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GENETIC EVIDENCE SUPPORTS THE NEW ANATOLIAN LUPINE ACCESSION, *LUPINUS ANATOLICUS*, AS AN OLD WORLD "ROUGH-SEEDED" LUPINE (SECTION *SCABRISPERMAE*) RELATED TO *L. PILOSUS*

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Abstract: A noteworthy wild lupine accession was recently discovered in southwestern Turkey and was proposed as a new separate Old World "smooth-seeded" species close to *L. micranthus* and named *L. anatolicus*. Its species status was controversial with respect to cytological and crossability data. In order to examine the position and the evolutionary relationships of this Anatolian accession relative to the Old World lupines, we investigated new data from seed coat micromorphology, and from internal transcribed spacer (ITS) nucleotide sequences of the nuclear ribosomal DNA repeat. The micromorphological seed coat pattern of *L. anatolicus*, as revealed by scanning electron microscopy, is characterized by pluricellular tubercles, which represent the typical and unique pattern of the Old World "rough-seeded" lupines (sect. *Scabrispermae*). In accordance with the micromorphological results, the genetic distances and phylogenetic relationships among the Old World lupines, estimated from ITS data, unambiguously support the new Anatolian lupine accession as part of the *L. pilosus-L. palaestinus* lineage within the strongly monophyletic group containing all the *Scabrispermae*. The results provided in this study, together with other lines of data available from the literature, are thus hardly compatible with the hypothesis that this new Anatolian lupine accession could be related to Old World "smooth-seeded" lupines (including *L. micranthus*); instead, it appears closely related to *L. pilosus*.

INTRODUCTION

Among the hundreds of taxa described in the genus *Lupinus* L. (*Fabaceae*, *Genisteae*), eleven to twelve species are presently recognized in the Old World (GLADSTONES 1974). Because of their limited number and their economic potential as protein and nitrogen suppliers (GLADSTONES 1981, 1984, BELTEKY et al. 1983), the Mediterranean and African lupines have been fairly well studied over recent decades. They have been studied in order to understand their biology, particularly their taxonomy and systematics which have long been in confusion (GLADSTONES 1974, 1984).

The Old World lupines are all annual, herbaceous, and predominantly autogamous, and were subdivided into two groups mainly on the basis of both their macroscopic (GLADSTONES 1974) and microscopic (HEYN & HERRNSTADT 1977, PLITMANN & HEYN 1984) seed coat structure: the "smooth-seeded" lupine group and the "rough-seeded" one of GLADSTONES (1974). These two groups also differ in other characters, such as cytology (PLITMANN & PAZY

1984, KAZIMIERSKI 1988, and references therein), serology (CRISTOFOLINI 1989), isozymes (WOLKO & WEEDEN 1990), seed storage proteins (SALMANOWICZ & PRZYBYLSKA 1994, PRZYBYLSKA & ZIMNIAK-PRZYBYLSKA 1995), alkaloids (NOWACKI 1963, WINK et al. 1995, AÏNOUCHE et al. 1996, AÏNOUCHE 1998), and ITS nuclear ribosomal DNA sequences (KÄSS & WINK 1997, AÏNOUCHE & BAYER 1999a, 1999b). Moreover, these groups are genetically well isolated from one another and all attempted interspecific hybridization between them have failed (KAZIMIERSKI 1961, PLITMANN & PAZY 1984). Consequently, PLITMANN & HEYN (1984) proposed to recognize the "rough-seeded" lupines as a separate section within the genus *Lupinus* named *Scabrispermae* PLITMANN et HEYN.

The Old World "smooth-seeded" lupine group is composed of five well-differentiated and genetically isolated species characterized by seeds with a smooth surface and a unicellular micromorphological seed-coat pattern (GLADSTONES 1974, HEYN & HERRNSTADT 1977). These species are distributed around the Mediterranean Sea where they often grow sympatrically in various areas. They display different chromosome numbers: 2n=50 for *L. albus* L., 2n=40 for *L. angustifolius* L., and 2n=52 for *L. luteus* L., *L. hispanicus* BOISS. et REUT. and *L. micranthus* GUSS. (GLADSTONES 1984, PLITMANN & PAZY 1984).

Sect. *Scabrispermae* contains species characterized by their overall morphological similarity and by the rough seed testa resulting from a pluricellular and prominent micromorphological seed coat pattern (HEYN & HERRNSTADT 1977, AÏNOUCHE 1998). They are primarily distributed in Northern Equatorial Africa and in the eastern part of the Mediterranean region and are ecogeographically isolated from one another (GLADSTONES 1974, PLITMANN & PAZY 1984). Six species are presently recognized: *L. pilosus* MURRAY (2n=42), *L. palaestinus* BOISS. (2n=42), *L. cosentinii* GUSS. (2n=32), *L. atlanticus* GLADST. (2n=38), *L. digitatus* FORSSK. (2n=36), and *L. princei* HARMS (2n=38) (GLADSTONES 1984).

