

Phylogeny of the genus *Lotus* (Leguminosae, Loteae): evidence from nrITS sequences and morphology

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Abstract: *Lotus* (120–130 species) is the largest genus of the tribe Loteae. The taxonomy of *Lotus* is complicated, and a comprehensive taxonomic revision of the genus is needed. We have conducted phylogenetic analyses of *Lotus* based on nrITS data alone and combined with data on 46 morphological characters. Eighty-one ingroup nrITS accessions representing 71 *Lotus* species are studied; among them 47 accessions representing 40 species are new. Representatives of all other genera of the tribe Loteae are included in the outgroup (for three genera, nrITS sequences are published for the first time). Forty-two of 71 ingroup species were not included in previous morphological phylogenetic studies. The most important conclusions of the present study are (1) addition of morphological data to the nrITS matrix produces a better resolved phylogeny of *Lotus*; (2) previous findings that *Dorycnium* and *Tetragonolobus* cannot be separated from *Lotus* at the generic level are well supported; (3) *Lotus creticus* should be placed in section *Pedrosia* rather than in section *Lotea*; (4) a broad treatment of section *Ononidium* is unnatural and the section should possibly not be recognized at all; (5) section *Heinekenia* is paraphyletic; (6) section *Lotus* should include *Lotus conimbricensis*; then the section is monophyletic; (7) a basic chromosome number of $x = 6$ is an important synapomorphy for the expanded section *Lotus*; (8) the segregation of *Lotus schimperii* and allies into section *Chamaelotus* is well supported; (9) there is an apparent functional correlation between styliodum and keel evolution in *Lotus*.

Key words: Leguminosae, Loteae, *Lotus*, nuclear ribosomal ITS sequences, morphology.

Résumé : Le genre *Lotus* (120–130 espèces) est le plus grand de la tribu des Loteae. La taxonomie des *Lotus* est compliquée, et une révision taxonomique complète du genre s'impose. Les auteurs ont conduit des analyses phylogénétiques des *Lotus*, sur la base des données nrITS isolément et combinées avec les données sur 46 caractères morphologiques. Les auteurs ont étudié 81 accessions nrITS d'un groupe interne représentant 71 espèces de *Lotus*; parmi celle-ci, 47 accessions représentant 40 espèces sont nouvelles. On retrouve des représentants de tous les autres genres de la tribu Loteae dans le groupe externe (pour trois de ces genres, on publie les séquences nrITS pour la première fois). Des 71 espèces du groupe interne, 42 n'ont pas été incluses dans des études morpho-phylogénétiques précédentes. Les plus importantes conclusions de cette étude sont: (1) l'addition de données morphologiques à la matrice nrITS conduit à une meilleure résolution phylogénétique des *Lotus*; (2) on confirme les constats antécédents à l'effet que les *Dorycnium* et *Tetragonolobus* ne peuvent pas être séparés des *Lotus* au niveau du genre; (3) le *L. creticus* devrait être placé dans la section *Pedrosia*, plutôt que la section *Lotea*; (4) le traitement général de la section *Ononidium* n'est pas naturel et la section devrait possiblement ne pas être reconnue du tout; (5) la section *Heinekenia* est paraphylétique; (6) la section *Lotus* doit inclure le *L. conimbricensis*; la section devient alors monophylétique; (7) le nombre de base de chromosomes $x = 6$ est une importante synapomorphie pour la section *Lotus* étendue; (8) la ségrégation du *L. schimperii* et alliés dans la section *Chamaelotus* est bien supportée; (9) il y a une apparente corrélation fonctionnelle entre l'évolution du styliodum et de la carène chez les *Lotus*.

Mots clés : Leguminosae, Lotae, *Lotus*, séquences de l'ITS nucléaire ribosomal, morphologie.

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Introduction

There is little agreement in the literature regarding generic limits of *Lotus* (e.g., Greene 1890; Taubert 1894; Brand 1898; Ottley 1944; Callen 1959; Gillett 1959; Hutchinson 1964; Polhill 1981, 1994; Isely 1981; Lassen 1986; Kirkbride 1994, 1999; Kramina and Sokoloff 1997, 2001; Talavera and Salgueiro 1999; Sokoloff 1999, 2000, 2003a, 2003b). The (lecto) type species, *Lotus corniculatus*, as well as its closest relatives are native to the Old World. Many species are confined to or common within the Mediterranean Region. There are several Old World taxa that are either included in *Lotus* or accepted as distinct genera by various

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taxonomic authorities. Among them, the mostly Mediterranean (also in other parts of Europe and western Asia) *Dorycnium* Mill. (8–10 species) and *Tetragonolobus* Scop. (5–6 species) are most important (Rikli 1901; Dominguez and Galiano 1979). Other problematic Old World genera variously included or excluded from *Lotus* are *Podolotus* Royle (1 species found in India, Pakistan, Afghanistan, Iran, and Oman; Rechinger 1984), *Pseudolotus* Rech.f. (1 species found in Pakistan, Iran, and Oman; Rechinger 1984; Ali and Sokoloff 2001), *Kebirita* Kramina & Sokoloff (1 species in the Sahara, northwestern Africa; Kramina and Sokoloff 2001), and *Benedictella* Maire (1 species in Morocco; Maire 1924).

