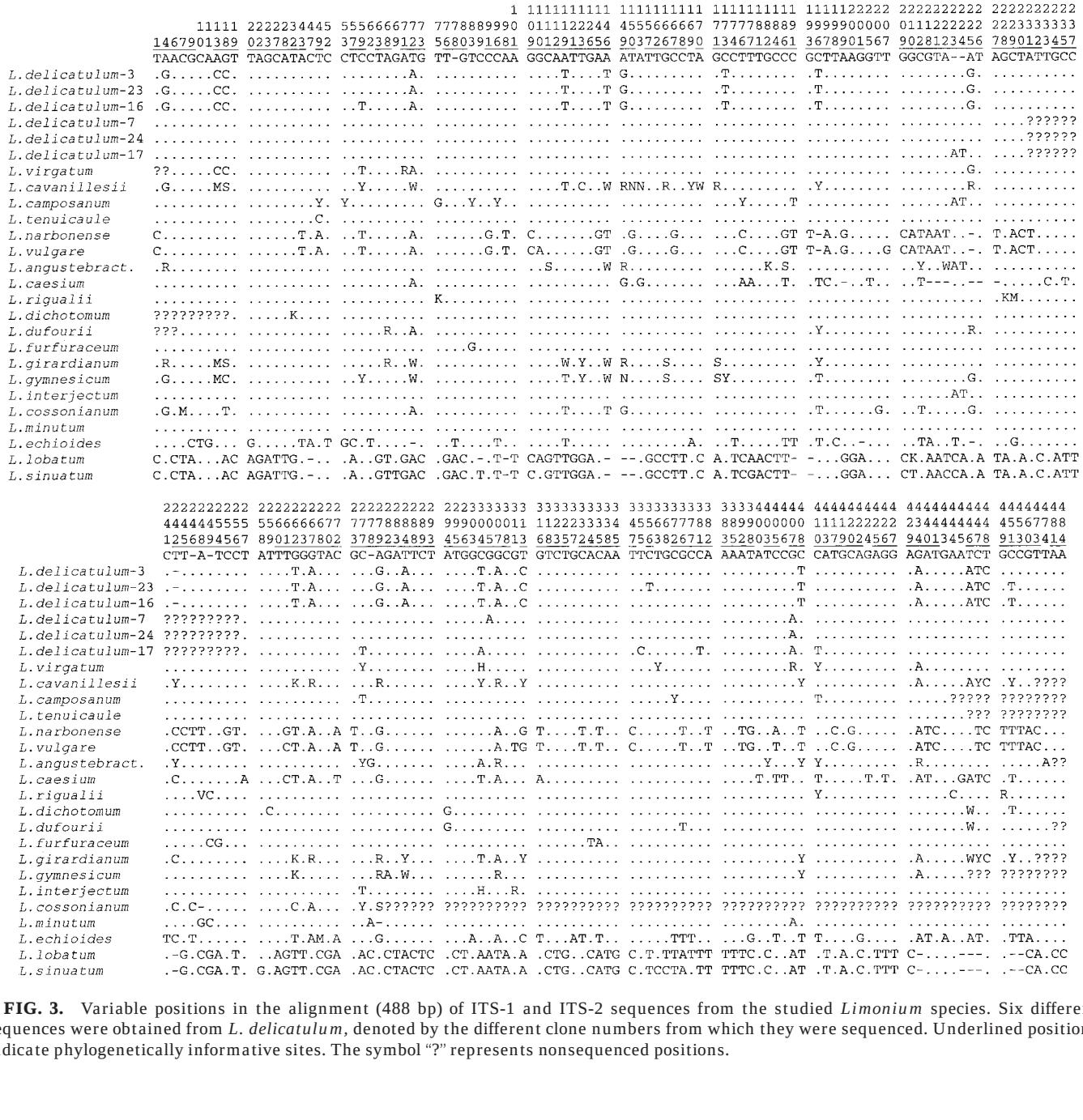

FIG. 3. Variable positions in the alignment (488 bp) of ITS-1 and ITS-2 sequences from the studied *Limonium* species. Six different sequences were obtained from *L. delicatulum*, denoted by the different clone numbers from which they were sequenced. Underlined positions indicate phylogenetically informative sites. The symbol "?" represents nonsequenced positions.

was extracted from the other species surveyed. A consensus sequence was obtained from each population analyzed in the corresponding species. In those species for which more than one population was examined, the consensus sequence was identical in all the corresponding populations. Therefore, ambiguities shown in the alignment in Fig. 3 were present in all the sequences from the corresponding species.

