# Phylogenetic relationships in Lupinus (Fabaceae: Papilionoideae) based on INTERNAL TRANSCRIBED SPACER SEQUENCES (ITS) OF NUCLEAR RIbOSOMAL DNA ${ }^{1}$ 

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#### Abstract

Internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA from 44 taxa of the genus Lupinus and five outgroup taxa were used for phylogenetic analysis. Lupinus appears as a strongly supported monophyletic genus, which is unambiguously part of the Genisteae. The lupines are distributed into five main clades in general accordance with their geographical origin. In the Old World, almost all the recognized taxonomic units are well resolved. The ITS data reveal an unexpectedly close relationship between the diverse sections Angustifoli and Lutei. The ITS results suggest a geographical division between the western New World lupines and the eastern ones. They also indicate the presence of some moderately to strongly supported groups of taxa, such as the Microcarpi-Pusilli group, the L. spariflorus-L. arizonicus group, the L. mexicanusL. elegans group in the western New World, and the notable L. multiflorus-L. paraguariensis group in the eastern New World. The latter group strongly suggests that the eastern South American compound- and simple-leaved perennial lupines derive from a common ancestor. However, apart from some exceptions, relationships within the genus still remain largely unresolved based on ITS data. The lack of resolution at the base of the genus is suggestive of a rapid initial radiation of the lupines subsequent to the dispersal of their common ancestor. Relative rate tests demonstrate the presence of rate heterogeneity of ITS sequences within Lupinus. In many pairwise comparisons between taxa, substitution rate inequalities are correlated with the habit (annual, perennial), suggesting some role for the generation time effects in the evolutionary history of lupines.


Key words: Fabaceae; Genisteae; ITS-nrDNA; Lupinus; phylogeny; relative rates of substitution.

Lupinus L. (Fabaceae) is a large and diverse genus comprising 200-500 (Dunn and Gillett, 1966) annual and perennial herbaceous species, as well as a few soft-woody shrubs and small trees (Dunn, 1984; Turner, 1995), which occur in a wide range of ecogeographical conditions in both the New and the Old World. Lupines are more diverse in the New World with over $90 \%$ of the species in

[^0]the genus. They are mainly distributed in alpine, temperate, and subtropical biomes of the western cordillera of the New World, from Alaska to South Argentina and Chile. They occur all along and on both sides of the Rocky and Andean mountains, with some of the species occurring particularly in the east-central part of South America (Dunn, 1984; Planchuelo Ravelo, 1984). Only 12-13 species are native to the Mediterranean region and Africa, with some populations extending to highlands of East African tropical areas (Gladstones, 1974; Amaral Franco and Pinto da Silva, 1978).

During the past several decades, much has been accomplished to improve the taxonomy and systematics of lupines. This is particularly true in the Old World because of the limited number of species and the growing economical interest in them as nitrogen and protein suppliers (Gladstones, 1974, 1980). Old World lupines are all annual, herbaceous, and predominantly autogamous. Their fruits and seeds are generally large, and their leaves are always digitate. Two distinct groups are recognized primarily on the basis of the seed coat texture: the smoothseeded and the rough-seeded species (Gladstones, 1974; Heyn and Herrnstadt, 1977). The smooth-seeded group comprises five species usually treated as members of four sections, Albi, Micranthi, Angustifoli, and Lutei (Gladstones, 1984). These taxa are distributed around the Mediterranean and exhibit variable chromosome numbers ranging from $2 n=40$ to 52 . Spontaneous autopolyploids are known from L. albus $(2 n=100)$ and L. luteus $(2 n$ $=104)$ (Kazimierski, 1984). The rough-seeded group contains six or seven species characterized by their great overall morphological resemblance and their typical sca-
brous-tuberculate testa, which is unique in the genus. This group is generally typified by L. pilosus Murr. and often designated at the sectional rank (Gladstones, 1984; Plitmann and Heyn, 1984). The rough-seeded species are mainly distributed in North Africa and in the Eastern part of the Mediterranean region. They are ecogeographically isolated from one another and display chromosome numbers ranging from $2 n=32$ to 42 (Plitmann and Pazy, 1984; Carstairs, Buirchell, and Cowling, 1992). Numerous isolated populations in North Africa are morphotaxonomically poorly known and several Mediterranean areas still need to be better investigated (Swiecicki, 1988; Clements, Buirchell, and Cowling, 1996). Thus, it is possible that new undiscovered lupin forms and/or species exist in these areas (Clements, Buirchell, and Cowling, 1996; Swiecicki, Swiecicki, and Wolko, 1996).

In the New World, Lupinus is notorious for being a very complex and difficult genus. Taxonomic confusion exists in the literature, where numerous taxa or groups are distinguished based on only a few minor and inconsistent morphological characters. Over 1700 names have been proposed for Lupinus (Dunn, 1984). Approximately 200 species clustered in 18 groups were suggested by Smith (1944) for North America. Taking into account new evidence from various approaches, it became clear to modern authors that the complexity of this genus resulted from its high morphological, breeding system, and ecogeographical diversity and the lack of clear diagnostic features to separate species (Dunn and Gillett, 1966; Dunn and Harmon, 1977; Dunn, 1984; Planchuelo Ravelo, 1984; and references therein). In recent floristic accounts, the New World lupines are treated as broadly defined polymorphic species (Welsh et al., 1987; Barneby, 1989; Riggins and Sholars, 1993). Although the New World lupines in general, particularly in Central and South America, still need to be investigated more extensively, a satisfactory number of groups and complexes are already recognized and others are still roughly circumscribed but represent a good basis for further modern analyses (Dunn, 1984; Planchuelo Ravelo, 1984; Broich and Morrison, 1995). One of the most remarkable groups of lupines in the New World is composed of $\sim 22$ perennial species with simple or unifoliolate leaves (e.g., $L$. crotalarioides Benth.), previously referred to the "Foliis integris" (Agardh, 1835) or "Simplicifolieae" group (Bentham, 1859), which occur mainly in the subtropical highlands of the east-central region of South America (Planchuelo and Dunn, 1984; Monteiro and Gibbs, 1986); four other representatives of this group are also found in the southeastern United States (Dunn, 1971). Other annual (e.g., L. bracteolaris) and perennial (e.g., L. multiflorus) compound-leaved lupines also grow in eastern South America, (Planchuelo Ravelo, 1984; Planchuelo and Dunn, 1984). In this region, some perennial taxa represent an intermediate condition and display a combination of both simple and compound leaves (e.g., L. paraguariensis); these taxa are usually regarded as close relatives of the unifoliolate or simple-leaved lupines (Planchuelo and Dunn, 1984; Monteiro and Gibbs, 1986). All of the remaining species from the two main New World lupine centers of diversity, the Andean region and the North and Central American regions, have digitate leaves. Most of the New World species cytologically investigated
display a common chromosome number of $2 n=48$ (Phillips, 1957; Dunn and Gillett, 1966), with some occasional individuals having $2 n=96$ (Phillips, 1957), except for L. texensis and L. subcarnosus Hook., which have $2 n=$ 36 (Turner, 1957). Apart from L. mutabilis, which also has $2 n=48$, to our knowledge there are no available chromosome counts on the South American lupines. The base chromosome number suggested for the New World species is $x=6$ and consequently the New World Lupines are regarded as a paleopolyploid series that behave as diploids (Dunn, 1984). This was also inferred from the disomic behavior of polyploid individuals in isozyme profiles (Wolko and Weeden, 1989).

In spite of their high diversity, the lupines have always been regarded as a natural and distinct group (Bentham, 1865; Polhill, 1976). However, no infrageneric classification of the lupines is presently available, and there is a great need to provide a clear overview of the whole genus. According to the most recent systematic review, Lupinus L . is included in the monotypic subtribe Lupininae (Hutch.) of the tribe Genisteae (Adanson) Bentham (Bisby, 1981). Nevertheless, its tribal position has often been disputed (Monteiro, 1986; Saint-Martin, 1986; Badr, Martin, and Jensen, 1994). Hence, the origin of Lupinus is also under debate and four different centers of origin have been proposed for the genus: Mediterranean-African region, North America, South America, and East Asia (summarized by Cristofolini, 1989).

Recent development and use of molecular data have increased significantly the understanding of plant systematics at various taxonomic levels (Soltis and Soltis, 1995). The chloroplast genome, in particular, has been extensively surveyed to reconstruct plant phylogeny (Chase et al., 1993; Olmstead and Palmer, 1994). Studies on both structural characters and nucleotide sequence variation in the chloroplast genome have provided very useful information in legume systematics (Doyle, 1995; Käss and Wink, 1995, 1996, 1997a; Liston, 1995; Sanderson and Liston, 1995; Doyle et al., 1997). Recent studies using chloroplast DNA data, obtained from both sequencing of the $r b c L$ gene (Käss and Wink, 1994, 1997b) or restriction site mapping (Badr, Martin, and Jensen, 1994), supported a common phylogenetic origin for Old World and New World lupines but did not provide enough phylogenetic resolution within the genus.

Among the nuclear markers recently available, the internal transcribed spacers (ITS) of the ribosomal DNA repeats (Jorgensen and Cluster, 1988; Hamby and Zimmer, 1992), ITS1 and ITS2 regions, have been used successfully in recent phylogenetic studies at lower taxonomic levels in many angiosperms (Baldwin, 1992; Baldwin et al., 1995), including: Astragalus (Wojciechowski et al., 1993; Sanderson and Wojciechowski, 1996); Dendroseris (Sang et al., 1994); Peonia (Sang, Crawford, and Stuessy, 1995); Antennaria (Bayer, Soltis, and Soltis, 1996); Gentianinae (Yuan and Küpfer, 1995); Gentiana (Yuan, Küpfer, and Doyle, 1996); Echium (Böhle, Hilger, and Martin, 1996); Silene (Desfeux and Lejeune, 1996); Kalmia, Leiophyllum, and Loiseleuria (Kron and King, 1996); Sarraceniaceae (Bayer, Hufford, and Soltis, 1996); and Bromus (Aïnouche and Bayer, 1997). Preliminary results presented at the Eighth International Lupin Conference (Ainouche and Bayer, in press), paralleling those of
the independent study of Käss and Wink (1997b), revealed a relatively larger amount of variation in Lupinus ITS sequences comparatively to that exhibited by the cpDNA (Käss and Wink, 1994, 1997b).