In the New World, species related to *Lotus* are most diverse in California. Recent studies based on nrITS sequences (Allan and Porter 2000; Allan et al. 2003) and morphology (Arambarri 2000a; Arambarri et al. 2005; Sokoloff 2006) clearly show that New World species are not closely related to Old World *Lotus*. According to nrITS data, Old World *Lotus* is closer to the Old World genera *Hammatolobium* and *Tripodion* than to New World Loteae (Allan et al. 2003; Degtjareva et al. 2003). Thus all New World species should be excluded from the genus *Lotus*; in our opinion (Sokoloff 1999, 2000; Sokoloff and Lock 2005), they form four different genera (*Hosackia* Douglas ex Benth., *Ottleya* D.D. Sokoloff, *Acmispon* Raf., and *Syrmatium* Vogel).

Phylogenetic studies of the tribe Loteae based on nrITS sequences and morphology show a clade containing *Dorycnium*, *Tetragonolobus*, and Old World species of *Lotus* studied so far (Allan and Porter 2000; Allan et al. 2003, 2004; Sokoloff 2003b, 2006). All analyses clearly show that *Tetragonolobus* is derived from within Old World *Lotus* (Allan and Porter 2000; Arambarri 2000b; Allan et al. 2003; Sokoloff 2006). It is logical to include *Tetragonolobus* within *Lotus*. In the molecular phylogenetic study by Allan et al. (2003), the four *Dorycnium* species analyzed did not form a clade. In the morphological cladistic study of Arambarri (2000b), *Dorycnium* is nested in the Old World *Lotus* clade as a close relative of *Lotus corniculatus* and its allies. Since morphological grounds for separation of *Dorycnium* from *Lotus* are equivocal, Sokoloff (2003a) has suggested following Polhill (1981) in placing all *Dorycnium* species in *Lotus*.

Of four monospecific and problematic Old World genera, nrITS data have only been published for *Kebirita* (Allan et al. 2003). Molecular and morphological data clearly show that *Kebirita* is distinct from Old World *Lotus* and deserves generic rank (Sokoloff 2006). Cladistic analyses based on morphological characters suggest that *Benedictella* should be included within *Lotus* (Sokoloff 2003b), but generic rank is supported for *Podolotus* and *Pseudolotus* (Sokoloff 2006).

Although recent phylogenetic data have provided a much better understanding of generic limits and relationships of *Lotus*, the sectional classification of the genus remains problematic. Different authors accept very different classification systems for *Lotus* species (e.g., Fig. 1). Only a few authors discuss all species worldwide while many sectional systems

are introduced in regional Floras. Recent phylogenetic (Allan and Porter 2000; Arambarri 2000b; Allan et al. 2003, 2004) and phenetic (Stenglein et al. 2004) studies clarified some problems; however, many problematic species and some sections were not included in these analyses. Phylogenetic trees based on morphology (Arambarri 2000b) and nrITS data (Allan et al. 2003, 2004) differ significantly in topology, but they also differ considerably in species sampling.

The objectives of this paper are (1) to increase taxon sampling in nrITS phylogenetic analyses of Loteae and (2) to conduct, for the first time, a combined phylogenetic analysis of *Lotus* based on morphological and nrITS data for the same set of species. Our study should help to clarify sectional limits in the genus *Lotus* and their phylogenetic relationships.