Although significant advances have been made in understanding the taxonomy and systematics of *Lupinus* in the Old World, several areas of the Mediterranean region and Africa still need to be explored as potential sources of wild populations in order to evaluate with more accuracy both their systematic and genetic diversity (SWIECICKI 1988).

Recently, a noteworthy wild lupine population, apparently with "smooth" seeds, was discovered in Anatolia, southwestern Turkey (SWIECICKI et al. 1994). This population displayed morphological similarities with both *L. pilosus* and *L. micranthus* but was found to be different from the wild lupines growing in the same region (DAVIS 1970) with respect to various morphological, physiological and chemical characters (PRZYBYLSKA & ZIMNIAK-PRZYBYLSKA 1995, SWIECICKI et al. 1996). Based on observations emphasizing seed features (size and macroscopic seed surface), the Anatolian population was designated as a new separate Old World smooth-seeded species, close to *L. micranthus*: *Lupinus anatolicus* W. SWIECICKI et W.K. SWIECICKI. Likewise, studying the natural variation within *L. pilosus* among accessions from various geographical origins, CLEMENTS et al. (1996) considered the Anatolian lupine of SWIECICKI et al. (1994) as no more than a smooth-seeded *L. pilosus* population. It is worth noting that the Balkan Peninsula and Turkey are regarded as centres of diversification of *L. micranthus* and *L. pilosus* and that these taxa were sometimes confused in earlier literature (see review of GLADSTONES 1974).

In order to verify the position of this Anatolian accession relative to the Old World lupines, we investigated new data from seed-coat micromorphology, and from the phylogenetic analysis of nuclear DNA nucleotide sequences. The micromorphological seed-coat pattern (examined by scanning electron microscopy) has proven to be a useful criterion to separate the

"rough-seeded" lupines (*Scabrispermae*) from the "smooth-seeded" ones, and to distinguish taxa among the latter group (HEYN & HERRNSTADT 1977, PLITMANN & HEYN 1984, AÏNOUCHE 1998). Recently, nucleotide sequences of the internal transcribed spacers (ITS1 and ITS2) region of the 18S-26S nuclear ribosomal DNA repeat proved to be a useful tool for determining the taxonomy and systematics of *Lupinus* in the Old World, and provided informative characters to resolve relationships among closely related taxa (KÄSS and WINK 1997, AÏNOUCHE & BAYER 1999a, 1999b). Thus, these independent approaches appeared to be of particular interest to elucidate the status of *L. anatolicus* and its evolutionary relationships relative to the Old World lupines.

MATERIAL AND METHODS

Plant material

Seeds of the new lupine species proposed by SWIECICKI et al. (1996), *Lupinus anatolicus*, were kindly provided by one of the authors (W.K. SWIECICKI). They were grown together with representatives of other "rough-" and "smooth-seeded" Old World lupines in the greenhouse at the University of Rennes-1 (France) under the same conditions.

Seed-coat micromorphology

The dry mature seed samples were dipped in 95% ethanol, air-dried, mounted on aluminum or brass stubs with double-stick carpet tape, then sputter coated with gold prior to viewing using a scanning electron microscope. The seed surface was examined (with a JEOL JSM-630 1 FXV) and a micrograph of a representative seed-coat pattern was taken at mid-seed. The hand-cut transverse section of the seed coat was also observed to assess the cellular structure of the surface pattern. The observations were made on three different seeds from the Anatolian reference sample (of W.K. Swiecicki) and were compared to the large database of micromorphological seed-coat patterns (including numerous Old and New World lupine species and populations) available from the senior author (AÏNOUCHE 1991, 1998) and from the literature (HEYN & HERRNSTADT 1977, BRAGG 1983, PLITMANN & HEYN 1984, MONTEIRO 1987).

Molecular analyses

DNA isolation, amplification and sequencing were conducted using the methods described in BAYER et al. (1996) and AÏNOUCHE & BAYER (1999a). Total DNA was extracted from about 10 mg of fresh leaf material using a modified CTAB method (DOYLE & DOYLE 1987), where 1.0% of β-mercaptoethanol (instead of 0.2%) was used in the extraction buffer.

A polymerase chain reaction (PCR) of the targeted nuclear DNA region was performed using the template DNA, the appropriate universal primers, dNTPs and Taq DNA polymerase in a total volume of 100 µl via 30 cycles of 1 min at 94 °C, 1 min at 48 °C and 2 min at 72 °C. A 7 min final extension at 72 °C followed 30 cycles. The external ITS-1 and ITS-4 primers designed by WHITE et al. (1990) were employed to amplify the ITS1 + 5.8S cistron + ITS2 region comprised between the 18S and 26S nrDNA genes. The purified double-stranded DNA was directly sequenced by the standard dideoxy chain termination technique (SANGER et al. 1977), using the fmol DNA Sequencing System of Promega with ^{32}P labelled primers as described by AÏNOUCHE & BAYER (1999a). The internal primers ITS-2 and ITS-3 (from

WHITE et al. 1990) were used to sequence separately the ITS1 and ITS2 regions respectively. For most taxa, end labelled ITS-1 and ITS-4 primers were also used to complete the sequence of the 5.8S cistron localized between the ITS regions. DNA fragments generated by the sequencing reactions were separated by electrophoresis on 6% acrylamide-8M urea gel then fixed and exposed to X-ray films.