The primary goal of this study was to evaluate whether ITS sequence variation may provide valuable data for elucidating taxonomic and phylogenetic relationships within Lupinus, using a simultaneous cladistic analysis of a large number of taxa to cover as well as possible the diversity of this complex genus. This study includes a more extensive taxon sampling (44 taxa) than did the previous molecular analyses on Lupinus (Badr, Martin, and Jensen, 1994; Käss and Wink, 1994, 1997b; Ainouche and Bayer, in press). More precisely the objectives were: (1) to ascertain the monophyly of the genus and its position relative to the tribe Genisteae; (2) to estimate the phylogenetic relationships between and within both the Old World and New World members; (3) to compare the ITS results with the data and hypotheses provided in earlier systematics studies on Lupinus. Also examined is the variance of ITS sequence divergence rates among both the annual and perennial taxa.

## MATERIALS AND METHODS

Plant material-Forty-four samples belonging to 41 species originating from the main geographic distribution areas of the genus Lupinus were used in this study. All of the Old World lupines (Gladstones, 1984), except L. princei from tropical East Africa, are represented in our sampling. Most of the remainder of the species come from North and Central America. These were selected to represent a broad range of the morphological, biological, and ecogeographical diversity present in these regions, based on the major groups and complexes reported by Dunn (1984). Few samples were available from South America, despite numerous attempts to obtain them. Only two annual species occurring in the Western part of South America (Andean regions) were obtained: L. mutabilis and L. microcarpus. Fortunately, despite the small number of samples (three) available, the major groups of lupines recognized in the east-central part of South America (Dunn, 1984; Planchuelo Ravelo, 1984; Planchuelo and Dunn, 1984; Monteiro and Gibbs, 1986) are relatively well represented in our sampling: L. bracteolaris (annual and compound leaved); L. multiflorus (perennial and compound leaved); and L. paraguariensis (perennial with combined simple and compound leaves).

Based on previous systematic studies (Bisby, 1981; Polhill, 1981) and on recent cpDNA data from Papilionoideae including Genisteae and Lupinus (Badr, Martin, and Jensen, 1994; Käss and Wink, 1995), five additional taxa were sequenced to represent the outgroup in this study including: Chamaecytisus mollis, Genista tinctoria, and Ulex parviflorus, which are members of the subtribe Genistinae (Genisteae); Crotalaria podocarpa (Crotalarieae), and Thermopsis rhombifolia (Thermopsideae), which belong to two more distant clades from the Genisteae in the $r b c L$ phylogeny of the Papilionoideae (Käss and Wink, 1995).

Most of the Old World lupine samples come from personal collections of natural populations from Algeria (North Africa). The other taxa were kindly provided by seed banks, Institutes of Agronomy, and botanical gardens, except for five New World taxa obtained directly from herbarium samples at ALTA. The ingroup and outgroup taxa here used are listed in Table 1 along with their place of origin and/or sources. All seed samples were grown and cultivated in the phytotron at the University of Alberta.

DNA isolation and PCR amplification of the ITS region-The procedure followed is that previously described in Ainouche and Bayer (1997). Total DNA was isolated from a single individual for each sam-
ple using a modified CTAB method (Doyle and Doyle, 1987), with $1 \%$ $\beta$-mercaptoethanol (instead of $0.2 \%$ ). The entire ITS region, comprising ITS1, 5.8S gene, and ITS2, was amplified via the polymerase chain reaction (PCR) using external "ITS1" and "ITS4" primers designed by White et al. (1990) and Taq DNA polymerase. The PCR mixtures were preheated to $94^{\circ} \mathrm{C}$ for 2 min prior to the addition of Taq DNA polymerase to denature proteases and nucleases. The PCR amplification was then performed via 30 cycles of 1 min of denaturation at $94^{\circ} \mathrm{C}, 1$ $\min$ at $48^{\circ} \mathrm{C}$ for primer annealing, and 2 min of extension at $72^{\circ} \mathrm{C}$ for each cycle. A $7-\mathrm{min}$ final extension at $72^{\circ} \mathrm{C}$ followed cycle 30 . The double-stranded PCR products were purified by differential filtration using Millipore Ultra-free-MC tubes ( 30000 NMWL filters) prior to sequencing.

DNA sequencing-The purified double-stranded DNA was directly sequenced by dideoxy chain termination and cycle sequencing (Sanger, Nicklen, and Coulson, 1977), using the fmol DNA Sequencing System of Promega (Madison, Wisconsin) with ${ }^{32} \mathrm{P}$-labeled primers and following the protocol of the manufacturer. The internal "ITS2" and "ITS3" primers (from White et al., 1990) were used to sequence separately the ITS1 and ITS2 regions, respectively. For most of the taxa, the external "ITS1" and "ITS4" primers were also used to sequence the ITS regions and the 5.8 S region. After 2 min at $95^{\circ} \mathrm{C}$, the sequencing mixtures were subjected to 30 cycles of 1 min at $95^{\circ} \mathrm{C}, 1 \mathrm{~min}$ at $58^{\circ} \mathrm{C}$ and 1 min at $72^{\circ} \mathrm{C}$ each one, followed by a last cycle of 3 min at $94^{\circ} \mathrm{C}$. The DNA fragments obtained were separated in $6.0 \%$ acrylamide- $8 \mathrm{~mol} / \mathrm{L}$ urea gels ( 0.4 mm thickness; $1 \times$ TBE buffer) at high voltage. The gels were fixed in $10 \%$ acetic acid for 20 min , washed with distilled water, and air dried. They were then used to expose Kodak Biomax-MR films.

Sequence alignment and analysis-The ITS region boundaries were determined by comparison with various published sequences available in GenBank. The DNA sequences were entered and aligned manually using MacClade 3.0 (Maddison and Maddison, 1992). The alignment was straightforward among the lupine taxa and required the introduction of a few small and unambiguous insertion/deletion events (indels), five of 1 bp and two of 2 bp . The inclusion of the outgroup taxa in the data matrix with Lupinus necessitated inference of relatively few additional indels to adjust the overall alignment in both ITS1 and ITS2 regions: five of 1 bp , three of 2 bp , and two of 4 bp .

Sequence length and base composition were calculated using Amplify 1.2 (Engels, 1993). The number of variable characters (potentially informative and autapomorphous characters) and the proportions of nucleotide differences (pairwise sequence divergence) among the taxa were determined for the ITS regions (ITS1, ITS2, and the combined ITS1 + ITS2 regions) employing the options "show data matrix" and "show distance matrix" of PAUP 3.1.1 (Swofford, 1993). The 5.8S cistron was also sequenced in all the outgroup taxa and some Lupinus species. All the sequences reported here have been deposited in the GenBank database under the accession numbers given in Table 1.

Phylogenetic analysis-Sequence data were analyzed using PAUP 3.1.1 (Swofford, 1993). The analyses were performed on a reduced data set where the groups of taxa exhibiting the same ITS sequence were each represented by no more than two taxa to show the exact number of synapomorphies supporting each of these groups. The others were later added in the resulting trees. Only the extratribal taxa (outside of the tribe Genisteae) Thermopsis rhombifolia and Crotalaria podocarpa were used as outgroups in the analyses. The extrasubtribal taxa (outside of the Lupininae), Ulex parviflorus, Genista tinctoria, and Chamaecytisus mollis, were treated as ingroup taxa in order to examine their position relative to Lupinus. Additionally, the outgroup was rooted "at an internal node with basal polytomy" to assess the monophyly of Lu pinus relative to all the extra-lupine taxa prior to rooting the phylogenetic trees using the option "make ingroup monophyletic and the outgroup paraphyletic with respect to the ingroup." Phylogenetically un-
informative characters (invariant and strictly autapomorphic sites) were excluded from all analyses.

Given the number of taxa included in this study, the maximum parsimony analyses were performed by heuristic searches using Fitch parsimony. Characters and character states were weighted equally. The "Tree-Bisection-Reconnection" (TBR) branch swapping option in conjunction with saving all minimal trees (MULPARS) and accelerated transformation (ACCTRAN) were used to search for the shortest topologies. Branches of zero length were collapsed. Three different regimes of stepwise addition sequences were employed: SIMPLE, CLOSEST, and RANDOM (100 replicates). The last strategy (RANDOM) was conducted to search for all possible undiscovered islands of most parsimonious trees (Maddison, 1991). These methods were performed on three different versions of the data sets in order to explore different treatments of gaps: (search 1) positions containing gaps were excluded; (search 2) indels were coded following the strategy suggested by Barriel (1994) to express the potential phylogenetic information contained in insertion/deletion zones; this required the conversion of indels ( $28 \mathrm{nu}-$ cleotide sites) into 25 multistate characters in the data matrix; (search 3) gaps were treated as missing data and any phylogenetically informative base substitutions at the locus were included in the analysis.

For each analysis, the strict consensus and $50 \%$ majority-rule consensus trees showing other compatible groups were generated (Margush and McMorris, 1981). Bootstrap (Felsenstein, 1985) and decay (Bremer, 1988; Donoghue et al., 1992) methods were used in order to examine the robustness of the various clades revealed in the consensus tree clades. Bootstrap values (B.V.) were estimated from 100 replicates of heuristic searches using only SIMPLE addition sequence with TBR swapping, MULPARS, and ACCTRAN options in effect. Decay analyses were performed using a converse constraint (ENFORCE CONVERSE command) method described in Baum, Systma, and Hoch (1994). In this procedure, multiple heuristic TBR searches using a random addition sequence of 100 replicates (MULPARS not in effect) were constrained to assess the strength of each clade (Decay index value $=$ D.I.).

The relative rate test (Sarich and Wilson, 1973) was used to examine the apparent heterogeneity of the ITS sequence divergence rates in Lu pinus, i.e., to test whether the rates of nucleotide substitutions are the same in two different taxa or lineages. Given the polytomy obtained at the base of the genus (Fig. 1), Chamaecytisus mollis, the closest outgroup to Lupinus, was used as the reference taxon in substitution rate comparisons between taxa. Sequence divergences for the tests were calculated only from substitutions using the method of Jukes and Cantor (1969), and relative rate test values were estimated following the procedure of Wu and Li (1985) and Li and Tanimura (1987).