Material and methods

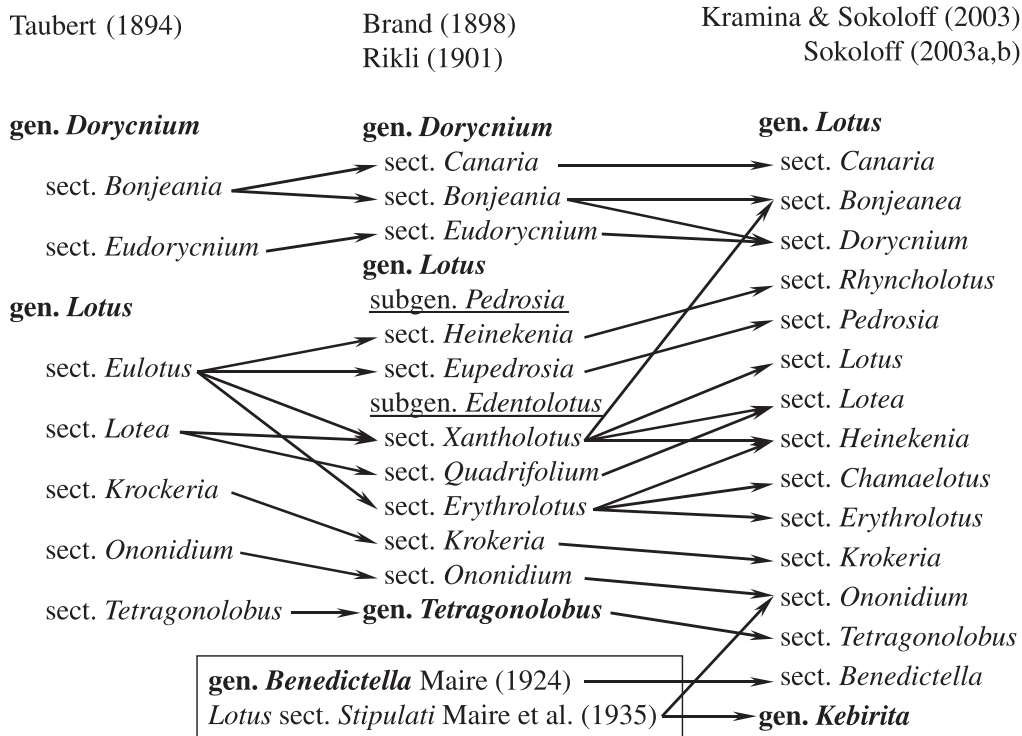
Complete sequences of ITS1 and ITS2 were generated for 51 accessions representing 44 species of the genus *Lotus* and related genera. In addition, GenBank data on the ITS region in 49 taxa of Loteae are used (Table 1). In total, 81 ingroup nrITS accessions representing 71 *Lotus* species were studied (i.e., more than half of the total number of *Lotus* species, which is estimated as 120–130). The taxon sampling covers all sections of *Lotus*. However, we were able to produce only ITS1 sequence of the rare endemic *L. benoistii* (Maire) Lassen from Morocco (monospecific section *Benedictella*). This sequence was not included in the main analyses. Except for *Lotus* and *Hammatolobium*, each genus of the tribe Loteae is represented by one species in the present study. Members of Robinieae (*Robinia*) and Sesbanieae (*Sesbania*) are used as outgroups because higher level molecular phylogenetic studies of legumes strongly support a close relationship of these two tribes to the Loteae (e.g., Wojciechowski et al. 2000; Lewis et al. 2005). In the Results and Discussion sections, the taxonomy of Kramina and Sokoloff (2003) and Sokoloff (2003a, 2003b) is used (see Table 2 for details) because it is the only recent system of *Lotus* that assigns each species worldwide to a particular section.

DNA was isolated from leaf tissue using the CTAB method of Doyle and Doyle (1987). PCR reactions were performed with universal primers (White et al. 1990). Both spacer regions were sequenced in their entirety for both strands. The sequences obtained were aligned manually using the SED editor of the VOSTORG package (Zharkikh et al. 1990).

A morphological data matrix was produced for the same set of species (Appendix A and supplementary data.²). A total of 46 characters were obtained mostly from original morphological observations. Literature data on chromosome numbers were also used (Grant 1965, 1995; Fedorov 1969; Goldblatt and Johnson 1996, 1998). Three multistate morphological characters were coded as additive while others were binary or multistate nonadditive. The following characters were coded as additive: flower number per partial inflorescence (18), flower size (22), and basic chromosome

²Supplementary data for this article are available on the journal Web site (<http://canjbot.nrc.ca>) or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Building M-55, 1200 Montreal Road, Ottawa, ON K1A 0R6, Canada. DUD 5039. For more information on obtaining material refer to http://cisti-icist.nrc-cnrc.gc.ca/irm/unpub_e.shtml.