ITS secondary structure and character weighting. The consensus secondary structure models for the ITS-1 and ITS-2 regions were obtained from the corresponding *Limonium* consensus sequences and compared with available models for angiosperms (Yeh and Lee, 1991; Liu and Schardl, 1994; Bakker *et al.*, 1995; Hershkovitz and Zimmer, 1996). The relevant features described by these authors are also present in both *Limonium* ITS regions.

From the ITS-1 and ITS-2 secondary structure models, the observed number of single and double compensatory and noncompensatory mutations were calculated, excluding outgroup species and considering ambiguities as uncertainties and polymorphisms separately. The test proposed by Dixon and Hillis (1993) was highly significant (χ^2 values ranged from 14.9 to 54.0, $df = 1$, $P < 0.001$). After assuming a linear scaling, a relative weighting scheme of 0.8:1.0 for stem vs loop characters was chosen for further analyses, as opposed to equal character state weights. However, the results obtained did not differ significantly between both alternatives, and we present the results from only the equal weights analyses.

ITS sequence divergence. Table 4 shows the average number of substitutions per site using JC distance for ITS-1–ITS-2 sequences in pairwise comparisons. ITS sequence divergence values ranged from 0.000 to 0.283 substitutions/site. The highest value corresponds to the typical divergence between *L. sinuatum* or *L. lobatum* and all the other species (average = 0.251). Levels of divergence between *L. narbonense* and *L. vulgare* sequences with respect to the remaining species were around 0.11 substitutions/site. Pairwise comparison of *L. echioides* with all the other ingroup species was slightly lower (approx 0.09). The average divergence was even lower, 0.06, when *L. caesium* was compared with these other ingroup species. When *L. narbonense* and *L. vulgare* were excluded, ITS divergence values for the ingroup species from section *Limonium* ranged from 0.00 to 0.05. A null divergence was found between *L. girardianum* and *L. cavanillesii*. However, the identity percentage between these species was 92.2, and the discrepancy was due to differing ambiguous positions.

Intraspecific ITS pairwise divergence values among *L. delicatulum* clones (Ldel) had range limits (0.00 to 0.05) similar to interspecific values among the remaining ingroup species. Two major types of sequences were observed in this species: type A, including Ldel7, Ldel17, and Ldel24, with pairwise sequence variation ranging from 0.004 to 0.008; and type B, including Ldel3, Ldel16, and Ldel23, whose range of pairwise variation was 0.000–0.008. Note that each clone pair Ldel7–Ldel3, Ldel17–Ldel16, and Ldel23–Ldel24, was obtained from the same individual. These two ITS types present in each *L. delicatulum* individual are closely related to two diploid, sexual species, *L. cossonianum* (type B) and *L. minutum* (type A), with different base chromosome numbers, $n = 8$ and $n = 9$, respectively.

Phylogenetic analyses. Of the 488 aligned positions from the whole ITS region, 198 sites were variable, of which 156 were parsimony informative (Fig. 3). The skewness test (Hillis, 1991) suggested nonrandom structure in this data set ($g1 = -2.70$, $P \ll 0.01$).

Maximum likelihood (ML) analysis of *Limonium* ITS sequences rendered exactly the same topologies for the different ts/tv ratios tested (from 0.5 to 4.0) with and without considering different character state weights (categories). However, the tree with the highest ML value (−2765.76) was that obtained for a ts/tv ratio of 1.0, without using categories (Fig. 4). Basal branches were all significantly different from zero. The group formed by all the other ingroup species apart from *L. narbonense* and *L. vulgare* was also well supported. Within this group there were only three significant branches, one corresponding to the terminal branch for *L. furfuraceum*. The two inner branches defined three groupings. The first group included *L. cossonianum*, *L. cavanillesii*, *L. girardianum*, and the three *L. delicatulum* type B sequences. The second group was composed of *L. gymnesicum* and *L. virgatum*. The third group included all the remaining species.

The NJ dendrogram derived from the JC distance matrix of Table 4 had the same topology as the one obtained by ML (Fig. 4), differing only in the support for some branching nodes. Bootstrap values were high at the basal nodes of the tree, involving outgroup species and the sister species *L. narbonense* and *L. vulgare*. The other ingroup species formed a monophyletic group with a bootstrap support of 82%. There were two well-supported subgroups within this group. The first subgroup was formed by *L. delicatulum* ITS type B sequences (with 87% bootstrap value). This subgroup is joined to other ingroup species, *L. girardianum*, *L. cavanillesii*, *L. gymnesicum*, and *L. cossonianum*, with bootstrap values lower than 50%, and to *L. virgatum* with 68% bootstrap support. The other subgroup was supported by a bootstrap value of 87% and included the remaining species analyzed, coincident with the last subgroup described for the ML tree. Here, only the nodes corresponding to the clade formed by two *L. delicatulum* ITS type A sequences and *L. minutum* had bootstrap supports higher than 50%.