## RESULTS

Characteristics of the ITS region in Lupinus-The main characteristics of the ITS region in Lupinus and outgroup taxa are summarized in Table 2 (distance matrix not shown). Length variation for the entire ITS region (including 5.8S cistron) ranged from 624 to 629 bp . The length of the combined ITS1 and ITS2 region in the lupine taxa surveyed ranged from 461 to 466 bp and the $\mathrm{G}+\mathrm{C}$ content varied from 58.9 to $62.1 \%$. The ITS1 region (234-238 bp) was slightly longer than ITS2 (227229 bp ). Of the 481 aligned positions, 28 sites involved gaps: 18 (or $7.3 \%$ ) in ITS1 and ten (or $4.2 \%$ ) in ITS2. Forty-five sites (from 100 variables sites) were potentially informative in ITS1, while 41 were recorded (from 85 variables sites) in ITS2. Within Lupinus, 25 and 17 sites were potentially informative in ITS1 and ITS2, respectively. The proportion of nucleotide differences between pairs of species of Lupinus for ITS1 + ITS2 ranged from

0 to $6.5 \%$. In fact, several samples surveyed in this work were found to have the same ITS sequence (see Fig. 1). With respect to ITS1, the pairwise sequence divergence varied from 0 to $8.1 \%$ in Lupinus; it ranged from 0 to $5.7 \%$ in ITS2. The ITS divergence values (including both ITS1 and ITS2) from pairwise comparisons between Lu pinus species and the extra-lupine taxa are: 6.5-11.3\% relative to the Genistinae, $12.9-14.8 \%$ relative to Crotalaria, and $14.5-16.3 \%$ relative to Thermopsis.

The 5.8 S cistron was also sequenced in 30 taxa: 25 from Lupinus and five from the outgroup (Table 1). The 5.8 S sequence was identical in size ( 163 bp ) for all these taxa and no gaps were involved to align the sequences. Compared to the ITS regions, the $\mathrm{G}+\mathrm{C}$ content was lower in the 5.8S gene (53.2\%). Among the seven variable nucleotide sites detected in the 5.8 S cistron within Lupinus, four were synapomorphic sites, and sequence divergence between pairs of species ranged from 0 to $3.1 \%$. The proportion of nucleotide differences between pairs of species increased to $4.3 \%$ when the outgroup taxa were taken into account.

Phylogenetic analysis-Only the potentially informative characters from the ITS1 and ITS2 regions were used in the analyses, since the 5.8 S sequence data were not available for all the taxa surveyed; the four informative sites found in that region only reinforced the monophyly of two clades already well supported based upon ITS data alone (see Fig. 2). When indels were removed (search 1), the phylogenetic analysis generated 1512 equally most parsimonious trees of 180 steps with a consistency index (CI) of 0.639 and a retention index (RI) of 0.758 . Search 2 , where gaps were converted into new multistate characters, resulted in 8514 most parsimonious trees of 218 steps with $\mathrm{CI}=0.633$ and $\mathrm{RI}=0.751$, while 13662 trees (205 steps; CI $=0.634 ; \mathrm{RI}=0.742$ ) were obtained with gaps scored as missing data (search 3). A consensus tree of the same topology emerged from the three heuristic searches performed under different gap treatments and regimes of stepwise addition sequences (Fig. 1). The 50\% majority-rule consensus trees resulting from these searches were very similar in their overall topology. They differed slightly from one another in the support of some clades, particularly in search 2 when gaps were coded (see below and Fig. 2). Some western New World lupines (Fig. 2, clade E), including L. concinnus, L. arcticus, and L. lepidus, varied in their relative, but poorly resolved, position (not shown). One of the shortest trees with a topology identical to that of the majority-rule tree (search 3) is shown (Fig. 2). The phylogenetic trees (Figs. 1, 2) supported the monophyly of the Genisteae ( 12 synapomorphies (SYN); D.I. = 8; B.V. $=97 \%$ ), including Lu pinus, and their sister relationship to Crotalaria $(\mathrm{SYN}=$ 9). According to Hillis and Bull (1993), the branches having $\sim 70 \%$ or more of bootstrap value are well supported. Thermopsis is placed as sister to the Crotalaria-Genisteae clade, whereas Lupinus appears as a well-supported monophyletic group (SYN $=15$; D.I. $=10$; B.V. $=$ $100 \%$ ) that is clearly separated from the Genistinae (Ulex, Genista, and Chamaecytisus). Within the Genisteae, Lupinus appears more closely related to the Genista-Chamaecytisus group than to Ulex.

At the intrageneric level, the parsimony analyses re-


| Taxon | Origin | Distribution ${ }^{\text {a }}$ | $\begin{gathered} \text { Life } \\ \text { history } \\ \text { trait } / 2 n^{\mathrm{b}} \end{gathered}$ | Source ${ }^{\text {/ Accession }}$ number ${ }^{\text {d }}$ | nrDNA GenBank accession number |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  |  |  |  | ITS 1 | ITS2 | ITS1 + 5.8S + ITS2 |
| Old World lupine taxa |  |  |  |  |  |  |  |
| Lupinus albus L. | Algeria | OW, Med | A/50 | INAE-DZ/M11 |  |  | GBANAF007481 |
| L. albus var. graecus Boiss. \& Sprun. | Greece | OW, Med | A/50 | INRAL-Fr/M12 | GBANAF007450 | GBANAF007451 |  |
| L. angustifolius L. | Algeria | OW, Med | A/40 | A.-K. A./M1 |  |  | GBANAF007477 |
| L. angustifolius L. subsp. reticulatus Desv. | Algeria | OW, Med | A/40 | A.-K. A./M2 | GBANAF007448 | GBANAF007449 |  |
| L. atlanticus Gladst. | Morocco | OW, Afr-Med | A/38 | USDA/384612 | GBANAF007432 | GBANAF007433 |  |
| L. cosentinii Guss. | ? | OW, Med | A/32 | INRAL-Fr/A16 | GBANAF007464 | GBANAF007465 |  |
| L. digitatus Forsk. | Egypt | OW, Afr-Med | A/36 | WADA/PI 26877 | GBANAF007430 | GBANAF007431 |  |
| L. hispanicus Boiss. \& Reuter | Portugal | OW, Med | A/52 | USDA/384555 | GBANAF007466 | GBANAF007467 |  |
| L. luteus L. | Algeria | OW, Med | A/52 | A.-K. A./M5 |  |  | GBANAF007478 |
| L. micranthus Guss. | Algeria | OW, Med | A/52 | A.-K. A./M8 |  |  | GBANAF007480 |
| L. palaestinus Boiss. | Middle-East | OW, Med-Afr | A/42 | INRAL-Fr/A15 |  |  | GBANAF007479 |
| L. pilosus Murr. | ? | OW, Med-Afr | A/42 | INAE-DZ./A13 | GBANAF007434 | GBANAF007435 |  |
| New World lupine taxa |  |  |  |  |  |  |  |
| L. affinis J. Agardh | Oregon, USA | NW, N.A. | A/48 | USDA/504315 |  |  | GBANAF007487 |
| L. albifrons Benth. | California, USA | NW, N.A. | $\mathrm{P} /$ ? | USDA/284707 | GBANAF007454 | GBANAF007455 |  |
| L. arcticus Wats. | Yukon, Canada | NW, N.A. | P/48 | Hb, ALTA/95826 |  |  | GBANAF007495 |
| L. argenteus Pursh | Washington, USA | NW, N.A. | P/48 | USDA/504374 | GBANAF007458 | GBANAF007459 |  |
| L. aridus Dougl. ex Lindl. | Oregon, USA | NW, N.A. | P/48 | USDA/504435 | GBANAF007446 | GBANAF007447 |  |
| L. arizonicus Wats. | USA | NW, N.A. | A/48 | USDA/577285 |  |  | GBANAF007483 |
| L. bracteolaris Desr. | Brazil | NW, S.A. | A/? | USDA/404349 |  |  | GBANAF007473 |
| L. concinnus J. Agardh | California, USA | NW, N.A. | A/48 | USDA/284715 | GBANAF007438 | GBANAF007439 |  |
| L. duranii Eastwood | California, USA | NW, N.A. | $\mathrm{P} /$ ? | Hb. ALTA/92238 |  |  | GBANAF007493 |
| L. elegans H.B.K. | Mexico F.D. | NW, C.A. | P/48 | USDA/185099 | GBANAF007462 | GBANAF007463 |  |
| L. excubitus M.E. Jones | California, USA | NW, N.A. \& C.A. | $\mathrm{P} /$ ? | Hb. ALTA/95550 |  |  | GBANAF007492 |
| L. hirsutissimus Benth. | California, USA | NW, N.A. | A/48 | Hb. ALTA/60637 |  |  | GBANAF007486 |
| L. latifolia J. Agardh | California, USA | NW, N.A. | $\mathrm{P} /$ ? | USDA/284720 | GBANAF007456 | GBANAF007457 |  |
| L. lepidus Dougl. ex Lindl. | Wyoming, USA | NW, N.A. | P/48 | Hb. ALTA/94855 |  |  | GBANAF007485 |
| L. leucophyllus Dougl. ex Lindl. | Oregon, USA | NW, N.A. | P/48 | USDA/504316 | GBANAF007442 | GBANAF007443 |  |
| L. littoralis Dougl. | Washington, USA | NW, N.A. | P/48 | USDA/504401 | GBANAF007452 | GBANAF007453 |  |
| L. luteolus Kellogg | California, USA | NW, N.A. | A/? | USDA/284721 |  |  | GBANAF007490 |
| L. mexicanus Cerv. ex Lag. | Mexico F.D. | NW, C.A. | P/48 | USDA/14748 | GBANAF007444 | GBANAF007445 |  |
| L. microcarpus Sims. var. densiflorus (Benth.) Jepson | USA | NW, N.A. \& S.A. | A/48 | USDA/15617 |  |  | GBANAF007489 |
| L. microcarpus Sims var. microcarpus | Maryland, USA | NW, N.A. \&S.A. | A/48 | USDA/241271 |  |  | GBANAF007488 |
| L. minimus Dougl. | Oregon, USA | NW, N.A. | $\mathrm{P} /$ ? | USDA/504439 |  |  | GBANAF007497 |
| L. multiflorus Desr. | Brazil | NW, S.A. | $\mathrm{P} /$ ? | USDA/508613 |  |  | GBANAF007475 |
| L. mutabilis Sweet | Peru | NW, S.A. | A/48 | INAE-DZ/S35 |  |  | GBANAF007484 |
| L. nanus Benth. | California, USA | NW, N.A. | A/48 | USDA/284729 | GBANAF007440 | GBANAF007441 |  |
| L. paraguariensis Chodat \& Hassler | S.C.-Brazil | NW, S.A. | $\mathrm{P} /$ ? | CENARGEN/BRA-02828 |  |  | GBANAF007476 |
| L. polyphyllus Lindl. | Washington, USA | NW, N.A. | P/48 | USDA/504404 |  |  | GBANAF007496 |
| L. pusillus Pursh | Oregon, USA | NW, N.A. | A/48 | USDA/504356 |  |  | GBANAF007491 |
| L. sericeus Pursh | Utah, USA | NW, N.A. | P/48 | USDA/356830 | GBANAF007436 | GBANAF007437 |  |
| L. sparsiflorus Benth. | Arizona, USA | NW, N.A. | A/48 | USDA/577289 |  |  | GBANAF007482 |
| L. succulentus Koch | California, USA | NW, N.A. | A/48 | USDA/284728 |  |  | GBANAF007494 |
| L. sulphureus Dougl. ex Hook | Washington, USA | NW, N.A. | P/48 | USDA/504367 | GBANAF007460 | GBANAF007461 |  |