Fig. 1. Historical changes in sectional classification of Old World *Lotus*.



number (46). We decided to use the additive coding because of the nature of the characters was such that some character states are intermediate between the others. For example, there are reasons to hypothesize that evolutionary transitions between basic chromosome numbers $x = 8$ and $x = 6$ were most likely performed through the intermediate number $x = 7$ (see also Grant 1991). It is reasonable to suppose that evolutionary transitions between large and small flowers occurred via mid-size flowers. All characters were not a priori polarized in our analyses. Maximum parsimony and Bayesian analyses were performed for a combined molecular-morphological data set as well as for the molecular data alone. No phylogenetic analysis of morphological data alone was performed because there are insufficient characters to produce a resolved phylogeny. Some characters, such as those of pollen morphology (Crompton and Grant 1993; Díez and Ferguson 1994) are relatively uniform among Old World *Lotus* species and offer little phylogenetic information at this level. Seed morphology (Arambarri 1999) and leaf epidermal microcharacters (Stenglein et al. 2004) offer significant and useful characters, but many species included in the present analyses have not yet been studied for these aspects.

Bayesian inference of phylogeny was explored using the MrBayes program (version 3.1; Ronquist and Huelsenbeck 2003). The evolutionary model implemented in MrBayes for morphological data are analogous to a Jukes-Cantor model with a variable number of states. For the analyses of molecular data, the GTR+I+ Γ model of nucleotide substitutions was selected by the Akaike Information Criterion in Modeltest (Posada and Crandall 1998). A total of 3 000 000 generations were performed and trees from first 2 200 000 generations were discarded. The number of generations to

be discarded was determined using a convergence diagnostic. Parsimony analysis involved a heuristic search conducted with PAUP* (version 4.0b8; Swofford 2000) using tree bisection-reconnection (TBR) branch swapping with character states specified as equally weighted. One hundred replicates with random addition of sequences were performed and all shortest trees were saved. Bootstrap (Felsenstein 1985) analysis was performed to assess the degree of support for particular branches on the tree. Bootstrap values were calculated from 100 replicate analyses with TBR branch swapping and random addition sequence of taxa. One thousand most parsimonious trees from each replicate were saved. In the parsimony analyses all gaps were treated as missing data.

Results

Analyses of nrITS sequences (Fig. 2)

The length of the ITS region (ITS1, 5.8S, and ITS2) ranged from 587 to 617 bp for the 99 accessions of the in-group and two outgroup taxa studied. The length of the ITS1 region varied from 210 to 239 bp and the ITS2 region from 194 to 229 bp. The 5.8S gene was 163–164 bp in length. The alignment of 101 ITS sequences resulted in matrix of 646 nucleotide positions after excluding 332 ambiguous positions. A total of 261 characters were parsimony-informative, 299 characters were constant, and 86 variable characters were parsimony-uninformative. Our study revealed a length polymorphism of the ITS1 spacer for two species, a 4 bp duplication in *Lotus cytisoides* and a 1 bp duplication in *Lotus preslii*.

In the maximum parsimony analysis, 20 004 shortest trees (1461 steps) were found, with a consistency index of 0.411

Table 1. GenBank accession numbers and sources of nrITS sequences used in this paper.

