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Relationships of *Lupinus* species based on variation in seed protein electrophoretic profiles

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The present work deals with application of seed protein diversity as revealed by SDS-PAGE to reassess the relationships of 27 samples represent six North American species and eight Old World species of *Lupinus* in the light of their chromosome counts and pervious taxonomic treatments. The relationships among the examined samples have been demonstrated as UPGMA and Neighbor joining (NJ) trees that agree with the taxonomy and ecogeographic distribution of the studied species. In both trees the 27 samples have been divided in two major groups; one small group comprised of the New World species and a large group comprised of the Old World species. The North American lupines are clearly delimited as separate identities with high levels of dissimilarity between them particularly in the UPGMA tree. In the NJ tree high levels of dissimilarity are observed between *L. sativus* and *L. sylvestris* and a cluster comprised of *L. mutabilis*, *L. succulentus*, *L. elegans* and *L. hartwegii*. The relationships among the Old World species, with few exceptions, correlate well with their morphology and intercrossing data. The morphologically diverse and genetically well-differentiated smooth-seeded species were separated as one group from the morphologically homogeneous and genetically less differentiated rough-seeded lupines. In the smooth seeded lupines, the separation of *L. albus* (2*n*=50) and *L. angustifolius* (2*n*=40) is congruent with their sectional delimitation, However, *L. micranthus*, (sect. *Micranthi*) and *L. luteus* and *L. hispanicus* (sect. *Lutei*) all have 2*n*=52. The rough seeded species are differentiated into two clusters; one includes the three samples of *L. consentinii* (2*n*=32) and the other comprises the two samples of *L. pilosus* (2*n*=42) and *atlanticus* (2*n*=38).

Key words: Fabaceae, *Lupinus*, seed protein electrophoreses, systematics.

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Introduction

The genus *Lupinus* L. (Fabaceae) comprises over 200 annual and perennial herbaceous species, as well as a few soft-woody shrubs and small trees (Dunn & Gillett, 1966; Dunn, 1984; Turner, 1995). The species of *Lupinus* (lupines) occur in a wide range of ecogeographical conditions in both the New World and the Old World with over 90% of the species in alpine, temperate, and subtropical biomes in the New World from Alaska to South Argentina and Chile. In the Old World only 12–13 species are native to the Mediterranean region and North Africa (Gladstones, 1974 &1998).

Old World lupines are all herbaceous annuals with large fruits and seeds and digitate leaves. In these species, two distinct groups have been recognized primarily on the basis of the seed coat texture: the smooth-seeded and the rough-seeded species (Gladstones, 1974; Heyn & Herrnstadt, 1977). The smooth-seeded group (Malacospermae) comprises five species usually treated as members of four sections viz *Albi*, *Micranthi*, *Angustifoli*, and *Lutei* (Gladstones, 1974 & 1998). These samples are mainly distributed around the Mediterranean and exhibit variable chromosome numbers ranging from 2*n*=40 to 2*n*=52 (Fedorov, 1974; Kazimierski, 1982). The rough-seeded group (Scabrispermae) contains six or seven species characterized by their morphological resemblance (Gladstones, 1974; 1998; Plitmann & Heyn, 1984). The species of this group are distributed in North Africa and in the Eastern part of the Mediterranean region and display chromosome numbers ranging from 2*n*=32 to 2*n*=42 (Fedorov, 1974; Plitmann & Pazy, 1984; Carstairs *et al.*, 1992).

In the New World, the species of *Lupinus* form a very complex and difficult group, the complexity of this group results from its high morphological, breeding system, and ecogeographical diversity and the lack of clear diagnostic features to separate species (Dunn & Gillett, 1966; Dunn, 1984). However, a remarkable group of lupines in the New World is composed of 22 perennial species with simple or unifoliate leaves, which occur mainly in the subtropical highlands of the east-central region of South America (Planchuelo-Ravelo, 1984; Planchuelo-Ravelo & Dunn, 1984; Monteiro & Gibbs, 1986). All of the remaining species have digitate leaves and from two main New World lupine centers of diversity, the Andean region and the North and Central American regions. Most of the New World species, cytologically investigated, display a common chromosome number of 2*n*=48 (Phillips, 1957; Dunn & Gillett, 1966), with some occasional individuals having 2*n*=96 (Phillips, 1957). The base chromosome number

suggested for the New World species is x=6 and consequently the New World Lupines are regarded as paleopolyploids that behave as diploids (Dunn, 1984).