The ITS nucleotide sequences of *L. anatolicus* were aligned with and compared to those of the Old World lupine species previously sequenced and used in a most extensive phylogenetic analyses of the genus *Lupinus* which included representative taxa from both the Old and the New World (AÏNOUCHE & BAYER 1999a): *L. albus, L. angustifolius, L. luteus, L. hispanicus* and *L. micranthus* from the "smooth-seeded" group; *L. cosentinii, L. digitatus, L. atlanticus, L. pilosus* and *L. palaestinus* for the "rough-seeded" group (Sect. *Scabrispermae*). Based on recent molecular systematic data from *Papilionoideae* DC. and *Genisteae* (ADANS.) BENTH. including *Lupinus* (BADR et al. 1994, DOYLE 1995, KÄSS & WINK 1997, AÏNOUCHE & BAYER 1999a), the ITS sequences of three additional taxa from the Genistoid alliance were introduced and aligned in the data matrix to represent the outgroup: *Genista tinctoria* L. and *Chamaecytisus mollis* (CAV.) GREUTER et BURDET from subtribe *Genistinae* (HUTCH.) F.A. BISBY (tribe *Genisteae*), and *Crotalaria podocarpa* DC. from tribe *Crotalarieae* (BENTH.) HUTCH. The proportion of nucleotide differences among pairs of taxa (pairwise distances) was calculated for the combined ITS1 and ITS2 sequences (the 5.8S sequence being not available for all the taxa) with PAUP version 3.1.1 (SWOFFORD 1993) using the "mean distance" adjusted for missing data.

In order to determine the position of *L. anatolicus* relative to the Old World lupines, the resulting data matrix of aligned nuclear DNA sequences (available from the senior author upon request) was subjected to a maximum parsimony analysis performed by branch-and-bound search using Fitch parsimony with PAUP 3.1.1. All characters were included in the analysis with gaps treated as missing data. Characters and character states were weighted equally. The branch-and-bound search was computed via stepwise addition of furthest sequences ("MULPARS" option in effect), and topological constraints were not enforced. Branches of zero length were collapsed to yield polytomies. *Crotalaria podocarpa*, the extratribal taxon (outside of the tribe *Genisteae*), has been designated as the outgroup in the analyses. The bootstrap method with branch-and-bound search (1000 replicates) was performed to estimate the robustness of clades (FELSENSTEIN 1985).

The references and sources of the taxa included in this study are presented in Tab. 1, including the accession numbers of all sequences reported here (deposited in GenBank Sequence Database: http://www.ncbi.nlm.nih.gov).

RESULTS

Grown in the greenhouse together with other representatives of the Old World lupines, *L. anatolicus* showed similarities and disimilarities with both *L. pilosus* and *L. micranthus* in morphology (mainly in quantitative characters) and in physiological behaviour (rhythm of plant growth and flowering). However, *L. anatolicus* appeared to resemble most in general morphology *L. pilosus*, from which it mainly differs by shortest plant height, less exuberant habit, slow early growth and late flowering time. In particular, seeds of *L. anatolicus* showed an overall external appearance very similar, in shape and somewhat in coloration, but of smaller size to that of *L. pilosus*. The macroscopic seed surface appeared smooth when seen with the naked eye or at low magnifications.

Table 1. List of *Lupinus* and outgroup taxa included in the molecular analyses. Presented are taxa, author's references, and GenBank Sequence Database accession numbers of ITS nrDNA sequences. Author's references: AKA = Abdelkader Aïnouche.

Taxon	Origin	Author's references	GenBank DNA-sequence accession number	
			ITS1 / ITS2	ITS1+5.8S+ITS2
Ingroup taxa				
Lupinus albus L.	Algeria	AKA/ INAE-DZ-M11		AF007481
L. anatolicus W. SWIECICKI et W.K. SWIECICKI	Turkey	AKA/ ANAT-SWIEC.		AF108085
L. angustifolius L.	Algeria	AKA/ M1		AF007477
L. atlanticus GLADST.	Morocco	AKA/ USDA-384612	AF007432 / AF007433	
L. cosentinii GUSS.	?	AKA/ INRAL-FR-A16	AF007464 / AF007465	
L. digitatus FORSSK.	Egypt	AKA/ WADA-PI 26877	AF007430 / AF007431	
L. luteus L.	Algeria	AKA/ M5		AF007478
L. micranthus GUSS.	Algeria	AKA/ M8		AF007480
L. palaestinus BOISS.	Middle East	AKA/ INRAL-FR-A15		AF007479
L. pilosus MURRAY	Middle East	AKA/ INAE-DZ-A13	AF007434 / AF007435	
Outgroup taxa				
Chamaecytisus mollis (CAV.) GREUTER et BURDET		AKA/ RBGKew-84327		AF007472
Crotalaria podocarpa DC.		AKA/ RBGKew-90928		AF007469
Genista tinctoria L.		AKA/ RBGKew-51334		AF007471