Table 1. Continued.

| Taxon | Origin |  | $\begin{gathered} \text { Life } \\ \text { history } \\ \text { trait } / 2 n^{\mathrm{b}} \end{gathered}$ | Source ${ }^{\text {c/Accession }}$ number ${ }^{\text {d }}$ | nrDNA GenBank accession number |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  | Distribution ${ }^{\text {a }}$ |  |  | ITS1 | ITS2 | ITS1 + 5.8S + ITS2 |
| L. texensis | Texas, USA | NW, N.A. | A/36 | USDA/577291 |  |  | GBANAF007474 |
| Outgroup taxa |  |  |  |  |  |  |  |
| Chamaecytisus mollis (Cav.) Greuter \& Burdet | ? | OW | P/ | RBG-Kew/84327 |  |  | GBANAF007472 |
| Crotalaria podocarpa D.C. | ? | OW | A/16 | RBG-Kew/90928 |  |  | GBANAF007469 |
| Genista tinctoria L. | ? | OW | P/48 | RBG-Kew/51334 |  |  | GBANAF007471 |
| Thermopsis rhombifolia (Nutt.) Richards var. ovata | Idaho, USA | NW | P/18 | USDA/10173 |  |  | GBANAF007468 |
| Ulex parviflorus Pourret | France | OW | P/32 | LB-UR-Fr/G53 |  |  | GBANAF007470 |
| ${ }^{\text {a }}$ Geographic distribution: Old World (OW); Mediterranean (Med); African (Afr); New World (NW); North America (N.A.); Central America (C.A.) and South America (S.A <br> ${ }^{\mathrm{b}} \mathrm{A}=$ annual, $\mathrm{P}=$ perennial; Chromosome numbers (2n) are from Dunn and Gillet (1966), Fedorov (1974), Gladstones (1984), and Dunn (1984). <br> ${ }^{\text {c }}$ Sources: Abdel-Kader Aïnouche (A.-K. A.); U.S. Department of Agriculture, Plant Introduction, Pullman, Washington (USDA); Institut National d'Agronomie d'El-Har |  |  |  |  |  |  |  |
| Algérie (INAE-DZ); Institut National de Recherche Agronomique, Lusignan-France (INRAL-Fr); Western Australian Department of Agriculture (WADA); Herbarium of of Alberta, Edmonton, ALTA, Canada (Hb. ALTA); Centro Nacional de Recursos Geneticos Biotecnologia, Brasilia-DF-Brasil (CENARGEN); Royal Botanic Gardens, Kew, |  |  |  |  |  |  |  |
| Kew); Laboratoire de Botanique U <br> ${ }^{\text {d }}$ Voucher specimens are availab Edmonton, Canada). | té de Rennes | (LB-UR-Fr). <br> tany of the Un | of | nnes (France), except |  | $\mathrm{mm}$ | iversity of Alberta, |

sulted in a largely unresolved polytomy at the base of the genus. Nevertheless, the lupine taxa are always resolved into five distinct clades in the strict consensus trees (Fig. 1). The Old World lupines are distributed into three different groups. The rough-seeded species are all members of the same strongly supported monophyletic group (clade $\mathrm{C} ; \mathrm{SYN}=10 ;$ D.I. $=8 ; \mathrm{B} . \mathrm{V} .=100 \%$ ). This group is supported by one additional synapomorphy (added in Fig. 2) and a decay value of 9 when indels are taken into account. Three small subclades appear within this group in the strict consensus tree: L. pilosus and L. palaestinus, which exhibit the same ITS sequence and form a moderately supported subgroup ( $\mathrm{SYN}=2$; D.I. $=2$; B.V. $=$ 85\%); L. atlanticus and L. digitatus, which are related by only one synapomorphy (D.I. $=1$; B.V. $=58 \%$ ) and differ by two nucleotide changes; and L. cosentinii. The smooth-seeded species are separated into two clades: one (clade B) is relatively well resolved (SYN = 3; D.I. = $2 ; \mathrm{B} . \mathrm{V} .=73 \%$ ) and contains four taxa, which are morphologically and cytologically well differentiated: L. angustifolius, L. angustifolius subsp. reticulatus, L. luteus, and $L$. hispanicus. The first two are sister group to the latter two. Clade D has less support (SYN = 1; D.I. = 1 ; B.V. $=49 \%$ ) and is composed of L. albus, L. albus var. graecus, and L. micranthus.
The New World taxa form two clades, A and E (Fig. 1). Clade A is strictly composed of lupines originating from east-central parts of South America (i.e., L. bracteolaris, and the sister taxa L. paraguariensis and L. multiflorus), and from southeastern United States (L. texensis). Six synapomorphies, a decay value of 4, and a bootstrap confidence of $87 \%$ support this clade. The remaining 28 New World taxa are in clade E (Fig. 2: SYN = $2 ;$ D.I. $=1$; B.V. $=33$ ), and they all originate in the western parts of the Americas. These taxa all also share a single base-pair insertion in the ITS1 region (added in Fig. 2) providing additional support to clade $E$ in search 2 (SYN = 3; D.I. = 2). Within this clade, only three monophyletic subgroups with moderate to strong support may be distinguished. One is composed of the sister taxa L. arizonicus and L. sparsiflorus (SYN = 3; D.I. $=2$; B.V. $=77 \%$ ); the relative position of these two taxa as sister group to the rest of the western New World species is weakly supported. Another subgroup contains L. mexicanus and L. elegans, which are united by two synapomorphies (D.I. $=2 ; \mathrm{B} . \mathrm{V} .=66 \%$ ) and differ from one another by two autapomorphic changes (shown in Fig. 2 ). The third and most strongly supported clade (SYN = 9; D.I. $=9$; B.V. $=100 \%$; Fig. 2) is composed of five annual taxa, including $L$. microcarpus, with identical ITS sequences. This subgroup is in fact more strongly supported by three additional phylogenetically informative characters if we consider one synapomorphic single basepair insertion in the ITS1 region and two synapomorphic mutations in the 5.8S cistron (indicated in Fig. 2). Sequence divergence among the remaining western New World taxa are generally low, resulting in a lack of resolution of phylogenetic relationships and no support for the subclades observed in Fig. 2, several of which contain taxa with identical ITS sequences (groups 1 to 4). Nevertheless, it may be pointed out that L. hirsutissimus is always positioned as sister to the L. microcarpus group


Fig. 1. The strict consensus tree of 36 species of Lupinus and five extra-lupine taxa based on the combined sequence data of ITS1 and ITS2. This topology emerged from all the heuristic searches performed under different gap treatments (indel events removed, coded as new multistate characters, or coded as missing) and regimes of stepwise addition sequences (SIMPLE, CLOSEST, and 100 replicates RANDOM). The dashed branches represent taxa that were not taken into account in the phylogenetic analyses and later mapped on the cladogram. The dashed boxes contain the taxa that have identical ITS sequences. In the western New World lupine clade, the taxa originating from Central or South America are distinguished from the North American ones by double brackets.
in the strict consensus trees (searches 1 to 3 ), and that $L$. concinnus is a strongly differentiated species.

ITS sequence variation and rate heterogeneity-Sequence divergence comparison among the main clades shows that the eastern New World lupine clade (A) has the highest average sequence divergence level of ITS ( $12.17 \%$ to the outgroup; 4.44-5.56\% to lupin clades) and that the western New World clade (E) displays the lowest values ( $10.96 \%$ to the outgroup; $3.08-4.44 \%$ to lupin clades). The Old World clades exhibit intermediate
values. The average sequence divergence is higher within the eastern New World clade (3.45\%) than within the western New World one ( $1.34 \%$ ), and higher than within any of the other clades. However, comparisons among only annual taxa or only perennial ones show unequal patterns of sequence divergence within each New World clade (Table 3; Fig. 2): $3.44 \%$ for annuals and $2.58 \%$ for perennials (a ratio of 1.33 to 1 ) in the eastern New World clade (A), and 2.3 and $0.44 \%$ (a ratio of 5.23 to 1 ), respectively, in the western New World one (E). Sequence divergence also varies within the Old World clades, ex-

Table 2. Characteristics and variation of the ITS nrDNA region in Lupinus.

| Taxa/sequence characteristics | nrDNA region |  |  |  |
| :---: | :---: | :---: | :---: | :---: |
|  | ITS 1 | ITS 2 | ITS $1+$ ITS 2 | 5.8S |
| Within Lupinus |  |  |  |  |
| Length range (bp) | 234-238 | 227-229 | 461-466 | 163 |
| \% of G + C content (mean) | 60.68 | 60.49 | 60.59 | 53.24 |
| Invariant characters | 191 | 198 | 389 | 156 |
| Variable characters | 55 | 37 | 92 | 7 |
| Potentially informative characters | 25 | 17 | 42 | 4 |
| Proportion (\%) of nucleotide differences between pairs of species |  |  |  |  |
| within Lupinus | 0-8.05 | 0-5.7 | 0-6.5 | 0-3.1 |
| between Lupinus and outgroups | 5.5-16.3 | 6.5-18 | 6.5-16.3 | 0.6-4.3 |
| between Lupinus and Genistinae | 5.5-12.1 | 6.5-10.9 | 6.5-11.3 | 0.6-3.1 |
| between Lupinus and Crotalaria | 12.4-15.9 | 12.5-15.1 | 12.9-14.8 | 2.5-3.7 |
| between Lupinus and Thermopsis | 12.8-16.3 | 15.4-18.0 | 14.5-16.3 | 2.5-3.7 |

clusively comprised of annual taxa: $0.56 \%$ within the rough-seeded lupin clade (C), 1.86 and $3.30 \%$ within the smooth-seeded lupin clades B and D, respectively.