TABLE 4

Average Number of Pairwise Substitutions per Site ($\times 10^{-2}$) Using Jukes and Cantor Distance for the ITS Region Sequences of *Limonium* Species

	Lvir	*Lcav*	*Lcam*	*Lten*	*Lnar*	*Lvul*	*Lang*	*Lcae*	*Lrig*	*Ldic*	*Lduf*	*Lfur*	*Lgir*	*Lgym*	*Lint*	*Ld 3*	*Ld 23*	*Ld 16*	*Ld 7*	*Ld 24*	*Ld 17*	*Lcos*	*Lm*	*Lech*	*Llob*
L. virgatum	—																								
L. cavanillesii	0.010	—																							
L. camposanum	0.023	0.020	—																						
L. tenuicaule	0.020	0.013	0.010	—																					
L. narbonense	0.155	0.136	0.155	0.150	—																				
L. vulgare	0.163	0.145	0.164	0.159	0.011	—																			
L. angustebract.	0.014	0.012	0.007	0.005	0.144	0.153	—																		
L. caesium	0.080	0.058	0.084	0.083	0.152	0.155	0.073	—																	
L. rigualii	0.018	0.017	0.005	0.005	0.155	0.164	0.007	0.084	—																
L. dichotomum	0.019	0.015	0.015	0.008	0.161	0.170	0.012	0.087	0.009	—															
L. dufourii	0.016	0.015	0.018	0.010	0.157	0.165	0.012	0.085	0.009	0.010	—														
L. furfuraceum	0.025	0.024	0.020	0.012	0.163	0.171	0.014	0.093	0.011	0.016	0.016	—													
L. girardianum	0.012	0.000	0.020	0.012	0.131	0.139	0.005	0.052	0.014	0.017	0.017	0.021	—												
L. gymnesicum	0.008	0.003	0.028	0.020	0.141	0.150	0.015	0.074	0.020	0.018	0.015	0.028	0.010	—											
L. interjectum	0.016	0.017	0.007	0.005	0.158	0.166	0.002	0.083	0.005	0.009	0.009	0.011	0.012	0.020	—										
L. delicatulum 3	0.034	0.007	0.059	0.050	0.150	0.158	0.040	0.073	0.053	0.050	0.045	0.060	0.007	0.010	0.050	—									
L. delicatulum 23	0.039	0.010	0.061	0.053	0.150	0.158	0.045	0.074	0.058	0.050	0.050	0.065	0.009	0.012	0.055	0.004	—								
L. delicatulum 16	0.034	0.007	0.061	0.053	0.145	0.153	0.045	0.074	0.058	0.050	0.050	0.065	0.009	0.010	0.055	0.004	0.004	—							
L. delicatulum 7	0.019	0.021	0.016	0.008	0.152	0.161	0.010	0.085	0.007	0.012	0.012	0.012	0.015	0.024	0.007	0.058	0.063	0.063	—						
L. delicatulum 24	0.017	0.018	0.013	0.005	0.150	0.158	0.007	0.083	0.005	0.010	0.010	0.009	0.012	0.021	0.005	0.055	0.061	0.061	0.002	—					
L. delicatulum 17	0.022	0.031	0.016	0.018	0.164	0.173	0.010	0.088	0.014	0.022	0.022	0.021	0.023	0.035	0.009	0.065	0.071	0.071	0.014	0.012	—				
L. cossonianum	0.051	0.021	0.072	0.063	0.171	0.181	0.038	0.074	0.059	0.058	0.042	0.067	0.021	0.025	0.062	0.024	0.024	0.029	0.059	0.059	0.064	—			
L. minutum	0.018	0.019	0.015	0.007	0.155	0.163	0.012	0.086	0.004	0.012	0.012	0.013	0.017	0.023	0.007	0.053	0.058	0.058	0.002	0.000	0.012	0.054	—		
L. echioides	0.131	0.106	0.120	0.124	0.158	0.164	0.120	0.128	0.124	0.120	0.124	0.131	0.101	0.112	0.128	0.116	0.116	0.116	0.130	0.128	0.138	0.123	0.126	—	
L. lobatum	0.350	0.354	0.369	0.366	0.372	0.380	0.351	0.380	0.357	0.353	0.356	0.354	0.343	0.354	0.354	0.361	0.359	0.366	0.336	0.339	0.345	0.418	0.354	0.392	—
L. sinuatum	0.355	0.363	0.379	0.376	0.382	0.398	0.364	0.393	0.366	0.363	0.365	0.362	0.352	0.364	0.363	0.370	0.367	0.375	0.345	0.349	0.355	0.434	0.362	0.409	0.018