Seed-coat micromorphology

The micromorphological seed-coat structure of *L. anatolicus* is presented in Fig. 1, along with the microtopographical pattern of the seed surface (Fig. 1 A) and the transverse section of the testa (Fig. 1 B). Also shown are the micromorphological seed-coat patterns of the two Old World lupine taxa to which *L. anatolicus* has been alternatively related by SWIECICKI et al. (1996) and CLEMENTS et al. (1996), *L. micranthus* (Fig. 1 C–D) and *L. pilosus* (Fig. 1 E–F), respectively. The transverse section of the lupine seed testa (Fig. 1 B, D, F) consists of two external unicellular layers overlaying an internal parenchymatous tissue. The inner layer is composed of osteosclereid (sclerified hypodermis or hourglass cells) separated by large intercellular spaces, while the outer layer is characterized by elongated palisade cells (also called macrosclereids or Malpighian cells) that are always longer than the osteosclereids at midseed: never more than three times longer in *L. micranthus* (Fig. 1 D), and at least five-six times longer in *L. anatolicus* (Fig. 1 B) and *L. pilosus* (Fig. 1 F). The elongated palisade cells exhibit two distinct portions: the inner portion is characterized by thickened irregular and heterogeneous walls, whereas the outer portion appears with smooth and homogeneous radial cell walls. The palisade cells are clearly fascicled in their upper part forming prominent pluricellular protuberances (or tubercles) separated by more or less wide spaces in *L. anatolicus* (Fig. 1 B) and *L. pilosus* (Fig. 1 F). The distinct thin cuticular membrane stretched over and between the tubercle tops contributes to the design of the typical pattern observed at high magnification on the seed testa surface of *L. anatolicus* and *L. pilosus* (Fig. 1 A and E). In *L. micranthus*, the outer portion of palisade cells is always compact (as in all