Pairwise comparison of the ITS nucleotide substitution rates using the relative rate test involved a representative subset of 17 annual and perennial lupin taxa (Table 4). Among the 136 tests performed, significant differences (at the 5 and $1 \%$ levels) were found in 28 pairwise comparisons indicating the presence of unequal evolutionary rates of ITS regions in Lupinus. No significant rate differences were observed between annual and perennial taxa in the eastern New World clade (A). In contrast, in the western New World clade, the highly homogeneous (in ITS substitution rate) perennial species showed significantly slower rates in comparison to several annual taxa (L. microcarpus species group, L. concinnus). Among the annual Old World taxa, the rate differences in general were not significant (eight of ten comparisons). However, the ITS sequences appear to have evolved significantly faster in L. micranthus than in L. albus (with a significance close to the $1 \%$ level) or L. angustifolius (at a level of 5\%).

## DISCUSSION

Outgroup relationships of Lupinus-The phylogenetic relationship of the genus Lupinus relative to the outgroup taxa is well resolved using ITS sequences. Its relationship to the Genisteae has often been discussed (Hutchinson, 1964; Plitmann, 1981; Polhill, 1981; Dunn, 1984; Saint-Martin, 1986; Badr, Martin, and Jensen, 1994). Relative to the extra-lupine taxa used in this study, the genus Lupinus is a well-supported monophyletic group that is unambiguously part of the Genisteae. Crotalaria is placed as sister to the Genisteae, while Thermopsis is more distantly related to Lupinus and sister to the Crotalaria-Genisteae group; this was confirmed when other more distant outgroups were introduced in the analyses, such as Sophora arizonica S. Wats., Caragana arborescens Lam., and Vicia americana Muhl. ex. Willd. (results not shown). Thus, ITS sequence data strongly support the views of Polhill (1976) and Bisby (1981), which include Lupinus in the tribe Genisteae (Adanson) Bentham, but as a distinct lineage (subtribe Lupininae (Hutch.) Bisby) from the Genistinae ("Cytisus-Genista
complex"). These results are highly congruent with the pattern of relationships indicated by both the serological data (Cristofolini and Feoli Chiapella, 1977; Cristofolini, 1989) and the recent molecular-based phylogenies of the Papilionoideae, including Lupinus (Doyle, 1995; Käss and Wink, 1995, 1997a, b; Doyle et al., 1997). Structural characteristics of the chloroplast genome and nucleotide sequence data from both $r b c L$ and the ITS-nrDNA regions are incompatible with the hypothesis of an independent origin of Lupinus from the rest of the Genisteae (Plitmann, 1981; Plitmann and Pazy, 1984; Badr, Martin, and Jensen, 1994). Moreover, the ITS results do not reveal a closer relationship of Lupinus to Crotalaria than to Genisteae, as previously suggested by Dunn (1984) and Gross (1986).

Phylogenetic relationships within Lupinus-The ITS sequence data were useful to resolve some relationships at lower taxonomic levels within the genus. The lupines investigated are distributed into five main clades, each of them strictly belonging exclusively to either the Old or New World (Figs. 1, 2).

Old World lupines-The rough-seeded lupines represent the most strongly supported clade in the Old World based on ITS sequences (clade C; Figs. 1, 2). The remarkable morphological homogeneity of this group was already demonstrated with respect to various sources of data: seed coat texture (Heyn and Herrnstadt, 1977); alkaloids .(Nowacki, 1963; Wink, Meibner, and Witte, 1995; Aïnouche et al., 1996); flavonoids (Williams, Demissie, and Harborne, 1983); seed storage proteins (Przybylska and Zimniak-Przybylska, 1995); protein serology (Cristofolini, 1989); and isozymes (Wolko and Weeden, 1990a). The proposition to recognize the rough-seeded species as a separate section Scabrispermae Plitm. \& Heyn (Plitmann and Heyn, 1984) is strongly reinforced by our nrDNA evidence and that of Käss and Wink (1997b).

Although they are differentiated by only a few nucleotide differences, the small subclades appearing within the rough-seeded group are consistent with the available geographical, cytological, and crossability data. Indeed, L. pilosus $(2 n=42)$ and L. palaestinus $(2 n=42)$, which exhibit the same ITS sequence, are able to artificially

Lupinus phylogeny based on ITS nrDNA sequences


Fig. 2. One of the 13662 equally parsimonious trees that is topologically identical to the $50 \%$ majority-rule consensus tree of 41 lupine and extra-lupine taxa, based on phylogenetic analysis of ITS sequence data (heuristic search, SIMPLE, gaps = missing, uninformative characters ignored; tree length $=205, \mathrm{CI}=0.634, \mathrm{RI}=0.742$ ). The numbers of character steps are presented above the branches, whereas decay indices and bootstrap confidence values ( 100 replicates) are given in bold face italic numbers below the branches (D.I.-B.V.\%). Three different informative insertions (f, $\mathrm{g}, \mathrm{h}$ ) from the ITS regions and four synapomorphies ( $\mathrm{m}, \mathrm{n}, \mathrm{p}, \mathrm{q}$ ) from the 5.8 S cistron are superimposed on the cladogram. A solid triangle corresponds to the insertion of a single base pair and a vertical bar to a synapomorphic character change. The dashed branches represent taxa that were not taken into account in the phylogenetic analysis and later mapped in the cladogram, each near the taxa that have the same ITS sequences. Annual species are indicated by open circles and perennial ones by solid circles. Chromosome numbers follow taxon names; open squares represent species in the aneuploid series, while solid squares correspond to taxa in the euploid series.

Table 3. Average percentage sequence divergence of ITS among and within the main Lupinus clades (A-E) appearing in the ITS phylogeny (Figs. $1,2) . n=$ number of taxa in each clade; ${ }^{\mathrm{a}}=$ annual taxa only; $\mathrm{p}=$ perennial taxa only.

|  | Outgroup | A | в | c | D | E | $\mathrm{A}^{\text {a }}$ | $\mathrm{A}^{\text {P }}$ | $\mathrm{E}^{\text {a }}$ | $\mathrm{E}^{p}$ |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Outgroup ( $n=5$ ) | 13.95 |  |  |  |  |  |  |  |  |  |
| A ( $n=4$ ) | 12.17 | 3.45 |  |  |  |  |  |  |  |  |
| B $(n=4)$ | 11.34 | 4.79 | 1.87 |  |  |  |  |  |  |  |
| C ( $n=5$ ) | 11.89 | 5.54 | 4.19 | 0.57 |  |  |  |  |  |  |
| D ( $n=2$ ) | 11.06 | 4.67 | 3.61 | 4.23 | 3.30 |  |  |  |  |  |
| $\mathrm{E}(n=27)$ | 10.96 | 4.40 | 2.88 | 3.75 | 3.15 | 1.34 |  |  |  |  |
| $\mathrm{A}^{\mathrm{a}}(\mathrm{n}=2)$ | - | - | - | - | - | - | 3.44 |  |  |  |
| $\mathrm{A}^{\mathrm{p}}(n=2)$ | - | - | - | - | - | - | - | 2.58 |  |  |
| $\mathrm{E}^{\mathrm{a}}(\mathrm{n}=12)$ | - | - | - | - | - | - | - | - | 2.30 |  |
| $\mathrm{E}^{\mathrm{p}}(n=16)$ | - | - | - | - | - | - | - | - | - | 0.44 |

cross with success, despite the existence in nature of reproductive barriers, which justify their treatment as distinct species (Kazimierski, 1961; Gladstones, 1974; Plitmann, Heyn, and Pazy, 1980; Pazy, Plitmann, and Heyn, 1981). These eastern Mediterranean species, L. pilosus and L. palaestinus, were shown to be reproductively isolated from the other rough-seeded species (Roy and Gladstones, 1988; Carstairs, Buirchell, and Cowling, 1992), although genome similarities were found between L. pilosus and L. atlanticus (Gupta, Buirchell, and Cowling, 1996). Likewise, the species originating from the desert and arid regions of North Africa, L. atlanticus $(2 n=38)$ and $L$. digitatus $(2 n=36)$, are sister taxa in this ITS phylogeny. This is not in exact concordance whith the ITS sequence data of Käss and Wink (1997b) who found that these two taxa are slightly more distantly related. Nevertheless, it has been demonstrated that L. atlanticus and L. digitatus intercross successfully and have a greater homology of chromosomes than to any other rough-seeded species (Roy and Gladstones, 1988; Carstairs, Buirchell, and Cowling, 1992; Gupta, Buirchell, and Cowling, 1996). Restricted to the Mediterranean region, L. cosentinii $(2 n=32)$ has an identical ITS sequence to that of the hypothesized recent common ancestor of the roughseeded lupines. This is in agreement with ITS results of Käss and Wink (1997b), but not with their rbcL data where $L$. cosentinii appeared to accumulate relatively more mutations. The latter species is more closely related to L. atlanticus and L. digitatus than to L. pilosus and $L$. palaestinus with regard to chromosome numbers and interspecific crossing ability (Roy and Gladstones, 1988; Carstairs, Buirchell, and Cowling, 1992). Geographically restricted to the tropical-subtropical areas of Eastern Equatorial Africa and having $2 n=38$ chromosomes, $L$. princei Harms. (here not analyzed) is unambiguously a member of the rough-seeded lupin group, as confirmed from both $r b c L$ and ITS data by Käss and Wink (1997b). These authors found that L. princei has an ITS sequence identical to that of L. digitatus. The latter result contrasts with the cytogenetic and interspecific crossing ability data, which all show that these species are well differentiated from one another (Carstairs, Buirchell, and Cowling, 1992; Gupta, Buirchell, and Cowling, 1996). Moreover, L. princei was demonstrated as the most genetically isolated taxon within the rough-seeded lupines (Gupta, Buirchell, and Cowling, 1996). Finally, relationships among the rough-seeded lupin subgroups still re-
main unresolved based upon ITS data and need additional informative characters to be further elucidated.