Species	GenBank No.	First publication of the sequence or voucher data
<i>Acmispon americanus</i> (Nutt.) Rydb. [= <i>Lotus unifoliolatus</i> (Hook.) Benth.]	AF450183	Allan et al. (2003)
<i>Anthyllis onobrychioides</i> Cav.	AF450210	Allan et al. (2003)
<i>Antopetitia abyssinica</i> A. Rich.	DQ166212	This paper; <i>Auquier 2598</i> (BE)
<i>Coronilla viminalis</i> Salisb.	DQ166213	This paper; Morocco, <i>Podlech 53755</i> (M)
<i>Cytisopsis pseudocytisus</i> (Boiss.) Fertig	AY325282	Degtjareva et. al. (2003)
<i>Dorycnopsis abyssinica</i> (A. Rich.) V.N. Tikhom. & D.D. Sokoloff	AF450235	Allan et al. (2003)
<i>Hammatolobium lotooides</i> Fenzl	AY325279	Degtjareva et. al. (2003)
<i>Hippocrepis emerus</i> (L.) Lassen	AF218531	Allan and Porter (2000)
<i>Hosackia crassifolia</i> Benth. [= <i>Lotus crassifolius</i> (Benth.) Greene]	AF218523	Allan and Porter (2000)
<i>Kebirita roudairei</i> (Bonnet) Kramina & D.D. Sokoloff (= <i>Lotus roudairei</i> Bonnet)	AF450200	Allan et al. (2003)
<i>Ornithopus micranthus</i> (Benth.) Arechav.	AY325277	Degtjareva et. al. (2003)
<i>Otleya oroboides</i> (Kunth) D.D. Sokoloff [= <i>Lotus oroboides</i> (Kunth) Ottley]	AF218510	Allan and Porter (2000)
<i>Podolotus hosackioides</i> Benth.	DQ166214	This paper; Afghanistan, 13 Apr. 1967, <i>Freitag s.n.</i> (KAS)
<i>Pseudolotus villosus</i> (Blatter & Hallb.) Ali & D.D. Sokoloff	DQ166215	This paper; Oman, <i>Redcliffe-Smith 3901</i> (K)
<i>Robinia pseudoacacia</i> L.	AF218538	Allan and Porter (2000)
<i>Scorpiurus vermiculatus</i> L.	AF218536	Allan and Porter (2000)
<i>Sesbania vesicaria</i> (Jacq.) Elliott	AF398761	Lavin et al. (2001)
<i>Syrmatium glabrum</i> Vogel [= <i>Lotus scoparius</i> (Nutt.) Ottley]	AF218521	Allan and Porter (2000)
<i>Tripodion tetraphyllum</i> (L.) Fourr.	AF218498	Allan and Porter (2000)
<i>Lotus</i> sect. <i>Benedictella</i> (Maire) Kramina & D.D. Sokoloff (1/1)		
<i>L. benoisstii</i> (Maire) Lassen	DQ372916	This paper; Morocco, 31 Mar. 1934, <i>Maire & Wilczek s.n.</i> (Z)
<i>Lotus</i> sect. <i>Bonjeanea</i> (Rchb.) D.D. Sokoloff (3/3)		
<i>L. hirsutus</i> L. [= <i>Dorycnium hirsutum</i> (L.) Ser.]	AY294292	Allan and Porter (2000)
<i>L. rectus</i> L. [= <i>Dorycnium rectum</i> (L.) Ser.]	AF218503	Allan and Porter (2000)
<i>L. strictus</i> Fisch. & C.A. Mey.	DQ160286	This paper; Asiatic Russia, 18 Sep. 2003, <i>Korolyuk s.n.</i> (MW)
<i>Lotus</i> sect. <i>Canaria</i> (Rikli) D.D. Sokoloff (3/1)		
<i>L. broussonetii</i> Choisy ex Ser. [= <i>Dorycnium broussonetii</i> (Choisy ex Ser.) Webb et Berth.]	DQ160278	This paper; plant cultivated at Royal Botanic Gardens, Kew, introduced from Canary Is., <i>Chase 16057</i> (K)
<i>Lotus</i> sect. <i>Chamaelotus</i> Kramina & D.D. Sokoloff (3/2)		
<i>L. glinoides</i> Del. (1)	DQ160282	This paper; Spain, Canary Is., <i>Nydegger 26086</i> (MHA)
<i>L. glinoides</i> Del. (2)	DQ166220	This paper; Egypt, 7 May 1962, <i>Bochantsev s.n.</i> (LE)
<i>L. schimperi</i> Steud. ex Boiss.	DQ166218	This paper; Oman, <i>McLeish 3458</i> (E)
<i>Lotus</i> sect. <i>Dorycnium</i> (Mill.) D.D. Sokoloff (5/2)		
<i>L. dorycnium</i> L. s.l. [= <i>Dorycnium herbaceum</i> Vill.]	AF218501	Allan and Porter (2000)
<i>L. graecus</i> L. [= <i>Dorycnium graecum</i> (L.) Ser.]	AF218500	Allan and Porter (2000)
<i>Lotus</i> sect. <i>Erythrolotus</i> Brand (1/1)		
<i>L. conimbricensis</i> Brot.	AF450186	Allan et al. (2003)
<i>Lotus</i> sect. <i>Heinekenia</i> Webb & Berth. (23/14)		
<i>Lotus arabicus</i> group		
<i>L. arabicus</i> L.	AF450176	Allan et al. (2003)
<i>L. lalambensis</i> Schweinf.	DQ166216	This paper; Saudi Arabia, <i>Collenette 7908</i> (E)
<i>L. lanuginosus</i> Vent.	DQ166221	This paper; Jordan, <i>Townsend 65/22</i> (LE)
<i>L. loricus</i> Rech.f., Aellen & Esfand.	DQ166233	This paper; Abu Dhabi, <i>Western 275</i> (E)
<i>L. quinatus</i> (Forssk.) J.B. Gillett	DQ166217	This paper; Yemen, <i>Thulin et al. 9374</i> (E)
<i>Lotus australis</i> group		
<i>L. australis</i> Andrews	AF450187	Allan et al. (2003)

Table 1 (continued).