Wagner parsimony analysis from the ITS data matrix identified 17,567 most-parsimonious trees, with length of 325 steps, and CI and RI values of 0.824 and 0.860, respectively. Despite the large number of MPTs, the consensus tree (Fig. 5) retained considerable resolution. For example, the basal relationships are coincident with those obtained in the previous analyses. Polytomies at inner branches were responsible for the large number of equally most-parsimonious trees encountered. However, the relationships among these ingroup species had features in common with those described above. In this analysis, *L. cossonianum* forms a clade with *L. delicatulum* type B sequences, which is not present in the previous analyses.

When parsimony analysis was performed with the additional data matrix of 43 presence–absence gap characters, the skewness index for the whole data set was -3.1 ($P \ll 0.01$). A total of 246 MPTs were obtained of 367 steps, with CI = 0.782 and RI = 0.825. The majority-rule consensus tree (not shown) obtained in this analysis was highly resolved not only at basal nodes but also at more internal branches. This topology showed basically the same relationships among *Limonium* species as in previous analyses, with most polytomies being coincident with those nodes in the NJ tree having low bootstrap support and the ML tree branches not significantly different from zero.

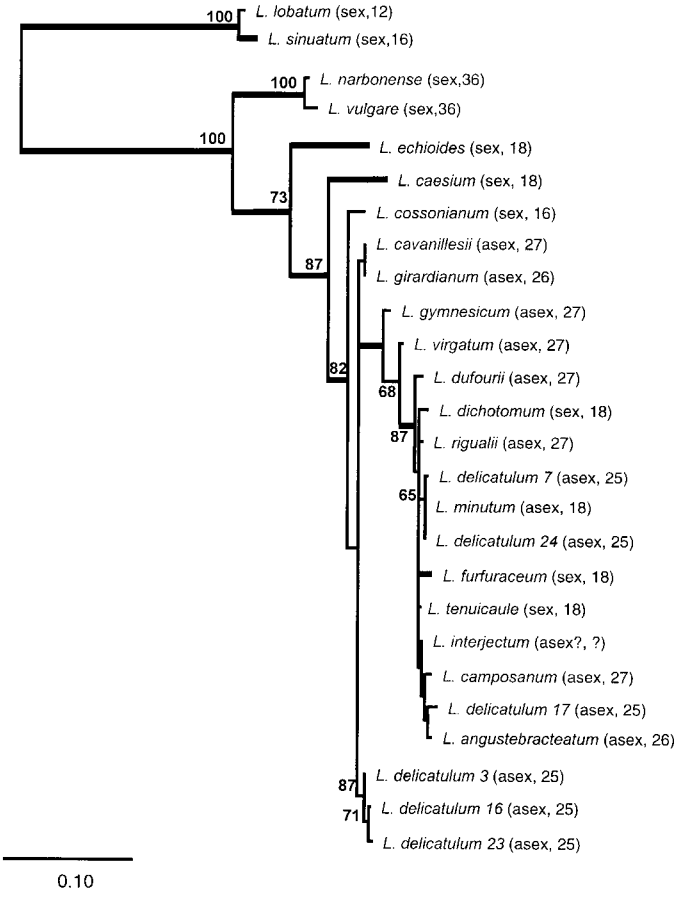

FIG. 4. Maximum likelihood tree derived from ITS sequence analysis of *Limonium* species. Thick branches are significantly different from zero length. Values above each node indicate bootstrap support higher than 50% for the corresponding branching point obtained with 1000 replicates using the neighbor-joining algorithm and JC estimate of nucleotide divergence. Reproduction system (sex, sexual; asex, asexual, apomictic) and number of chromosomes are indicated between parentheses next to each species.