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 (Adan.) Benth. (Bisby, 1981). Nevertheless, its tribal position has often been disputed (Monteiro, 1986; Saint-Martin, 1986; Badr *et al*., 1994). 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 (Cristofolini, 1989).

In recent years, molecular markers derived from DNA using electrophoretic techniques have provided powerful markers for the study of several aspects in all biological fields including systematic and genetic relationships of plant species and cultivars. However, biochemical evidence derived from electrophoretic separation of proteins is also currently used to in several fields of plant science. The variation in the electrophoretic pattern of seed proteins in polyacrylamide gel in the presence of sodium dodecyle sulphate (SDS-PAGE) have been found useful in the study of systematics and evolution of plant species (Ladizinsky & Hymowitz, 1979; Vaughan, 1983). These proteins are particularly abundant in the seeds of legumes and have provided valid source of evidence that have successfully been used for addressing the systematic relationships at the species level in many genera of the family Fabaceae. For example, to differentiate between species in *Trifolium* (Badr, 1995), *Phaseolus* (Schmit *et al*., 1996), *Sesbania* (Badr *et al*., 1998), *Lathyrus* (El-Shanshoury, 1997; Badr *et al*., 2000) and *Astragalus* (Al-Nowaihi *et al*., 2002) and also in plants from other families such as Solanaceae (Khalifa, *et al*. 1998), *Mentha* from family Lammiaceae (Badr *et al*., 2003) and *Artemisia* from family Asteraceae (Mohamed, 2004). The present work deals with the application of seed protein diversity as revealed by SDS-PAGE to reassess the taxonomic relationships of 27 samples representing 14 species, including eight (17 samples) from the Old World lupines and six (ten samples) from the New World lupines in the light of their pervious taxonomic treatments.

Materials and Methods

Seeds samples of the studied species were kindly provided by the IPK gene bank, Gatersleben, Germany and the FAL gene bank in Braunschweig, Germany. The *Lupinus* samples included in this study are presented as accessions, geographic origin, source, Gene Bank accession number and life history and chromosome numbers (2*n*) are listed in Table 1.

Table 1. List of *Lupinus* samples included in this study, and their geographic origin, source, Gene Bank accession number and life history and chromosome numbers (2*n*), Ch. No. = Chromosome number, A = Annual, P= Perennial.

Species	**Origin**	**Source and Gene bank ID**	**Longevity & Ch. No.**
Lupinus albus L. (1)	Italy	FAL-3912	A-50
Lupinus albus L. *ssp. albus* (2)	Egypt	IPK. 260/83	A-50
Lupinus angustifolius L. (1)	Breeder	FAL-10244	A-40
Lupinus angustifolius L (2)	Breeder	FAL-10245	A-40
Lupinus angustifolius L. ssp. *angustifolius* (3)	Algeria	IPK 1130/83	A-40
Lupinus atlanticus Gladst.	Morocco	FAL-22344	A-38
Lupinus consentinii Guss. (1)	Morocco	FAL-22344	A-32
Lupinus consentinii Guss. (2)	Portugal	FAL-22355	A-32
Lupinus consentinii Guss. (3)	Spain	FAL-48723	A-32
Lupinus elegans H. B. K.	Russia	FAL-47206	P-48
Lupinus hartwegii Lindl.	Germany	FAL-45093	P-48
Lupinus hispanicus Boiss & Reuter ssp. *bicolor* (1)	France	IPK 525/85	A-52
Lupinus hispanicus Boiss & Reuter ssp. *hispanicus* (2)	Spain	IPK 529/84	A-52
Lupinus hispanicus Boiss & Reuter ssp. *hispanicus* (3)	Spain	IPK 529/85	A-52
Lupinus luteus L. (1)	Sisily	IPK 342/76	A-52
Lupinus luteus L. (2)	Sweden	FAL-41160	A-52
Lupinus micranthus Guss.	----------	FAL-45068	A-52
Lupinus mutabilis Sweet (1)	Bolivia	FAL-58022	A-48
Lupinus mutabilis Sweet (2)	----------	FAL-58023	A-48
Lupinus mutabilis Sweet (3)	Bolivia	FAL-58022	A-48
Lupinus pilosus Murr. (1)	Israel	FAL-22376	A-42
Lupinus pilosus Murr. (2)	Turkey	FAL-22375	A-42
Lupius sativus L. (1)	----------	FAL-25104	
Lupius sativus L. (2)	----------	FAL-27444	
Lupinus succulentus Koch	----------	FAL-45070	
Lupinus sylvestris L. (1)	----------	FAL-50773	
Lupinus sylvestris L. (2)	----------	FAL-50778	