Species	GenBank No.	First publication of the sequence or voucher data
<i>L. cruentus</i> Court	AF450182	Allan et al. (2003)
<i>Lotus discolor</i> group		
<i>L. discolor</i> E. Mey.	DQ160288	This paper, <i>Lisocuski B-3330</i> (BE)
<i>L. goetzei</i> Harms	DQ166235	This paper; Kenya, <i>Gillett 16179</i> (LE)
<i>L. mlanjeanus</i> J.B. Gillett	DQ166232	This paper; Malawi, <i>J.D. & E.G. Chapman 8807</i> (E)
<i>L. wildii</i> J.B. Gillett	DQ160287	This paper; Zimbabwe, <i>Bayliss 10166</i> (E)
<i>Lotus gebelia</i> group		
<i>L. aegaeus</i> (Griseb.) Nym.	DQ160276	This paper; Turkey, <i>Khokhryakov & Mazurenko 1135</i> (MHA)
<i>L. gebelia</i> Vent.	AF450188	Allan et al. (2003)
<i>L. michauxianus</i> Ser.	AF450206	Allan et al. (2003)
<i>Lotus</i> sect. <i>Krokeria</i> (Moench) Ser. (1/1)		
<i>L. edulis</i> L.	AF450184	Allan et al. (2003)
<i>Lotus</i> sect. <i>Lotea</i> (Medik.) DC. (10/8)		
<i>L. cytisoides</i> L. (A)	DQ160280	This paper; Cyprus, <i>Seregin & Sokoloff 280</i> (MW)
<i>L. cytisoides</i> L. (B)	DQ166241	This paper; Cyprus, <i>Seregin & Sokoloff 280</i> (MW)
<i>L. halophilus</i> Boiss. & Spruner	DQ160283	This paper; Greece, <i>Raus 9307</i> (MHA)
<i>L. longisiliquosus</i> R. Roem.	AF218526	Allan & Porter (2000)
<i>L. ornithopodioides</i> L.	AF450205	Allan et al. (2003)
<i>L. peregrinus</i> L.	AF450177	Allan et al. (2003)
<i>L. polyphyllus</i> Clarke	DQ160289	This paper; Egypt, 06 Apr. 1962, <i>Bochantsev s.n.</i> (LE)
<i>L. weilleri</i> Maire	AF450180	Allan et al. (2003)
<i>Lotus</i> sect. <i>Lotus</i> (30/19)		
<i>Lotus angustissimus</i> group		
<i>L. angustissimus</i> L.	DQ166243	This paper; Australia, Norfolk Island, introduced, 14 Oct. 1999, <i>Waterhouse 5510</i> (NSW)
<i>L. castellanus</i> Boiss. & Reut. (1)	DQ160272	This paper; Portugal, <i>Malato-Beliz & Guerra 13585</i> (MW)
<i>L. castellanus</i> Boiss. & Reut. (2)	DQ166223	This paper; Spain, <i>Segura Zubizarreta 15112</i> (LE)
<i>L. castellanus</i> Boiss. & Reut. (3)	DQ166238	This paper; Spain, <i>Segura Zubizarreta 38111</i> (MHA)
<i>L. cf. castellanus</i> (4)	DQ160275	This paper; Turkey, 17 Oct. 1999, <i>Majorov s.n.</i> (MW)
<i>L. parviflorus</i> Desf. (1)	DQ166230	This paper; Spain, <i>Segura Zubizarreta 34567</i> (MHA)
<i>L. parviflorus</i> Desf. (2)	AF450194	Allan et al. (2003)
<i>L. praetermissus</i> Kuprian. (1)	DQ166227	This paper; European Russia, 20 July 1993, <i>Kramina s.n.</i> (MW)
<i>L. praetermissus</i> Kuprian. (2)	DQ168370	This paper; Ukraine, <i>Tzvelev et al. 1630</i> (LE)
<i>L. subbiflorus</i> Lag. (syn. <i>L. suaveolens</i> Pers.) (1)	DQ166239	This paper; cultivated at the Botanic Garden of Moscow University, 1998 <i>Kramina s.n.</i> (MW)
<i>L. subbiflorus</i> Lag. (2)	DQ166237	This paper; Australia, <i>Kodala et al. 163</i> (NSW)
<i>L. subbiflorus</i> Lag. (3)	DQ166231	This paper; Italy, <i>Iberite 15222</i> (MHA)
<i>L. subbiflorus</i> Lag. (4)	DQ168369	This paper; France, <i>Dutartre 570</i> (MHA)
<i>Lotus corniculatus</i> group		
<i>L. alpinus</i> (DC.) Schleicher ex Ramond	DQ160274	This paper; Spain, <i>Segura Zubizarreta 43694</i> (MHA)
<i>L. borbasii</i> Ujhelyi	DQ166226	This paper; Czech Republic, 14 May 1961, <i>Smejkal 1441</i> (MHA)
<i>L. corniculatus</i> L.	AF218527	Allan and Porter (2000)
<i>L. delortii</i> Timb.-Lagr. ex F.W. Schultz	DQ166228	This paper; Spain, <i>Sandwith 4772</i> (LE)
<i>L. glaber</i> Mill.	DQ166225	This paper; Slovakia, 16 July 1974, <i>Chrték & Křisa s.n.</i> (LE)
<i>L. japonicus</i> (Regel) K. Larsen 'Gifu'	AJ512882 (ITS1) AJ512942 (ITS2)	Nanni et al. (2004)
<i>L. japonicus</i> (Regel) K. Larsen 'Miyakojima'	AJ512881 (ITS1) AJ512943 (ITS2)	Nanni et al. (2004)