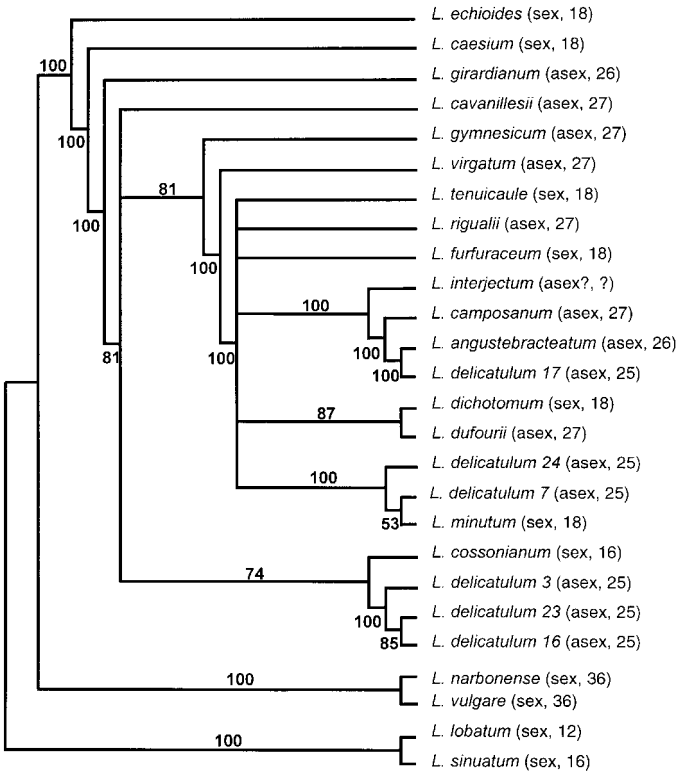

FIG. 5. Majority-rule (50%) consensus tree from parsimony analysis of ITS sequences. Values next to each node correspond to the percentage of the 17,567 MP trees supporting it. Reproduction system (sex, sexual; asex, asexual, apomictic) and number of chromosomes are indicated between parentheses next to each species.

Sexually reproducing species. To gain some insight into the evolution of this group of species without the possibly disturbing effects of reticulate evolution, we performed the same analyses previously described on a subset of *Limonium* ITS sequences, those corresponding to the 11 sexually reproducing species indicated in Table 1. In this case, the topologies obtained by the three phylogenetic reconstruction methods were identical. The ML tree (Fig. 6) presented most inner branches significantly different from zero. The two branches not differing from zero corresponded to the nodes with bootstrap support lower than 80% in the NJ and MP analyses. The MP analysis provided 15 equally MPTs, with 254 steps (CI = 0.890, RI = 0.887). The 50% majority-rule consensus for these 15 MPTs presented only one polytomy, coincident with one of the unsupported branches in the ML reconstruction, joining one out-

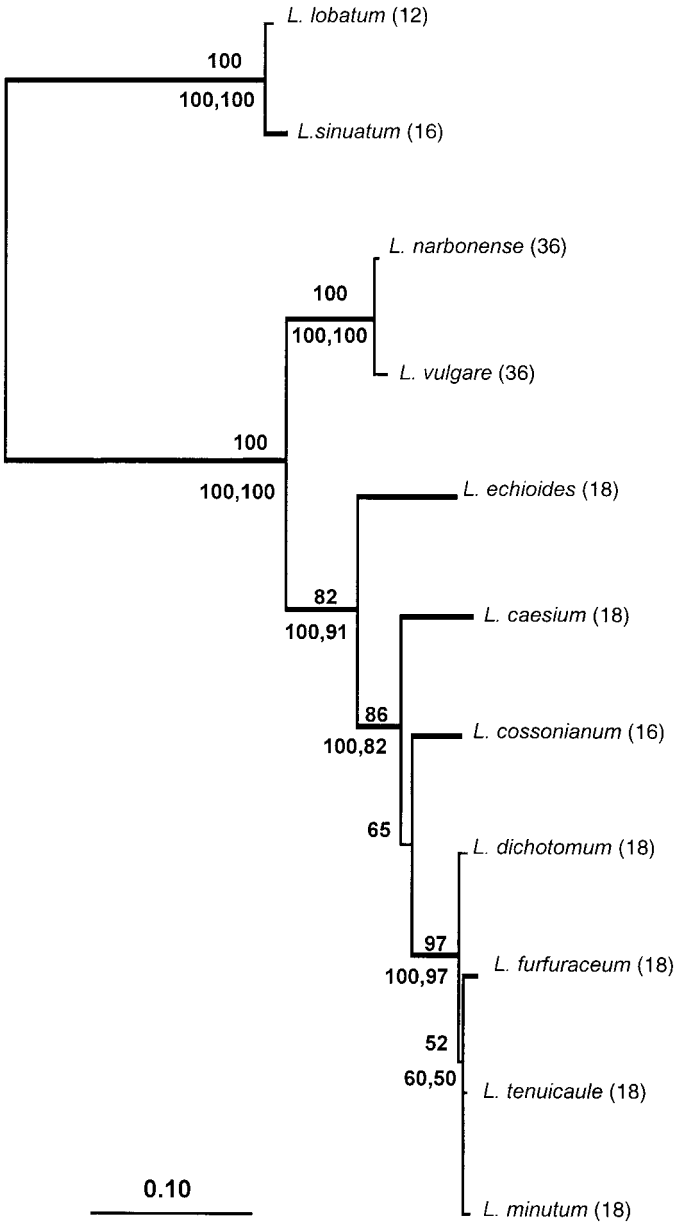

FIG. 6. Maximum likelihood tree obtained from ITS sequences of sexually reproducing *Limonium* species. Thick branches are significantly different from zero length. Values above each node indicate bootstrap support (higher than 50%) for the corresponding branching point obtained with 1000 replicates using the neighbor-joining algorithm and JC estimate of nucleotide divergence. The tree topology is identical to 1 of the 15 most-parsimonious trees obtained with the same data set. Numbers below each branch indicate the percentage of times the corresponding node appears in the 15 MP trees and in 1000 bootstrap replicates, respectively. The number of chromosomes is indicated between parentheses next to each species.