Seed protein was extracted at the college of Education, Ain Shams University and electrophorased at the faculty of Science, Tanta University in 2002-2003. For protein extraction, 5.0 g of mature seeds were mixed with an equal weight of pure, clean, sterile fine sand, powdered using a mortar and pestle. From seed powder 0.5 g were homogenized with 0.2 M Tris-HCl buffer, pH=8 for 1 h in sterilized Eppendorf tubes. The extract was centrifuged at 12000 g for 10 min and the supernatant (protein extract) was transferred to new tubes and immediately used for electrophoresis. For electrophoresis, 40 ml of the extract were mixed with an equal volume of a sample buffer (0.125 M Tris-HCl, pH = 6.8, 2% SDS, 10% sucrose, 0.5% β-mercaptoethanol), denatured by boiling for 5 min in a water bath, cooled and 0.1% bromophenol blue as a tracking dye was added. For separation of protein components, 20 µl of this mixture were loaded in 12.6% gel slabs, which was prepared as described by Lammeli (1970). Electrophoresis was carried out in Tris-Glycine buffer (pH=8.3) at 4°C and 125 volt for 2 h using a Pharmacia low-molecular weight protein mixture as standard marker. Gels were then stained in 0.1% Comassie Brilliant Blue R-250 for 1 h, destained and photographed while gels were wet and stored for subsequent examination. The bands produced in the electropherogram were scored and their molecular weights were calculated by comparison to the standard protein marker.

For data analysis, the total number of the recorded protein bands in the gel profile of each taxon was scored and coded as binary characters i.e. absent = 0 and present = 1, for creating a data matrix for computation. The relationships between the samples studied were expressed by using the coefficient of similarity proposed by (Dice, 1945). The equation for this coefficient is included in the computer program NTSYS-pc (Rohlf, 1993), which has been used for data analysis. Construction of the trees illustrating the relationships between the studied samples was performed using the unweighted pair group method using arithmetic average (UPGMA) proposed by Sokal & Michener (1958) and the Neighbour joining (NJ) method (Saitou. & Nei, 1987) as implemented in the NTSYS-pc program (Rohlf, 1993).

Results

The neighbour joining (NJ) tree illustrating the relationships between the studied samples of *Lupinus* is illustrated in Fig. 1. In this tree the 27

samples are divided in two major groups, one small group comprised of the New World species and a large group comprised of the Old World species. In the first group, the two samples of *L. sativus* and the two samples of *L. sylvestris* are clearly distinguished, as two separate clusters from the three samples of *L. mutabilis*, *L. succulentus*, *L. elegans* and *L. hartwegii*. The two samples of *L. sativus* and the two samples of *L. sylvestris* are clearly differentiated from each other and *L. elegans* and *L. hartwegii* are distinguished from *L. succulentus* and the samples of *L. mutabilis* at lower levels of similarity.