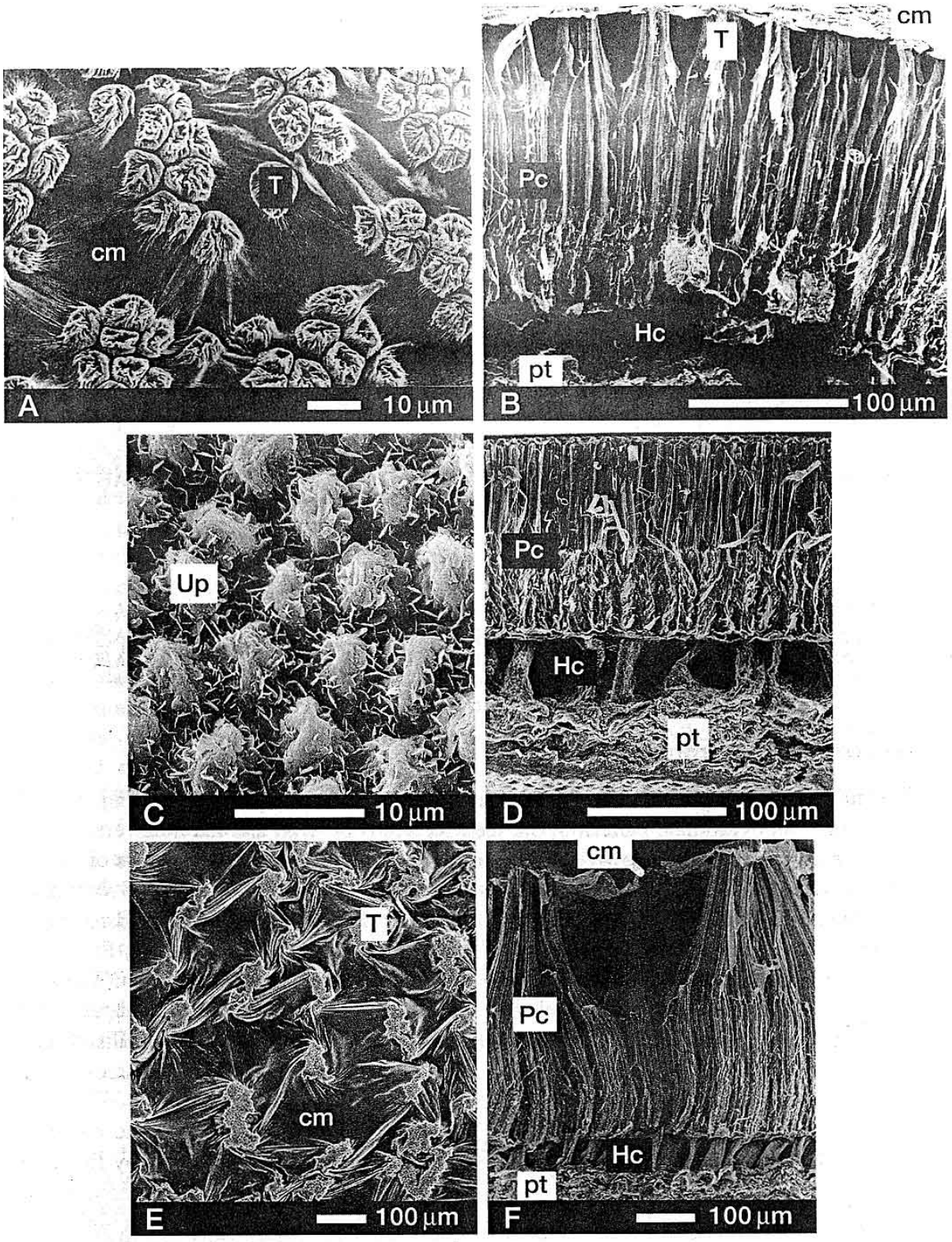

Fig. 1. Seed-coat micromorphological patterns of three Old World lupines. A–B – *Lupinus anatolicus*, C–D – *L. micranthus*, E–F – *L. pilosus*; A, C and E – seed-surface patterns; B, D and F – tranverse section of testa; cm – cuticular membrane; Pc – palisade cells; Hc – hypodermis cells; T – fascicled palisade cells or tubercles (pluricellular pattern); Up – unicellular pattern; pt – parenchymatous tissue.

Table 2. Pairwise divergence between sequences of combined ITS1-ITS2 nrDNA region from eleven Old World *Lupinus* taxa and three outgroups. The divergence between taxa is estimated (as percentage) by the "mean distance" (adjusted for missing data) using PAUP program. In boldface type are the divergence values between *L. anatolicus* and each of the other Old World lupine taxa.

Taxon no.	1	2	3	4	5	6	7	8	9	10	11	12	13
Taxon													
1 *Lupinus albus*	-												
2 *L. anatolicus*	**4.1**	-											
3 *L. angustifolius*	2.4	**4.3**	-										
4 *L. atlanticus*	3.4	**1.5**	4.1	-									
5 *L. cosentinii*	3.0	**1.1**	3.7	0.4	-								
6 *L. digitatus*	3.4	**1.5**	4.1	0.4	0.4	-							
7 *L. hispanicus*	2.6	**5.2**	1.7	4.5	4.1	4.5	-						
8 *L. luteus*	2.8	**5.2**	2.4	4.5	4.1	4.5	1.5	-					
9 *L. micranthus*	3.3	**5.9**	4.3	5.4	5.0	5.4	4.8	4.8	-				
10 *L. palaestinus*	3.4	**0.6**	3.7	0.9	0.4	0.9	4.5	4.5	5.2	-			
11 *L. pilosus*	3.4	**0.6**	3.7	0.9	0.4	0.9	4.5	4.5	5.2	0.0	-		
12 *Chamaecytisus mollis*	6.9	8.6	6.9	8.0	7.6	8.0	8.0	8.2	8.9	8.0	8.0	-	
13 *Crotalaria podocarpa*	13.1	15.5	12.9	14.4	14.4	14.4	14.2	14.6	13.8	14.8	14.8	12.4	-
14 *Genista tinctoria*	8.9	11.3	9.5	10.6	10.2	10.6	10.2	10.2	10.2	10.6	10.6	8.0	14.2

the "smooth-seeded" taxa; AÏNOUCHE 1991, 1998) and provides a micromorphological seed surface pattern represented by small protuberances (or "papillose pattern" according to LERNSTEN 1981) with overlaying epicuticular substances (Fig. 1 C). Each protuberance (or papilla) corresponds to the top of a single palisade cell.

ITS nuclear rDNA data

The entire ITS1 + 5.8S + ITS2 region of the 18S-26S nrDNA repeat has a length of 628 bp in *L. anatolicus*. The 5.8S sequence found in *L. anatolicus* is identical in length (163 bp) and nucleotide content to that of all the Old World lupines, with the exception of *L. micranthus* which displays one autapomorphous change at its 5'-end boundary. Thus, nearly all the nucleotide substitutions are within the ITS regions. The combined ungapped ITS regions are 465 bp in length: 236 bp for the ITS1 region, and 229 bp for the ITS2. These data are in general accordance with those obtained from our previous studies on Old World lupines (AÏNOUCHE & BAYER 1999a, and references therein). The proportion of nucleotide differences in pairwise sequence comparisons between *L. anatolicus* and the Old World lupines ranged from 4.1 to 5.9% with the smooth-seeded taxa; it ranged from 0.6 to 1.5% with the rough-seeded ones (see Tab. 2). The least divergent (0.6%) species from *L. anatolicus* are *L. pilosus* and *L. palaestinus*, whereas the most distant one (5.9%) is *L. micranthus*.