Table 1 (concluded).

Species	GenBank No.	First publication of the sequence or voucher data
<i>L. krylovii</i> Schischk. & Serg.	AF450209	Allan et al. (2003)
<i>L. palustris</i> Willd.	AF450195	Allan et al. (2003)
<i>L. peczoricus</i> Miniaev et Ulle	AF450191	Allan et al. (2003)
<i>L. preslii</i> Ten. (A)	DQ166229	This paper; Algeria, 22 July 1968, <i>Bochantsev s.n.</i> (LE)
<i>L. preslii</i> Ten. (B)	DQ166236	This paper; Algeria, 22 July 1968, <i>Bochantsev s.n.</i> (LE)
<i>L. schoeleri</i> Schweinf.	DQ166224	This paper; cultivated at the Botanic Garden of Moscow University, 16 Sep. 1994 <i>Kramina s.n.</i> (MW)
<i>L. stepposus</i> Kramina	DQ166242	This paper; Ukraine, 28 June 1989, <i>Kramina 14-4</i> (MW)
<i>Lotus pedunculatus</i> group		
<i>L. pedunculatus</i> Cav.	DQ166222	This paper; Spain, 18 July 1972, <i>Segura Zubizarreta s.n.</i> (LE)
<i>L. uliginosus</i> Schkuhr (1)	DQ160273	This paper; Denmark, <i>Larsen 29349</i> (LE)
<i>L. uliginosus</i> Schkuhr (2)	AF450197	Allan et al. (2003)
<i>Lotus</i> sect. <i>Ononidium</i> Boiss. (4/3)		
<i>L. garcinii</i> DC.	DQ166234	This paper; Iran, <i>Leonard 5899</i> (LE)
<i>L. ononopsis</i> Balf.f.	DQ166219	This paper; Yemen, <i>Miller et al. 10097</i> (E)
<i>L. simonae</i> Maire, Weiller & Wilczek	DQ160285	This paper; Morocco, <i>Podlech 49444</i> (M)
<i>Lotus</i> sect. <i>Pedrosia</i> (Lowe) Christ (29/11)		
<i>L. arenarius</i> Brot.	AF218528	Allan and Porter (2000)
<i>L. assakensis</i> Brand	DQ160277	This paper, Morocco, <i>Podlech 40448</i> (M)
<i>L. azoricus</i> P.W. Ball	AY294293	Allan et al. (2004)
<i>L. campylocladus</i> Webb & Berth.	AF450196	Allan et al. (2003)
<i>L. creticus</i> L.	DQ160279	This paper; Portugal, June 2001, <i>Severova s.n.</i> (MW)
<i>L. emeroides</i> R.P. Murray	AY294295	Allan et al. (2004)
<i>L. eriosolen</i> (Maire) Mader & Podlech	DQ160281	This paper; Morocco, <i>Podlech 52619</i> (M)
<i>L. jacobaeus</i> L.	AY294299	Allan et al. (2004)
<i>L. jolyi</i> Battand.	DQ166240	This paper; Morocco, <i>Lewalle 11581</i> (LE)
<i>L. lancerottensis</i> Webb & Berth.	AY294300	Allan et al. (2004)
<i>L. maroccanus</i> Ball	AF450181	Allan et al. (2003)
<i>L. pseudoreticus</i> Maire, Weiller & Wilczek	DQ160284	This paper; Morocco, <i>Podlech 52358</i> (M)
<i>Lotus</i> sect. <i>Rhyncholotus</i> (Monod) D.D. Sokoloff (3/2)		
<i>L. berthelotii</i> Masf.	AY294306	Allan et al. (2004)
<i>L. maculatus</i> Breif.	AY294308	Allan et al. (2004)
<i>Lotus</i> sect. <i>Tetragonolobus</i> (Scop.) Benth. & Hook.f. (5/2)		
<i>L. maritimus</i> L. [= <i>Tetragonolobus maritimus</i> (L.) Roth.]	AF218505	Allan and Porter (2000)
<i>L. tetragonolobus</i> L. (= <i>Tetragonolobus purpureus</i> Moench)	AF218506	Allan and Porter (2000)