group species (*L. caesium*) to the other ingroup species (apart from *L. narbonense* and *L. vulgare*) belonging to section *Limonium*. The same topology was obtained when only diploid species were analyzed in a similar way (results not shown).

DISCUSSION

Intraspecific Variation

We have detected intraspecific polymorphism in 21 species of *Limonium* with two molecular markers, RFLPs of chloroplast DNA and nuclear rDNA ITS sequences, despite the relatively small intraspecific sampling performed for most species. *L. furfuraceum*, a sexual species with a very conspicuous morphology and endemic to a small area in southeastern Spain, presented two different cpDNA haplotypes but no variability was found in its rDNA. Estimated sequence divergence between the two haplotypes (0.44%) falls within the reported values for intraspecific cpDNA variation in angiosperms (Soltis *et al.*, 1992). Moderate levels of cpDNA variation may therefore be present within *Limonium* species, which could have been overlooked due to the sampling strategy used in this study. However, this pattern of variation can be explained in other ways (e.g., Rieseberg and Brunsfeld, 1992). A likely explanation is that those individuals with cpDNA haplotype B obtained their cytoplasm by introgression from an unidentified *Limonium* species (as haplotype A was also present in the other population of the species analyzed) but introgression would not have been suspected based only on nuclear DNA. A second possibility is that *L. furfuraceum* is of recent hybrid origin and that it was formed by reciprocal crosses between two species with different cpDNAs. Thus, it could be argued that both ancestors acted as maternal progenitors but the offspring would have the same rDNA (perhaps the sum of both parents but see later). However, *L. furfuraceum* is a sexual, diploid species showing no signs of reduced pollen fertility or low seed production. A scenario involving diploid hybrid speciation has been invoked for several taxa of the related genus *Armeria* (Fuertes *et al.*, 1999) but basic data on experimental hybrids in *Limonium* that could support this hypothesis are missing.

L. delicatulum represents another case in which intraspecific variability has been detected but with an opposite rDNA–cpDNA pattern of variability. This is an asexual species, with apomictic reproduction, endemic to the southeast of the Iberian Peninsula. Mertens (1993) summarized the possible explanations for this pattern of association in asexual polyploids formed through hybridization. The association of several rDNA genotypes with a single cpDNA haplotype could be explained if a single species with a distinct cpDNA haplotype were the maternal parent in all cases of hybridization, with the different ITS types from both parents. These might remain as different rDNA intraindividual arrays in the genome (Bobola *et al.*, 1992). On the other hand, within-species polymorphism can be explained as a remnant of the polymorphism in the ancestral species or as due to the accumulation of

mutations without further homogenization. In other *Limonium* species some ambiguities in rDNA sequences have been detected (Fig. 3) that could actually represent intraspecific polymorphisms. An apomictic reproductive system (Table 1) is correlated with ITS sequences showing more than 10 ambiguously scored positions (Fig. 3), with only a few exceptions to this rule: *L. dufourii*, *L. interjectum*, and *L. virgatum* presented fewer than 6 ambiguities. If the results obtained in the more detailed analysis of *L. delicatulum* are similar for the other apomictic species and they actually represent polymorphic sites resulting from the simultaneous presence in each individual of two relatively divergent ITS sequence types, then it may be possible to determine the putative ancestral hybridizing species. Consequently, it will be necessary to investigate the levels of intraspecific and intraindividual variability in these species by a strategy similar to that followed with *L. delicatulum*.