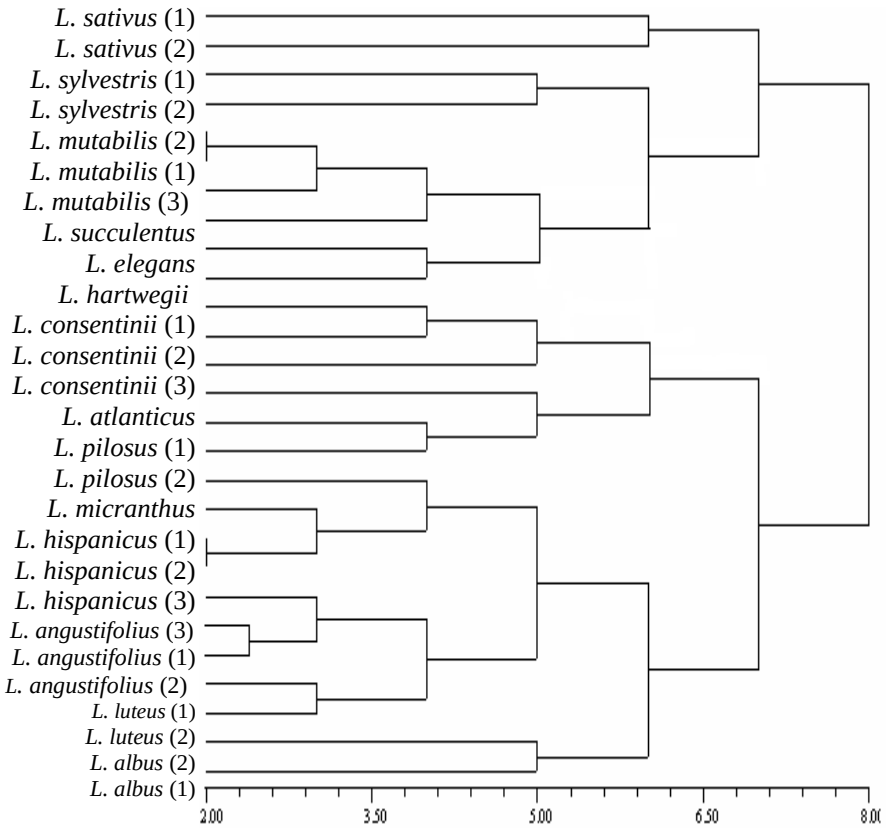

Fig. 1. The NJ tree based on variation in seed protein electrophoretic data, illustrating the relationships between the studied samples of *Lupinus*.

The second large group, which is comprised of the Old World species, is divided into two major subgroups, one comprised of 11 samples representing the smooth seeded species *L. albus, L. luteus, L. angustifolius, L. hispanicus* and *L. micranthus*. In this subgroup the two samples of *L. albus* are clearly distinguished from each other and from another cluster in which the two samples of *L. luteus* are differentiated from the three samples of *L. angustifolius*, and these two species were differentiated from the three samples of *L. hispanicus* and *L. micranthus*. The other subgroup of the Old World species comprises samples representing the rough-seeded species *L. pilosus*, *L. consentinii*, and *L. atlanticus*. In this subgroup, the two samples of *L. pilosus*; the three samples representing *L. consentinii* and *L. atlanticus* are separated as three separate clusters at relatively high levels of similarity.

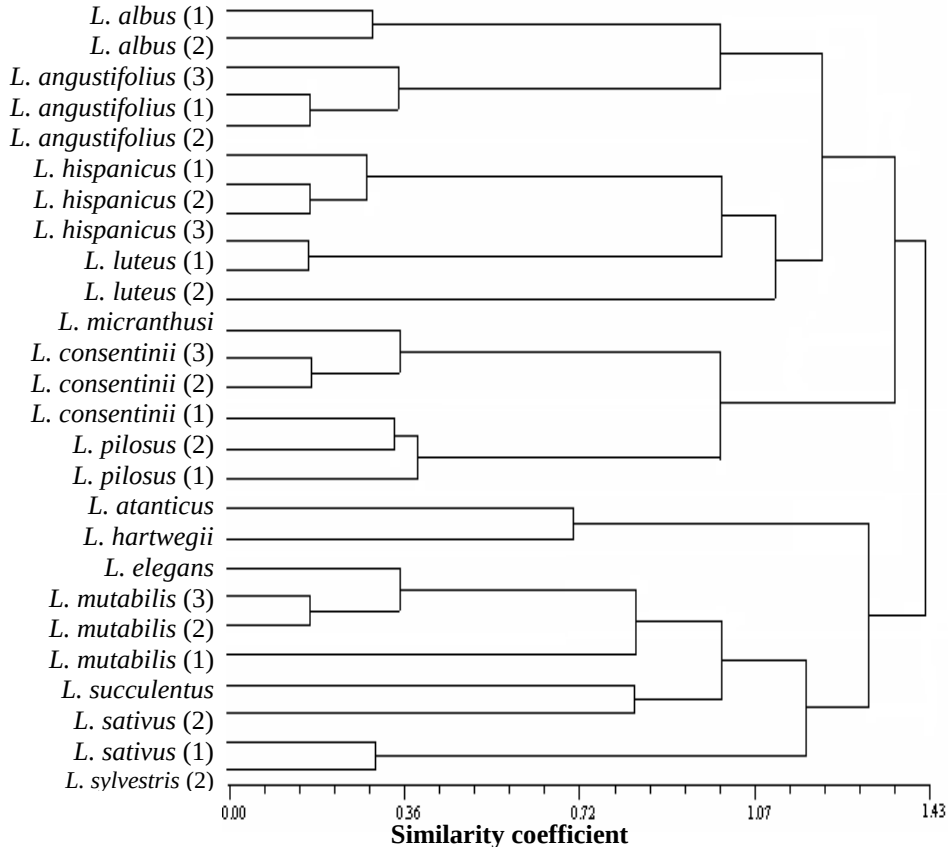

Fig. 2. The UPGMA tree, based on variation in seed protein elector-phoretic data illustrating the relationship between the studied samples of *Lupinus*.