The smooth-seeded lupines, all Mediterranean, are resolved into two distinct clades ( B and D ) in the strict consensus tree (Fig. 1). Within these clades, all the species and sections presently recognized (Gladstones, 1974, 1984) are morphologically well defined. This is largely in accordance with biochemical data from: alkaloids (Nowacki, 1963; Wink, Meibner, and Witte, 1995), leaf flavonoids (Williams, Demissie, and Harborne, 1983), seed proteins (Ainouche, 1988a, 1991; Salmanowicz and Przybylska, 1994; Przybylska and Zimniak-Przybylska, 1995), and isozymes (Wolko and Weeden, 1990b).

Within smooth-seeded sections, no ITS nucleotide differences were found between members of the same species. Lupinus albus var. graecus and L. angustifolius subsp. reticulatus, previously recognized at the species level (L. graecus Boiss. \& Sprun. and L. reticulatus Desv.), are now regarded as no more than forms, varieties, or subspecies of L. albus L. $(2 n=50)$ and L. angustifolius L. $(2 n=40)$, respectively (Gladstones, 1974, 1984; Amaral Franco and Pinto da Silva, 1978). Lupinus luteus and L. hispanicus (sect. Lutei), which both have $2 n=52$ chromosomes, are sister taxa and are well differentiated by seven nucleotide changes (shown in Fig. 2 ). Often growing sympatrically, these species are separated (though not completely) by reproductive barriers due to partial chromosome nonhomology (Kazimierski, 1982, 1988). Surprisingly, sections Lutei and Angustifoli are members of a monophyletic group (clade B, Fig. 1), although they are conspicuously different in morphology and cytology. This was also seen in the rbcL analysis of Käss and Wink (1997b). Such an unexpected relationship was, however, previously suspected when a "foveolate" seed coat pattern, different from the common L. angustifolius type, but similar to that of L. luteus, was found in some North African populations of L. angustifolius (Ainouche, 1988b, 1991). Instead, L. luteus was suggested as being closer to $L$. micranthus based on similar chromosome numbers and some morphological affinities (Gladstones, 1984), a relationship that is not corroborated by our ITS results nor by crossing data (Kazimierski, 1988). Lupinus luteus, represented on both sides of the Mediterranean, is the most derived species in clade B with respect to ITS sequence (Figs. 1, 2). Gladstones (1974) suggested the Iberian Peninsula as the place of

|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| 1. L. paraguariensis ${ }^{\text {c }}$ |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 2. L. multiflorus | $-1.69^{\text {ns }}$ |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 3. L. bracteolaris | $-1.39^{\text {ns }}$ | 0.00 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 4. L. texensis | $-0.46{ }^{\text {ns }}$ | -0.93 ns | $+1.00^{\text {ns }}$ |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 5. L. angustifolius | -2.52* | $-1.40{ }^{\text {ns }}$ | $-1.40^{\text {ns }}$ | -2.11* |  |  |  |  |  |  |  |  |  |  |  |  |
| 6. L. luteus | $-1.36{ }^{\text {ns }}$ | $-0.22^{\text {ns }}$ | $-0.21^{\text {ns }}$ | $-0.97{ }^{\text {ns }}$ | $+1.55^{\text {ns }}$ |  |  |  |  |  |  |  |  |  |  |  |
| 7. L. pilosus | $-1.50{ }^{\text {ns }}$ | $-0.42^{\text {ns }}$ | $-0.41^{\text {ns }}$ | $-1.13{ }^{\text {ns }}$ | $+0.99^{\text {ns }}$ | $-1.22^{\text {ns }}$ |  |  |  |  |  |  |  |  |  |  |
| 8. L. micranthus | $-0.55^{\text {ns }}$ | $+1.58{ }^{\text {ns }}$ | $+0.65{ }^{\text {ns }}$ | $-0.18{ }^{\text {ns }}$ | +1.99* | $+0.84{ }^{\text {ns }}$ | $+1.01{ }^{\text {ns }}$ |  |  |  |  |  |  |  |  |  |
| 9. L. albus | -2.79** | $-1.78{ }^{\text {ns }}$ | $-1.78{ }^{\text {ns }}$ | -2.37* | $-0.30^{\text {ns }}$ | $-1.70^{\text {ns }}$ | $-1.27^{\text {ns }}$ | -2.52* |  |  |  |  |  |  |  |  |
| 10. L. sparsiflorus | $-1.66^{\text {ns }}$ | $-1.68^{\text {ns }}$ | $-0.64{ }^{\text {ns }}$ | $-1.30^{\text {ns }}$ | $+1.02^{\text {ns }}$ | $-0.51{ }^{\text {ns }}$ | -0.21 ns | $-1.22^{\text {ns }}$ | $+1.09^{\text {ns }}$ |  |  |  |  |  |  |  |
| 11. L. concinnus | $-1.32^{\text {ns }}$ | $-0.20{ }^{\text {ns }}$ | $-0.20^{\text {ns }}$ | $-0.94{ }^{\text {ns }}$ | $+1.15^{\text {ns }}$ | $+0.02^{\text {ns }}$ | $+0.23{ }^{\text {ns }}$ | $-0.80{ }^{\text {ns }}$ | $+1.21^{\text {ns }}$ | $+0.48{ }^{\text {ns }}$ |  |  |  |  |  |  |
| 12. L. mutabilis | -2.52* | -1.40 ns | $-1.48^{\text {ns }}$ | $-2.11^{*}$ | 0.00 | $-1.42^{\text {ns }}$ | $-0.98{ }^{\text {ns }}$ | -2.09* | $+0.37{ }^{\text {ns }}$ | $-0.89^{\text {ns }}$ | $-1.65^{\text {ns }}$ |  |  |  |  |  |
| 13. L. affinis | $-0.73{ }^{\text {ns }}$ | $+0.39^{\text {ns }}$ | $+0.41{ }^{\text {ns }}$ | $-0.37 \mathrm{~ns}$ | $+1.81^{\text {ns }}$ | $+0.66{ }^{\text {ns }}$ | +0.83 ns | $-0.19^{\text {ns }}$ | +2.28* | $+1.20^{\text {ns }}$ | $+0.67^{\text {ns }}$ | +2.26* |  |  |  |  |
| 14. L. nanus | $-2.79 * *$ | $-1.68^{\text {ns }}$ | $-1.78^{\text {ns }}$ | -2.36* | $-0.30^{\text {ns }}$ | $-1.64{ }^{\text {ns }}$ | $-1.24{ }^{\text {ns }}$ | -2.26* | 0.00 | $-1.14{ }^{\text {ns }}$ | $-1.88{ }^{\text {ns }}$ | $+0.57 \mathrm{~ns}$ | -2.44* |  |  |  |
| 15. L. sericeus | -2.75** | $-1.68{ }^{\text {ns }}$ | $-1.78{ }^{\text {ns }}$ | -2.37* | $-0.30^{\text {ns }}$ | $-1.77{ }^{\text {ns }}$ | $-1.02^{\text {ns }}$ | -2.38* | 0.00 | $-1.24^{\text {ns }}$ | -2.10* | $-0.98{ }^{\text {ns }}$ | $-2.65 * *$ | 0.00 |  |  |
| 16. L. polyphyllus | -2.79** | $-1.68{ }^{\text {ns }}$ | $-1.78{ }^{\text {ns }}$ | -2.36* | $-0.30^{\text {ns }}$ | $-1.64{ }^{\text {ns }}$ | $-1.24{ }^{\text {ns }}$ | -2.26* | 0.00 | $-1.14{ }^{\text {ns }}$ | $-1.88{ }^{\text {ns }}$ | $+0.57^{\text {ns }}$ | -2.44* | 0.00 | 0.00 |  |
| 17. L. mexicanus | $-2.52^{* *}$ | $-1.40{ }^{\text {ns }}$ | $-1.48^{\text {ns }}$ | -2.11* | 0.00 | $-1.32^{\text {ns }}$ | $-0.96{ }^{\text {ns }}$ | -1.99* | $+0.33^{\text {ns }}$ | $-0.82^{\text {ns }}$ | $-1.50{ }^{\text {ns }}$ | 0.00 | -2.10* | $+0.57^{\text {ns }}$ | $+0.56^{\text {ns }}$ | $+0.57^{\text {ns }}$ |

${ }^{\text {a }}$ Relative rate test values are estimated following the procedure of Wu and Li (1985) and Li and Tanimura (1987).

origin of sect. Lutei, whereas that of sect. Angustifoli is somewhere in the Mediterranean.

The two other smooth-seeded sections, Albi $(2 n=50)$ and Micranthi $(2 n=52)$, are always placed as sister taxa in the strict consensus trees (clade D; Fig. 1). A relationship between L. micranthus and L. albus was conjectured by Gladstones (1974), and the Balkan Peninsula was suggested as the possible common center of origin of these taxa based on some morphological, ecogeographical, and chromosome number affinities. However, the alliance of these taxa results from only one synapomorphy and additional informative characters must be considered to assess the reliability of that relationship. Noteworthy is the large difference in number of nucleotide substitutions accumulated in the ITS sequences of $L$. micranthus and $L$. albus (Fig. 2; Table 3); the latter species shows a significantly slower ITS evolutionary rate (Table 4). The position of $L$. micranthus in relationship to other species has been the subject of debate in the literature; recent investigations based on flavonoids (Williams, Demissie, and Harborne, 1983), alkaloids (Ainouche, unpublished data), protein serology (Cristofolini, 1989), and isozymes (Wolko and Weeden, 1990a) suggested an intermediate position of this species between the smooth-seeded and the rough-seeded lupines of the Old World. However, further isozyme data suggested a close affinity with the smooth-seeded species (Wolko and Weeden, 1990b).