Using *Crotalaria podocarpa* as the outgroup, the phylogenetic analysis (branch-and-bound search) of the ITS1+ITS2 sequence data generated two maximum parsimonious (MP) trees of 154 steps with a consistency index of 0.909 and a retention index of 0.863. The strict consensus tree of these two MP trees is presented in Fig. 2. Although the New World lupines are not taken into account in this work, the topology shown in Fig. 2 is conform to the general topology previously obtained by most extensive ITS-based phylogenies of *Lupinus*, including all the Old World taxa and numerous representatives from the New World (KÄSS & WINK

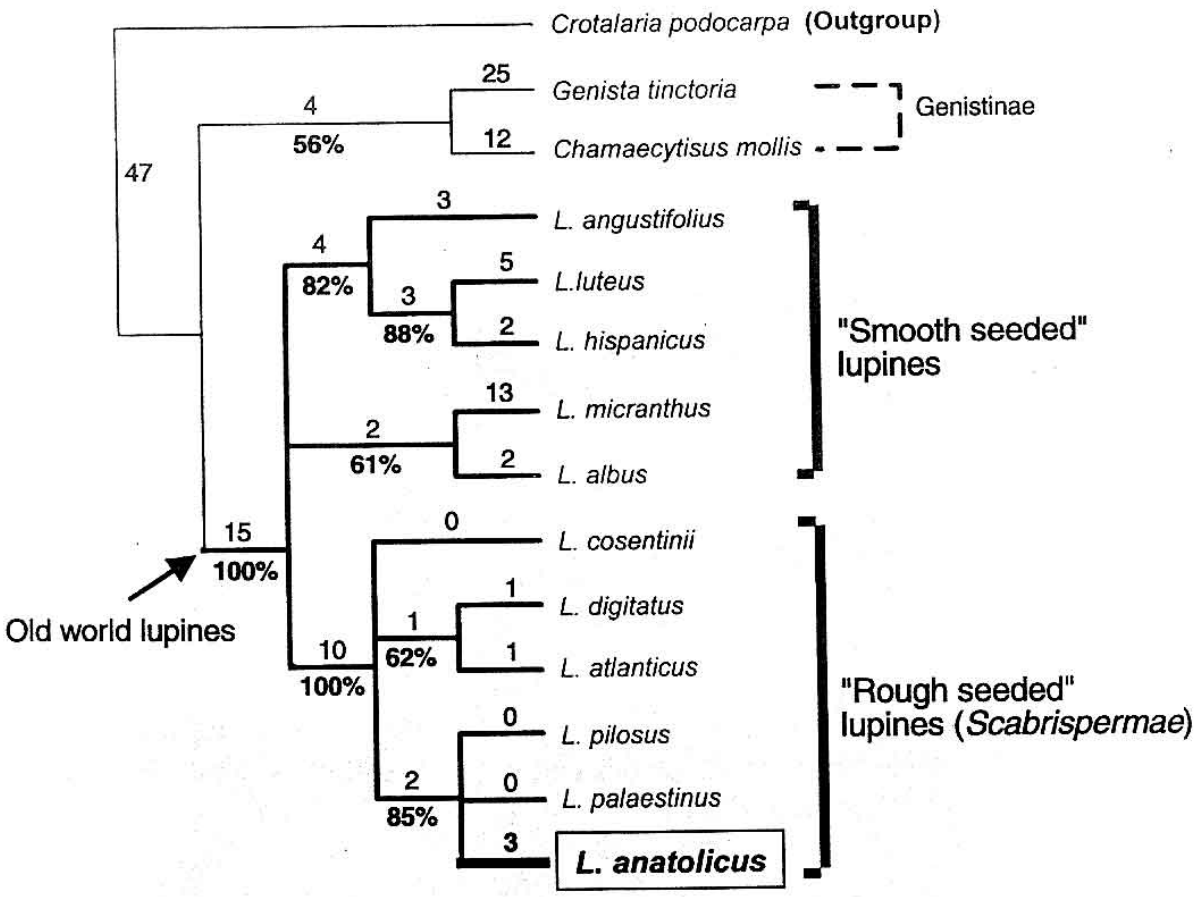

Fig. 2. Phylogenetic position of *Lupinus anatolicus* W. SWIECICKI et W.K. SWIECICKI relative to the Old World lupine taxa. Presented is the strict consensus of two maximum parsimonious trees inferred from combined ITS1+ITS2 nrDNA sequence data of eleven lupine species and three extralupine taxa. *Crotalaria podocarpa* was designated as the outgroup in the analysis. A branch-and-bound search was computed with gaps coded as missing data and all characters weighted equally, via stepwise addition of furthest sequences and "MULPARS" option in effect. Tree length = 154 steps; consistency index = 0.909; retention index = 0.863. The number of synapomorphies are above each branch, and bootstrap values (1000 replicates) are given below the branches.

1997, AÏNOUCHE and BAYER 1999a). The Mediterranean and African taxa are distributed into three distinct clades, whose relationships remained unresolved within the monophyletic genus *Lupinus*. The Old World "smooth-seeded" lupines fall into two clades, one containing *L. albus* and *L. micranthus* and the other *L. angustifolius*, *L. luteus* and *L. hispanicus*, while all the *Scabrispermae* ("rough-seeded" taxa) are in the same monophyletic group that is strongly supported by 10 base-pair changes and a bootstrap value of 100%. *Lupinus anatolicus* is clearly positioned in the *Scabrispermae* clade where it is related to the subclade comprising *L. pilosus* and *L. palaestinus*. This subclade is well supported by two synapomorphies and a bootstrap value of 85%. Moreover, *L. anatolicus* diverged from its close relatives, *L. pilosus* and *L. palaestinus* which both exhibit the same ITS sequence, by three strictly autapomorphous mutations, two transitions and one transversion localized in the ITS2 region.