In the UPGMA tree illustrating the relationships between the studied samples of *Lupinus* (Fig. 2), the New World and the Old World species are delimited as two separate groups. In the New World group, the two samples of *L. sativus* and the two samples of *L. sylvestris* are clearly distinguished, as two separate clusters from *L. succulentus* and the three samples of *L. mutabilis*. The two samples of *L. sativus* are differentiated from each other and *L. succulentus* is differentiated from the samples of *L. mutabilis*. Meanwhile, the above species are clearly separated from a cluster comprised of *L. elegans* and *L. hartwegii*, which are also differentiated from each other.

The large group comprising the Old World lupines is divided, in the UPGMA tree, into two subgroups; one comprising the smooth-seeded species *L. albus*, *L. angustifolius*, *L. hispanicus*, *L. luteus*, and *L. micranthus*. In this subgroup the samples representing each of the first four species are clearly distinguished as separate cluster, while the single sample representing *L. micranthus* appeared distinct from the other samples. The second subgroup comprising the-rough seeded species is differentiated into two clusters; one includes the three samples of *L. consentinii* and the other the two samples of *L. pilosus* and *L. atlanticus*.

Discussion

The relationships between the examined species of *Lupinus* based on the analysis of seed protein data and expressed as a NJ and UPGMA trees is congruent with their systematic treatments and ecogeographic distribution. The New World species are clearly separated together from the Old World species, this separation is clearly supported by the morphological and cytological diversity among the two groups (Dunn & Gillett, 1966; Dunn, 1984; Turner, 1995). Most of the New World species display a common chromosome number of $2n$=48 (Phillips, 1957; Dunn & Gillett, 1966), with some occasional polyploid individuals having $2n$=96 (Phillips, 1957). The Old World species, on the other hand, exhibit variable chromosome numbers ranging from $2n$=32 to $2n$=52 (Gladstones, 1984; Plitmann & Heyn, 1984; Plitmann & Pazy, 1984; Carstairs *et al*., 1992).The separation of the New and Old World lupines as two distinct groups was also indicated by evidence obtained from the chloroplast RFLP (Badr *et al*., 1994) and by variation in internal transcript spacer sequence (Ainouche & Bayer, 1999).

The grouping of the ten samples representing six species of the New World lupines as a single group is congruent with the difficulties to separate the New World species of *Lupinus* due to the few diagnostic features to separate them. However, the six species are clearly delimited as separate identities with high levels of dissimilarity between them particularly in the UPGMA tree. In the NJ tree, high levels of dissimilarity are observed between *L. sativus* and *L. sylvestris* and the cluster comprised of *L. mutabilis*, *L. succulentus*, *L. elegans* and *L. hartwegii*. However, with data on the small number of the New World species examined in the present study, it is difficult to discuss further the relationship of the New World lupines.

Meanwhile, 17 samples representing eight of the Old World species have been included in this study allowing detailed discussion of their relationships. The smooth-seeded lupines of the Old World, are delimited as a distinct group from the rough seeded species, these are all Mediterranean, and morphologically well defined species and have been recognized as different sections (Gladstones, 1974 & 1984) The separation of the smooth-seeded and rough-seeded species is largely in accordance with biochemical data from alkaloids (Wink *et al*., 1995), leaf flavonoids (Williams *et al.*, 1983), electrophoretic pattern of seed globulins (Przybylska & Zimniak-Przybylska, 1995), isozymes polymorphism (Wolko & Weeden, 1989; 1990) and nuclear DNA content variation (Naganowska *et al.*, 2003).

Within the smooth-seeded species, variation in seed protein data supports the sectional delimitation of the studied species. The analysis of these data, by the UPGMA tree building method, distinguished *L. albus*, *L. angustifolius*, *L. micranthus* that have been placed in separate sections (*Albi*, *Angustifoli*, and *Micranthi* respectively), and grouped *L. luteus* with *L. hispanicus* that have been placed together in sect. *Lutei*. The separation of *L. albus* (2*n*=50) and *L. angustifolius* (2*n*=40) is congruent with the variation in their chromosome number (Gladstones, 1974 and 1984; Amaral Franco & Pinto da Silva, 1978). However, *L. micranthus*, (sect. *Micranthi*) and *L. luteus* and *L. hispanicus* (sect. *Lutei*) all have 2*n*=52 (Kazimierski, 1982); the latter two species were also found as sister samples, well differentiated from other smooth seeded species by seven nucleotide changes in the ITS sequence (Ainouche & Bayer, 1999).