In summary, intraspecific rDNA or cpDNA types of *L. delicatulum* and *L. furfuraceum* were paraphyletic. They showed levels of intraindividual (*L. delicatulum*) and intrapopulation (*L. furfuraceum*) divergence similar to those detected between other *Limonium* species, reinforcing the introgression or hybridization hypothesis for the origins of these taxa. Furthermore, when multigene families are studied, the special mechanisms involved in their evolution (unequal crossing over, gene conversion, etc.) may override the classical factors (selection, mutation, and genetic drift) as agents regulating genetic variation, resulting in unexpected variation patterns based on the biology of the species. These factors tend to homogenize orthologous and paralogous genome regions and can obscure the parental rDNA source, as could be the case in *L. furfuraceum*. Although it has been demonstrated that these mechanisms are active in asexual species (Hillis *et al.*, 1991; Crease and Lynch, 1991), they could fail to act on multicopy units contributed by the parental species if the hybridization event was recent and/or the rDNAs occur at different loci in the parental taxa and interlocus gene conversion was not operating (Baldwin *et al.*, 1995). This could be the case in *L. delicatulum*. A similar explanation has been suggested for ITS polymorphism in other plant species (Suh *et al.*, 1993; Karvonen and Savolainen, 1993; Campbell *et al.*, 1997).

The results here reported illustrate the importance of analyzing intraspecific variability in studies of both cpDNA–RFLP and ITS sequences. In some cases, conspecific samples rendered identical genotypes; in others, intraspecific variability has been detected through pooled DNA samples from different individuals of the same population. These phenomena should prevent reporting these ITS sequences as unique sequences, representative of the corresponding species.

Phylogeny Reconstruction

The analysis of cpDNA using multiple methods of phylogenetic reconstruction has revealed a relatively stable phylogenetic structure. Weighted parsimony is considered the best choice among all parsimony methods for RFLP analysis (Albert *et al.*, 1992; Felsenstein, 1992; Holsinger and Jansen, 1993; Huelsenbeck and Hillis, 1993). Although the selection of weights remains controversial (Swofford *et al.*, 1996), those weights that correct for possible violations of the assumptions of the phylogenetic inference methods should be considered.

The presumed uniparental (maternal) inheritance of cpDNA in *Limonium* (Clegg, 1987; Harris and Ingram, 1991) and absence of intermolecular recombination prevent reticulation in cpDNA phylogenies. However, caution is needed in the interpretation of results in which lineage sorting, hybrid origin, or introgression may have resulted in the transfer of cpDNA from one lineage to another (Soltis *et al.*, 1992; Doyle, 1992). In section *Limonium*, polyploidy and apomixis are common, numerous hybrids occur naturally, and, as a consequence, reticulate evolution seems to be the rule rather than the exception. Therefore, an *a posteriori* search of inconsistencies with other types of data is necessary before any conclusion on the phylogeny of these species can be drawn. This should preferentially be done from the nuclear genome since hybridization is less permeable to this biparentally inherited DNA (McDade, 1992).

Sizes of ITS-1 and ITS-2 in the *Limonium* species studied were similar to those reported for other flowering plants, with ITS-1 longer than ITS-2 (Baldwin *et al.*, 1995). Within section *Limonium*, the ITS region has evolved primarily by point mutations, which conforms with other studies on closely related plants (Soltis and Kuzoff, 1995; Baldwin *et al.*, 1995). The conservation of ITS sequences is presumably due to their role in the production of mature rRNA, and this functionality depends on evolutionarily conserved secondary structural motifs. Inference of nonindependence at directly opposing sites in these secondary structures can be determined empirically (Dixon and Hillis, 1993). However, in our case the use of differential character weights for stem vs loop positions did not lead to different results in the analysis of ITS sequences from *Limonium*. It has been demonstrated that rRNA processing mechanisms could be labile enough to allow readjustments of intrastrand RNA pairing, which could imply mutations at nonpaired positions (cryptic nonindependence) (Olsthoorn *et al.*, 1994). This pattern of substitutions could have important implications for phylogenetic analysis but it could also mean that selection for compensatory mutations might be weaker for these spacers than for nrDNA coding regions, alleviating the concern about nonindependence of characters.

None of the methods currently available for phylogenetic analysis is ideal, and it is advisable to use different approaches for data analysis (Holsinger and Jansen, 1993; Swofford *et al.*, 1996). In this case, the relationships obtained among *Limonium* ITS sequences using ML, NJ, and parsimony approaches are, in general, congruent for well-supported groups.

Evolution in Limonium

Several base chromosome numbers ranging from $n = 6$ to $n = 9$ have been reported in *Limonium* (Erben, 1979). He suggested on the basis of chromosome morphology that in subgenus *Limonium* the chromosome number $n = 8$ is not ancestral but was derived from $n = 9$ karyotypes through chromosome fusion. Thus, only taxa with $n = 8$ have two long metacentric chromosomes in their diploid complement that are thought to have evolved from smaller ones present in the $n = 9$ genomes. The position of *L. cossonianum* ($2n = 16$) in the ITS phylogeny suggests that the $n = 8$ karyotype is derived within section *Limonium*, as taxa having $n = 9$ appear basal in the nuclear phylogeny, supporting the hypothesis of chromosomal evolution by descending aneuploidy in section *Limonium* (Erben, 1979). The relationships of the remaining species suggest that the base chromosome numbers present in *Limonium* species could have changed more than once in the evolutionary history of *Limonium* through similar cytological rearrangements.