DISCUSSION AND CONCLUSION

The aim of this work was to elucidate the status of the new smooth-seeded lupine species, *L. anatolicus* (SWIECICKI et al. 1996), using two independent sources of data, seed-coat micromorphological characters and molecular mar' rs, that have proven to be useful for resolving taxonomy and systematics of Old World lupines (HEYN & HERRNSTADT 1977, AÏNOUCHE 1998, AÏNOUCHE & BAYER 1999a, and references therein).

Seed-coat topography and structure were demonstrated to be significant characters in taxonomic studies of several *Papilionoideae* (HEYN & HERRNSTADT 1977, LERSTEN 1981, SAINT-MARTIN 1986). It has been stressed that seeds of the majority of taxa appeared smooth when examined with the naked eye or at low magnifications. Their micromorphological seed-coat patterns were revealed and accurately defined only after higher magnifications (LERSTEN 1981). The basic transectional structure of the seed testa viewed in *L. anatolicus* is similar to that of the lupines previously surveyed (HEYN & HERRNSTADT 1977, BRAGG 1983, SERRATO VALENTI et al. 1989, AÏNOUCHE 1991, 1998) and typical of the general pattern of *Papilionoideae* (CORNER 1951, GUNN 1981). The palisade cells differentiation into two radial portions (inner and outer) was also previously observed in other *Lupinus* species: *L. texensis* HOOK., *L. subcarnosus* HOOK. and *L. havardi* WATSON (BRAGG 1983), *L. angustifolius* (SERRATO VALENTI et al. 1989) and in many other Old and New World lupines (AÏNOUCHE, unpubl.). The micromorphological seed-coat pattern of *L. anatolicus*, as revealed by combining SEM observation of a transverse section with surface topography of the seed testa, is defined by pluricellular protuberances or tubercles (Fig. 1 A–B). This micromorphological pattern is clearly distinct from that of *L. micranthus* (Fig. 1 C–D), as well as from all patterns exhibited by the "smooth-seeded" lupines. The latter are characterized by a smooth macroscopic seed surface and by various but always unicellular submicroscopic seed-coat patterns (HEYN & HERRNSTADT 1977, AÏNOUCHE 1991, 1998). Instead, *L. anatolicus* displays a tuberculated micromorphological seed-coat pattern, which is typical of the *Scabrispermae* (i.e. the "rough-seeded" species of GLADSTONES, including *L. pilosus* and *L. palaestinus*) and is unique in the genus *Lupinus*. Therefore, based upon micromorphological data, *L. anatolicus* clearly appears related to *Scabrispermae*, despite the macroscopic "smooth" appearance of its seeds. Comparatively to all other accessions previously surveyed in *L. pilosus* (HEYN & HERRNSTADT 1977, PLITMANN & HEYN 1984, AÏNOUCHE 1991, 1998), the narrower spaces observed between the tubercles in *L. anatolicus* explain the apparent macroscopic smoothness of its seed surface, a feature that is accentuated by the small size of the seeds.

Based upon ITS-nrDNA sequence data, genetic distances and phylogenetic relationships among the Old World lupines clearly distinguished the Anatolian accession from all the Old World "smooth-seeded" taxa and especially from *L. micranthus,* which was the most distant taxon. Instead, the ITS-based phylogeny unambiguously supports *L. anatolicus* as a member of the strongly supported monophyletic group containing all the *Scabrispermae*, in agreement with micromorphological data. Moreover, ITS data indicate that among *Scabrispermae*, *L. anatolicus* shares a common ancestor with the two closely related eastern Mediterranean taxa, *L. pilosus* and *L. palaestinus* (Fig. 2).

Interestingly, different morphological wild types have been observed within *L. pilosus* in relation with geographical origins of accessions (GLADSTONES & CROSBIE 1979, GLADSTONES 1984, CLEMENTS et al. 1996). Among the wild types described, the "smooth-seeded" macroscopic character was found by CLEMENTS et al. (1996) in Syrian natural variants referred to *L. pilosus* that appeared similar to the Anatolian lupine accession in morphology, flowering

time and chromosome number (2n=42). Moreover, although SWIECICKI et al. (1996) failed to obtain viable seeds in crosses between the Anatolian accession and each of *L. micranthus* (2n=52) and *L. pilosus* (2n=42), CLEMENTS et al. (1996) reported that both the latter Anatolian and Syrian lupine lines artificially crossed with other *L. pilosus* accessions.