Despite their differences in morphology and cytology, sections *Lutei* and *Angustifoli* were found as members of a monophyletic group, based on the

analysis of variation in ITS sequence (Ainouche & Bayer, 1999). This is reflected in the NJ tree presented in this study and was also seen in the tree based on *rbcL* analysis (Käss & Wink, 1997). *L. luteus* was suggested as being closer to *L. micranthus* based on similar chromosome numbers and some morphological affinities (Gladstones, 1984 and 1998), a relationship that is not corroborated by ITS results (Ainouche & Bayer, 1999) or by crossing data (Roy& Gladstones, 1988; Gupta *et al*., 1996). *Lupinus luteus* is represented on both sides of the Mediterranean and has been considered the most derived species of the smooth seeded clade with respect to ITS sequence (Ainouche & Bayer, 1999). Gladstones (1974) suggested the Iberian Peninsula as the place of origin of sect. *Lutei,* whereas that of sect. *Angustifoli* is somewhere in the Mediterranean.

The position of *L. micranthus* in relationship to other species has been the subject of debate in the literature. Investigations based on flavonoids (Williams *et al*., 1983), serology (Cristofolini, 1989), and isozymes (Wolko & Weeden, 1989; 1990) indicated an intermediate position of this species between the smooth-seeded and the rough-seeded lupines of the Old World. This is clearly supported by the position of *L. micranthus* in the UPGMA tree based on our seed protein analysis.

The rough-seeded lupines have been delimited as a separate group from the smooth-seeded species in both the NJ and UPGMA trees. This is supported by remarkable morphological homogeneity of this group and has been demonstrated by various sources of data including seed coat texture (Heyn & Herrnstadt, 1977); alkaloids (Wink *et al*., 1995; Ainouche *et al*., 1996), flavonoids (Williams *et al*., 1983), seed globulin proteins (Przybylska & Zimniak-Przybylska, 1995), protein serology (Cristofolini, 1989) and isozymes (Wolko & Weden, 1989 & 1990). The analysis of seed protein data presented here added to the above evidence support the proposition to recognize the rough-seeded species as a separate section *Scabrispermae* Plitm. & Heyn (Plitmann & Heyn, 1984) that was also strongly reinforced by nrDNA evidence (Käss & Wink, 1997; Ainouche & Bayer, 1999).

The rough seeded species are differentiated into two clusters; one includes the three samples of *L. consentinii* (2*n*=32) and the other comprises the two samples of *L. pilosus* (2*n*=42) and *atlanticus* (2*n*=38). The relationships as expressed by seed protein data may be regarded congruent with their cytological differences. The rough-seeded lupines, which are distributed in the eastern Mediterranean region, were shown to be

reproductively isolated (Roy & Gladstones, 1988; Carstairs *et al*., 1992), although genome similarities were found between *L. pilosus* and *L. atlanticus* (Gupta, *et al*., 1996). Meanwhile, species originating from the arid regions of North Africa, *L. atlanticus* and *L. digitatus*, both with 2*n*=36, were found as sister samples in the ITS phylogeny presented by Ainouche & Bayer (1999), but were found slightly more distantly related in the ITS sequence data of Käss & Wink (1997). Nevertheless, it has been demonstrated that these two species intercross successfully and have a greater homology of chromosomes than to any other rough-seeded species (Roy & Gladstones, 1988; Carstairs *et al*., 1992; Gupta *et al*., 1996).

The Mediterranean, *L. cosentinii* (2*n*=32) was found to exhibit an identical ITS sequence to that of the hypothesized recent common ancestor of the rough-seeded lupines. This is in agreement with ITS results of Käss & Wink (1997) but not with their *rbcL* data where *L. cosentinii* appeared to accumulate relatively more mutations (Käss & Wink, 1997). This species was found 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 & Gladstones, 1988; Carstairs, *et al*., 1992). This evidence supports the results based on seed protein data, as reported here, where the three samples representing *L. cosentinii* are clearly isolated from the other rough-seeded *L. pilosus* and *L. atlanticus* in both the NJ and UPGMA trees.

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