The fact that two ITS types have been found within *L. delicatulum* is consistent with a predicted allopolyploid origin of a taxon having an odd chromosome number ($2n = 25$). Interestingly, one type of the nuclear sequences of *L. delicatulum* stands near *L. cossonianum* ($2n = 16$), whereas the other shows a close relationship with *L. minutum* ($2n = 18$), suggesting that these or other closely related taxa could be involved in the formation of the triploid taxon. Thus, Erben's (1979) hypothesis on the origin of polyploid taxa in *Limonium* is supported for *L. delicatulum* by the phylogenetic analysis of the ITS data.

Comparison of Phylogenetic Relationships and Taxonomy in Limonium

In the absence of a previous phylogenetic analysis of section *Limonium* based on other independent characters (although see Lledó *et al.*, 1998), our results can be discussed only in relation to the current classification of the group, which is based mainly on morphological and karyological characters (Boissier, 1848; Pignatti, 1971; Erben, 1993). This is an important limitation (Donoghue and Cantino, 1988; Doyle *et al.*, 1990), especially in cases of striking disagreements (Doyle and Doyle, 1993).

The current splitting of section *Limonium* does not agree with the results of the phylogenetic analyses of nuclear and organellar markers. In fact, they suggest that the largest section of the genus (section *Limonium*) does not constitute a monophyletic assemblage. The basal position of the two analyzed species of subsection *Limonium* (*L. vulgare* and *L. narbonense*) was strongly supported in all analyses. The same results were obtained using *rbcL* sequences with five *Limonium* species (Lledó *et al.*, 1998). Levels of sequence divergence suggest that molecular differentiation between taxa of subsection *Limonium* and the other species from section *Limonium* is larger than expected based on exomorphic features. In addition, morphological and anatomical data suggest that subsection *Limonium* is a group of well-knit species (Bokhari, 1973) not linked by intermediates to the remaining section *Limonium*. The distinctiveness of subsection *Limonium* is so remarkable that it should probably be granted a sectional status. Because *L. vulgare* is the type species of the genus (and hence of section *Limonium*), whatever nomenclatural rearrangement that could be proposed must also involve the other sectional names.

Although few sexual taxa belonging to subsections *Hyalolepidae* and *Steirocladae* were available for study, no clear distinction between them has arisen. This agrees with Bokhari's (1973) results based on anatomical and morphological features. He stated that the characters used by Boissier (1848) to separate subsections *Hyalolepidae* and *Steiroclade* were inefficient, and he suggested that these two subsections should be combined. Furthermore, he proposed that subsections *Densiflorae* and *Dissitiflorae* should be merged. This could not be directly addressed in this work since no sexual taxa from subsection *Densiflorae* could be analyzed and only one species from subsection *Dissitiflorae* was available. However, the combined analysis of sexual and apomictic taxa does not support the recognition of subsections *Densiflorae* and *Dissitiflorae* as natural segregates within *Limonium*. When diploid and polyploid taxa are analyzed together, none of the four subsections appears as monophyletic, although interspecific hybridization may have blurred boundaries between distinct lineages. Nevertheless, *Limonium* taxa belonging to these four subsections are apomictic and, presumably, have a hybrid origin from ancestors belonging to either subsection. Therefore, there is little sense in recognizing them as distinct unless other hybrid subsections are created. Taking into account the great taxonomic complexity of the genus, this is clearly an unsatisfactory solution.

Members of sections *Polyarthrion* and *Schyzymenium* are successive sisters to section *Limonium* (excluding *L. narbonense* and *L. vulgare*) but their monophyly should be checked by the analysis of additional taxa and markers. *L. caesium* has been excluded sometimes from subgenus *Limonium* [recognized by some authors and included with other species within subgenus *Siphonantha* (Pau, 1898) or *Myriolepis* (Pignatti, 1971)]. If

this is followed, then subgenus *Limonium* would appear paraphyletic. The isolated taxonomic position of *L. echioides* was previously suggested by Baker (1953) and Bohkari (1973). They pointed out that section *Schizhymenium* was heterogeneous and that the differences between *L. echioides* and the other two taxa included within the section (*L. owerinii* and *L. cabulicum*) were so remarkable that *L. echioides* should be accommodated in a section of its own. Molecular analysis of the latter speciesis needed before a reassessment of the placement of *L. echioides* within section *Limonium* can be made.

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