Therefore, both micromorphological and molecular data from this study, together with cytological and crossing data (CLEMENTS et al. 1996), provide evidence that the Anatolian lupine taxon of SWIECICKI et al. (1996) is closely related to *L. pilosus* (Sect. *Scabrispermae*). However, as shown by independent studies using diverse approaches and different samples of *L. pilosus*, the Anatolian lupine accession differs significantly from other *L. pilosus* wild types to which it has been compared (with exception of the "Syrian accessions" in morphological and physiological aspects), based on various lines of data: morphology (including the apparent smoothness of seeds), flowering time, chemical composition, isozymes and seed storage proteins electrophoretic patterns (PRZYBYLSKA & ZIMNIAK-PRZYBYLSKA 1995, SWIECICKI et al. 1996). Recently, OBERMAYER et al. (1999) demonstrated that not only the genome size varied significantly within *L. pilosus*, but also that the genome of *L. anatolicus* was smaller than the smallest genome found in *L. pilosus*. Also the ITS sequence data reveal a clear divergence (3 nucleotide differences) between this Anatolian accession and a representative sample of the most common wild type of *L. pilosus* analyzed in this study (Fig. 2). Such divergence is remarkable since no ITS sequence differences were usually found within lupine species (KÄSS & WINK 1997, AÏNOUCHE & BAYER 1999a). Furthermore, the molecular divergence between *L. anatolicus* and one sample of *L. pilosus* contrasts with the ITS sequence identity exhibited by *L. pilosus* and *L. palaestinus*. The two latter taxa, which greatly resemble one another in morphology and have the same chromosome number (2n=42), are still incompletely isolated genetically when artificially crossed under particular conditions (KAZIMIERSKI 1961, PAZY et al. 1981). They are, however, considered as two distinct species isolated from one another in nature by different reproductive barriers such as ecological requirements, prevalent selfing and partial genetic incompatibility (PAZY et al. 1981, GLADSTONES 1984). Moreover, *L. pilosus* was shown to successfully cross (artificially) with other ecogeographically isolated "rough-seeded" species such as *L. atlanticus* (2n=38) and *L. cosentinii* (2n=32) indicating that all these taxa, which are differentiated from one another by only a few ITS nucleotide changes (Fig. 2), still are genomically close to each other while they evolve independently in nature (ROY & GLADSTONES 1988, CARSTAIRS et al. 1992, GUPTA et al. 1996).

The above data, and others, which revealed distinct genotypes within *L. pilosus* (GLADSTONES 1984, CLEMENTS et al. 1996), indicate that adaptive and genetic differentiation processes are currently ongoing in this taxon, following the fragmentation and isolation (geographic and reproductive) of populations. The genetic isolation of natural populations within *L. pilosus* is favoured and maintained by their prevalent selfing pollination system, despite potential gene flow between them (PLITMANN et al. 1980, PAZY et al. 1981). The considerable natural variation emphasized in *L. pilosus* led the authors to suggest possible taxonomic implications at the infraspecific level (GLADSTONES 1984, CLEMENTS et al. 1996).

Finally, both micromorphological and ITS sequence results unambiguously support the new Anatolian lupine accession of SWIECICKI et al. (1996) as part of the *L. pilosus*-*L. palaestinus* lineage within the strongly monophyletic group of *Scabrispermae*. Accordingly, these results are hardly compatible with the hypothesis that this lupine accession could be related to the Old World "smooth-seeded" lupines (including *L. micranthus*).

Moreover, most combined data from this study (including macromorphological observations) and from the literature cited above (especially cytological and crossing data) demonstrate a close relationship of this Anatolian accession to *L. pilosus*, which considerably weakens the species status of *L. anatolicus*. As pointed out by CLEMENTS et al. (1996), it seems indeed premature to make this Anatolian lupine a new separate species. Nevertheless, it is worth noting that this accession conspicuously displays multiple genetic differences when independently compared to each of the different populations surveyed in *L. pilosus*, and that these differences appeared strikingly more important than those observed between *L. pilosus* and *L. palaestinus* (PRZYBYLSKA & ZIMNIAK-PRZYBYLSKA 1995, SWIECICKI et al. 1996, OBERMAYER et al. 1999, including the ITS data from this study).

However, further investigations over a wide and representative sampling of the natural diversity of *L. pilosus* and of the Anatolian lupines are needed in order to determine whether: (1) the new Anatolian lupine accession of SWIECICKI et al. (1996) and the "smooth-seeded Syrian variants" of CLEMENTS et al. (1996) (and possible other undiscovered relative populations) belong to the same lupine wild type, and fall within the range of the wide natural variation of *L. pilosus*; or (2) whether this Anatolian lupine could represent a distinct natural entity deriving from within the *L. pilosus-L. palaestinus* lineage.

This study additionally confirms that seed-coat micromorphology provides useful diagnostic features to distinguish the *Scabrispermae* from the "smooth-seeded" taxa in *Lupinus*, and that phylogenetic analysis of ITS sequence data from the nuclear ribosomal DNA repeat is efficient to assess the phylogenetic position of a given accession relative to the lupine taxa currently circumscribed in the Old World.

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