Although the Mediterranean and North African lupin clades always appear close to one another in the maximum parsimony trees and seem to form a paraphyletic grade (Fig. 2), relationships among them cannot be inferred with certainty from our ITS data due to insufficient resolution at the base of the genus. This was also the case with the rbcL and ITS analyses of Käss and Wink (1997b). However, the tree length increased by only one step more than the most parsimonious trees when the monophyly of the smooth-seeded lupines (clades B and C) was constrained. Finally, ITS data are highly congruent with Lupinus taxonomy in the Old World, and ITS sequence divergence among taxa correlates well with morphology and intercrossing data. The morphologically diverse and genetically well-differentiated smooth-seeded species display higher sequence divergence values than the morphologically homogeneous and genetically less differentiated rough-seeded lupines (Table 3).

New World lupines-One remarkable result emerging from this study is that the New World lupines are distributed into two distinct clades (A and E; Figs. 1, 2) in accordance with their geographic origin: the eastern New World lupines in clade A and the western New World ones in clade E.

The eastern New World lupines (clade A) represent a well-supported monophyletic group. Within this clade, the major groups presently recognized in the Atlantic subregion of South America (Planchuelo and Dunn, 1984; Planchuelo Ravelo, 1984; Monteiro, 1986) are here represented by three well-differentiated species: L. bracteolaris (annual; digitate leaves; $2 n=$ ?); L. multiflorus (perennial; digitate leaves; $2 n=48$ ); and L. paraguariensis (perennial with combined simple and compound leaves; $2 n=$ ?). The sister relationship between the two latter taxa (SYN $=2$; D.I. $=2 ;$ B.V. $=69 \%$ ), additionally
supported by two shared mutations in the 5.8 S cistron (see Fig. 2), demonstrate the close relationships between the digitate- and simple-leaved perennial lupine groups of the Atlantic South American subregion, an association previously suggested from morphological data (Planchuelo and Dunn, 1984). Two other digitate-leaved perennial lupines, native from the same subregion, and producing one unifoliolate first leaf above the cotyledons, $L$. albescens Hooker and Arnott. and L. aureonitens Gilles (Planchuelo and Dunn, 1984), were demonstrated as closely related to L. paraguariensis, based on both rbcL and ITS data (Käss and Wink, 1997b).The simple-leaved lupines growing in southeastern North America (unfortunately not available for our study) are interpreted by Dunn (1971) as a postglacial introduction from Brazil and would then be potentially related to this clade. A noteworthy feature of the ITS-based phylogenies (Fig. 2) is the inclusion of L. texensis (annual with digitate leaves and $2 n=36$, from Texas) in clade A as a closely related taxon to the eastern South American lupine group, which was not detected in previous phylogenetic studies of Lu pinus. It is then probable that other close relatives of $L$. texensis (in morphology and cytology) in Texas and adjacent areas, such as L. subcarnosus Hook., L. leonensis Wats., and L. havardii Wats. (Dunn, 1984), and some morphologically similar taxa in East Argentina and Uruguay, such as members of the L. gibertianus C. P. SmithL. linearis Desr. complex (Dunn, 1984), would be part of the same lineage (clade A). Interestingly, the latter species were suggested to be potentially related to $L$. angustifolius (Mediterranean), based on morphological and other biological similarities (Dunn, 1984; Planchuelo and Dunn, 1984). Moreover, it is noteworthy that L. paraguariensis, L. bracteolaris, L. multiflorus, and their close relatives display a uniform "simple-foveolate" seed coat pattern (Bragg, 1983; Monteiro, 1987; Aïnouche, unpublished data). As seen above, this pattern is also present in some Mediterranean smooth-seeded taxa as L. luteus and L. angustifolius (clade B).

Following traditional interpretations, Dunn (1984) considered that "the simple-leaved lupines are the primitive part of the genus" since they share supposedly primitive characters such as: habitat (subtropical highlands), perennial condition and woodiness, simple leaves, outcrossing, large-flowering, insect pollination, and other anatomical characters. Whether the perennial simple-leaved taxa are primitive or rather derived from the annual or perennial compound-leaved lupines is not resolved based on the conservative estimate of phylogeny (Fig. 2). Resolution of this question is of great importance in passing judgement on Dunn's hypothesis. Nevertheless, ITS data are hardly compatible with the view that the simple-leaved lupines derive from the Crotalarieae as previously suggested by Dunn (1984) and Gross (1986).

Therefore, the ITS data suggest that most of the eastern South American lupine groups and some southeastern North American ones, containing both annual and perennial, simple- and compound-leaved species, are derived from a common ancestor. This is in accordance with some previous assumptions based on thorough morphotaxonomic and geographical studies (Planchuelo Ravelo, 1984; Planchuelo and Dunn, 1984). A broader sampling
among potential close relatives is needed to circumscribe more accurately this distinct eastern New World clade.

Despite the lack of resolution at the base of the strict consensus tree, it may be pointed out that the eastern New World lupines are always placed closer to the Old World ones than to the western New World species in the most parsimonious trees generated by the different searches (Fig. 2). There are also shared morphological, micromorphological, and cytological features (reported above), which show affinities between Old World and eastern New World lupines. However, no conclusions may be drawn from present ITS data about close phylogenetic relationships between the Old World and eastern New World lupines, especially because only one additional step was required in a constraint analysis to force the monophyly of the New World lupines (clades A and E).

The western New World lupine clade (clade E; Figs. 1,2 ) is less well supported than the eastern one. However, it is present in $100 \%$ of the topologies generated by the different searches and is additionally supported by one synapomorphic indel shown in Fig. 2. This clade contains only digitate compound-leaved species usually $2 n=48$ (Phillips, 1957; Dunn and Gillett, 1966; Dunn, 1984). Thus, the western New World lupines represent a distinct euploid series contrasting with the Old World and the eastern New World aneuploid clades. It is noteworthy that the lupine species occurring in Central America, L. mexicanus and L. elegans (in Mexico), and in South America, L. mutabilis (Andean, Peru) and L. microcarpus (N. and S. America), are placed together with the western North American ones. There are also serological (Nowacki and Prus-Glowacki, 1971; Cristofolini, 1989), isozyme (Wolko and Weeden, 1990b), and molecular data (Käss and Wink, 1997b) indicating close affinities of the Andean species, L. mutabilis, and its relatives (e.g., L. bogotensis Benth.) to the North American ones.

Among the three monophyletic subclades with moderate to strong support distinguished within the western New World lupines, the best supported one is composed of five taxa exhibiting the same ITS sequence: L. microcarpus var. microcarpus, L. microcarpus var. densiflorus, L. affinis, L. luteolus, and L. pusillus (Figs. 1, 2). Presently, these taxa are considered taxonomic complexes of herbaceous winter annuals occurring in arid areas from southwestern Canada, to Mexico, with some (e.g., L. mi-crocarpus-L. densiflorus complex) extending their ranges into western regions of South America (Dunn, 1984; Planchuelo Ravelo, 1984). These taxa, and their relatives, were previously referred to as subg. Platycarpos in earlier literature (Watson, 1873); they correspond to the two distinct supraspecific groups Microcarpi and Pusilli of Smith (1944). ITS data demonstrate that these annual taxa (with sessile cotyledons, connate-perfoliate) form in fact a highly homogeneous group derived from a common ancestor. Their similar and apparently singular micromorphological seed coat pattern also suggests a common history (Ainouche, unpublished data). Despite weak support, L. hirsutissimus Benth., an annual species with petioled cotyledons from the dry and rocky areas of California and Baja, is always placed as sister taxon to the L. microcarpus group in the phylogenetic trees (Figs. 1, 2 ), while it is usually considered morphologically closer
to L. sparsiflorus (Smith, 1944; Riggins and Sholars, 1993).

One moderately supported subgroup in clade E corresponds to the well-defined $L$. arizonicus $-L$. sparsiflorus complex of winter annuals with petioled cotyledons endemic to arid areas extending from California, Nevada, and Arizona to Baja and Mexico (Smith, 1944; Dunn, 1984; and references therein). These species are relatively well differentiated from one another by 4-bp changes (Fig. 2), which is consistent with their reproductive isolation (Wainwright, 1978). Among the remaining annual species, Lupinus concinnus, with petioled cotyledons, sometimes morphologically confused with L. sparsiflorus (Riggins and Sholars, 1993), is here differentiated by seven autapomorphies, but there is no evidence of its relationship to any of the other taxa.

Another moderately supported monophyletic assemblage contains the perennial taxa $L$. mexicanus and $L$. elegans, which suggests that also their close relatives native to Central America (e.g., the perennial species $L$. exaltatus Zucc. and the annuals L. campestris Ch. and Schl., L. bilineatus Benth., L. hartwegii Lindl.) might have a common history (Dunn, 1984). A close relationship between $L$. hartwegii (close to L. mexicanus complex) and L. elegans was also shown from serological (Cristofolini, 1989) and isozyme data (Wolko and Weeden, 1990b), whereas L. aschenbornii Schauer (from Costa Rica) was sister taxon to L. elegans based on ITS data (Käss and Wink, 1997b).

All the remaining taxa in clade E (except for the annuals $L$. mutabilis, L. nanus, and $L$. succulentus) are representatives of perennial lupine complexes present in North America and Mexico (Dunn, 1984). These herbaceous and shrubby perennial species are characterized by a great morphological variability and intergradation. They cover a remarkably wide ecogeographical diversity ranging from the Arctic Circle in Alaska (e.g., L. arcticus) to Baja and Mexico (e.g., L. latifolius), and from sea level (e.g., L. littoralis) to subalpine and alpine slopes and meadows (e.g., L. lepidus, L. argenteus). Contrasting with this morphological and ecogeographical diversity, a much lower degree of ITS divergence is observed among these taxa, including several assemblages of species each with identical ITS sequences (groups 1 to 4, in Fig. 2) but without any reliable support. Moreover, both annual (e.g., L. nanus, L. succulentus) and perennial (e.g., L. latifolius) species may have the same ITS sequence (group 2). Lupinus nanus is predominantly self-incompatible and outcrossing as most of the perennial lupines, while the annual taxa are generally self-compatible and predominantly autogamous (Juncosa and Webster, 1989). Such a close relationship between annual and perennial lupines is not exceptional within the New World lupines. This may indicate that the annual and perennial habits have evolved independently many times within these lupines. Serological differences were not detected between annual and perennial North American lupines (Cristofolini, 1989). Dunn and co-workers (in Dunn, 1984) demonstrated that the L. mexicanus-L. exaltatus complex (in Mexico) contains both annual and perennial species, which are morphologically nearly indistinguishable and interfertile but completely different in their life history.

With few exceptions, the ITS sequences do not provide
enough informative characters to resolve relationships among the western New World lupines. The low divergence found especially among the perennial taxa and their annual close relatives seems congruent with common morphological intergradation, the stability of the chromosome number, and the lack of strong genetic barriers to interbreeding (Dunn and Gillett, 1966; Dunn, 1984; Welsh et al., 1987; Barneby, 1989). This indicates that these taxa are currently undergoing active processes of diversification and speciation. In contrast, higher levels of sequence divergence and more rapid substitution rates of ITS regions (Tables 3, 4) have been observed among annual taxa (to be discussed in next section).

## Sequence divergence and evolutionary rates of the

 ITS regions in Lupinus-Our results demonstrate variance of ITS sequence divergence both among and within the main lupine clades (Table 3). The relative rate tests performed detect unequal rates of ITS evolution within Lupinus (Table 4), indicating that the ITS regions violate the assumption of rate constancy among different lineages (Zuckerkandl and Pauling, 1965). Substitution rate heterogeneity between evolutionary lineages or taxa is not exceptional in plants and numerous examples have been documented at various taxonomic levels (Systma and Gottlieb, 1986; Shilling and Jansen, 1989; Doyle, Doyle, and Brown, 1990; Wilson, Gaut, and Clegg, 1990; Bousquet et al., 1992; Gaut et al., 1992, 1996, 1997; Li and Bousquet, 1992; Gaut, Muse and Clegg, 1993; Suh et al., 1993; Gielly and Taberlet, 1996; Eyre-Walker and Gaut, 1997). It is now widely accepted that the rate of molecular evolution (or the molecular clock) varies not only among DNA regions, coding and noncoding sequences, synonymous and nonsynonymous sites, but also between different lineages (Wu and Li, 1985; Britten, 1986; Li and Tanimura, 1987; Wolfe, Li, and Sharp, 1987; Bousquet et al., 1992; Gaut et al., 1992, 1996, 1997; Li, 1993).Despite the presence of ITS rate inequalities within Lupinus, the molecular clock cannot be rejected in a large proportion of pairwise comparisons ( $79.5 \%$ ) among the species tested, and different patterns of rate variation are noteworthy among the main clades (Table 4; Fig. 2). It is noteworthy, for example, that no significant ITS substitution rate differences are evident among the most strongly supported clades and subclades (revealed in the ITS phylogenies), which are composed of the eastern New World lupines, the Old World rough-seeded ones, the Lutei section, and the L. microcarpus group of western New World taxa. Moreover, it is also significant that most of the species or groups of species on long branches (with rapid ITS substitution rates) are annuals, while most of the perennial ones (except for those of clade A) display short branches and slower substitution rates. This is particularly apparent in the western New World clade (E). Such correlation between plant life history and rates of molecular evolution is generally explained by the generation time effects, according to the neutral theory (Shilling and Jansen, 1989; Wilson, Gaut, and Clegg, 1990; Gaut et al., 1992; Doyle, Lavin, and Bruneau, 1992; Suh et al., 1993; Böhle, Hilger, and Martin, 1996). However, rate inequalities are not always correlated with differences in habit in this and other studies: e.g., Microseris (Wal-
lace and Jansen, 1990); Microseridinae (Jansen et al., 1991); Krigia (Kim and Jansen, 1994); monocotyldons (Gaut et al., 1992). It has been suggested that not only the generation time, but also several other factors may influence rates of molecular evolution, including evolutionary history, selection, speciation rates, DNA replication or repair, and metabolic rates ( Wu and $\mathrm{Li}, 1985$; Britten, 1986; Bousquet et al., 1992; Li, 1993; Martin and Palumbi, 1993; Eyre-Walker and Gaut, 1997; Gaut et al., 1997). For example, the habit or generation time may have changed over the evolutionary history of lineages and it may be expected that some patterns of relative rate variation might reflect these changes (Wilson, Gaut, and Clegg, 1990 out et al., 1992; Gielly, 1994). Accordingly, the annual growth habit and the low rates of substitution in ITS displayed by some taxa (e.g., L. nanus and $L$. succulentus) in the western New World clade (E) might be interpreted as a recent acquisition of a short generation time. In contrast, the eastern New World perennial species, L. paraguariensis, which appears to have homogeneous substitution rates relative to, not only its perennial and annual close relatives in clade $A$, but also most of the annual lupines of the Old World and the eastern New World (Table 4), exhibits significantly more rapid substitution rate in comparison to the highly homogeneous western New World perennials and their annual close relatives in clade E. Thus, similar to the above argument, it may be tentatively hypothesized that the evolutionary history of L. paraguariensis might have included short generation times, which would explain its high ITS substitution rate. Gaut et al. (1992) suggested that the rapid substitution rate of the rbcL gene found in some perennial grass species (e.g., Puccinellia distans and Neuracshne sp.) might reflect the recent acquisition of the perennial generation time. However, the data also show other patterns, which remain obscure, such as the intriguing very slow substitution rate exhibited by $L$. albus.

Therefore, there is some evidence from ITS data, which argue for a role for the generation time effects in the evolutionary history of Lupinus. However, there is still a great need to clarify the evolutionary forces and mechanisms that influence nucleotide substitution rates in plant systems (Gaut et al., 1997). Thus, any conclusion in this context should be tested across different DNA regions (including different genomes as well), to accumulate as much data as possible, to be reliably assessed (Li and Tanimura, 1987; Gaut, Muse, and Clegg, 1993; Eyre-Walker and Gaut, 1997).

Phylogenetic utility of ITS sequences at the intrageneric level in Lupinus-Based on a simultaneous cladistic analysis of a large number of taxa, representative of a broad range of the lupine diversity, the ITS-based phylogeny presented here provides a general and objective overview of the resolved and unresolved relationships within the genus. Most of parallel ITS results of the independent study of Käss and Wink (1997b) are concordant with ours, despite their partitioned analyses of subsets of taxa instead of a generally preferred complete data set analysis. The increased taxon sampling included in this study ( 44 taxa vs. 29 sequenced by Käss and Wink, with 20 taxa common to the two independent stud-
ies) reveals some novel relationships especially within the New World, undetected in the previously published molecular analyses of Lupinus (Badr, Martin, and Jensen, 1994; Käss and Wink, 1997b). However, although some strongly to moderately supported groups are evident at different levels within Lupinus in the ITS phylogenetic tree topologies, relationships both among and within the main clades and groups (discussed above) still remain largely unresolved, particularly at the base of the genus. Despite the appreciable number of variable sites found in the ITS regions of lupines $(\sim 20 \%)$ in comparison to other taxonomic groups (Baldwin et al., 1995), the ITS potential of phylogenetic information has been considerably reduced due to the high proportion of autapomorphic mutations (Table 2) and a significant level of homoplasy. Additionally, most of the synapomorphies are positioned along the terminal branches supporting groups of taxa, while only very few have accumulated at the base of the genus to resolve the more ancestral relationships. Interestingly, such a lack of resolution at the base of Lupinus is also observed in chloroplast DNA-based phylogenies reconstructed from both restriction site and rbcL sequence data (Badr, Martin, and Jensen, 1994; Käss and Wink, 1994, 1997b). This basal star topology (i.e., unresolved polytomy) suggests that the lupines might have undergone an initial rapid radiation (Sang et al., 1994; Soltis and Soltis, 1995; Yuan and Küpfer, 1997). The present situation of the genus Lupinus, where the species are distributed into different major groups in general accordance with their geographical, morphological, micromorphological, biological, cytological, phytochemical, and genetic diversity, seems compatible with a rapid initial evolutionary diversification pattern of the genus. The herbaceous life form and short generation time might have contributed to a rapid radiation of the genus, as has been suggested for other taxonomic groups based on ITSnrDNA or cpDNA data (Baldwin et al., 1995; Soltis and Soltis, 1995; Yuan and Küpfer, 1997).

Conclusion-The ITS sequence data presented here provide novel information for taxonomy and systematics of the genus Lupinus. They lend strong molecular-based support to several previous assumptions based on diverse lines of data, including the monophyly of Lupinus and its close relationship to the Genisteae. They also provide new insights into the taxonomy and systematics of the taxonomically difficult New World lupines, including: (1) their apparent eastern-western geographic subdivision, (2) the relationship of southeastern North American annual species to both the annual and perennial, simple- and compound-leaved southeastern South American lupines, and (3) the recognition of the Platycarpos group. Moreover, life history has apparently influenced rates of ITS sequence evolution in Lupinus, and the data are suggestive of a rapid initial radiation of the genus. The ITS data failed, however, to resolve a number of relationships at the intrageneric level. The ITSbased phylogeny represents at least a basic framework, which could help initiate further more accurate investigations to elucidate phylogenetic relationships within the genus and their implications for biogeography and character evolution. Several questions remaining to be addressed include the clarification of relationships
among the main monophyletic groups and elucidation of their interrelationships. Thus, there is still a great need to improve the phylogeny of the genus Lupinus, at both the basal and internal levels, using more informative and appropriate characters, either from additional molecular data (nuclear and plastid) or in combination with a cladistic analysis of morphological data, and based on a broader representation of the New World diversity in the sampling. Additionally, the characterization of the molecular rate heterogeneity may have important implications for phylogenetic reconstruction and understanding of the evolutionary history.

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