Note: Sections of *Lotus* are indicated. Numbers after sectional names show total number of species in a section / number of species studied here.

and a retention index of 0.745. A strict consensus of all shortest trees is shown in Fig. 2. The Bayesian tree (not shown) is generally similar to the strict consensus.

The genus *Lotus* (including *Tetragonolobus* and *Dorycnium*) is revealed as a clade both in the Bayesian and parsimony analyses. A group containing *Hammatolobium*, *Tripodion*, plus *Cytisopsis* is well supported as a clade sister to *Lotus*. The problematic genera *Podolotus* and *Pseudolotus* do not group with the *Lotus* clade. In the Bayesian analysis, *Pseudolotus* is sister to another monospecific Old World genus, *Antopetitia* (tree not shown), while in the parsimony analysis, the position of *Pseudolotus* is unresolved. On the Bayesian tree, *Podolotus* is poorly supported as sister to a large clade comprising all New World taxa plus Old World *Dorycnopsis*, *Antopetitia*, *Pseudolotus*, and *Kebirita* (tree not shown). In the strict consensus of shortest trees, *Podolotus* is sister to *Hippocrepis* plus *Scorpiurus*, but this grouping has a bootstrap support of less than 50%.

Basally branching nodes within the *Lotus* clade are poorly supported in both the Bayesian and parsimony trees. In the Bayesian tree, as well as in the strict consensus of shortest trees, members of section *Chamaelotus* (*L. schimperi* and *L. glinoides*) are sister to the rest of *Lotus*, but posterior probability and bootstrap support for this grouping are very low.

Species of section *Lotus* fall into two clades. Clade A is highly supported but relationships are unresolved. Clade A includes three species of annuals, namely *L. parviflorus* and *L. subbiflorus* of section *Lotus* plus *L. conimbricensis* (sect. *Erythrolotus*). Clade B comprises the rest of the sampled species of section *Lotus*. Within this clade, members of the *L. corniculatus* group form a strongly supported subclade. Relationships within the *L. corniculatus* group are well resolved. The second subclade of clade B contains the perennials *L. uliginosus* and *L. pedunculatus* plus the annuals (biennials) *L. angustissimus*, *L. praetermissus*, and *L. castellanus*, and a

putative new taxon labeled “*L. cf. castellanus*”. The sister group relationship between clade A and clade B is not supported by analyses of nrITS sequences, which is unexpected given that these clades contain members of section *Lotus*, a group that was traditionally thought to be natural on the basis of morphological evidence (e.g., Kramina 1999; Valdés 2000). In the tree inferred from the Bayesian analysis, clades A and B group together with species traditionally included in *Dorycnium* sections *Bonjeanea* and (*Eu*)*Dorycnium* (*L. rectus*, *L. hirsutus*, *L. dorycnium*, *L. graecus*). *Lotus strictus* which was only recently classified as *Dorycnium* (Lassen 1986) also falls here. In the parsimony analysis, clades A and B, former members of *Dorycnium* plus section *Chamaelotus* form an unresolved polytomy at the base of the *Lotus* clade.

Clade C comprises members of sections *Canaria*, *Heinekenia*, *Ononidium*, *Krokeria*, *Tetragonolobus*, *Lotea*, *Pedrosia*, and *Rhyncholobus*. The only sampled member of the section *Canaria* is sister to the rest of this large clade in the tree inferred from the Bayesian analysis, however, bootstrap support for this grouping in the parsimony analysis is poor, and the grouping is also not present in strict consensus. Section *Heinekenia* is not monophyletic according to analyses of nrITS sequences. Its members fall into two clades (D and E) forming a grade within clade C. Clade D is composed entirely of members of section *Heinekenia*. Clade E contains seven species of section *Heinekenia* plus two of section *Ononidium* (*L. garcinii* and *L. ononopsis*). Relationships of *L. garcinii* and *L. ononopsis* within clade E are not resolved; more data are needed to determine if they are sister taxa.

Clade F includes members of sections *Tetragonolobus*, *Krokeria*, and *Lotea* plus *L. simonae*. The two GenBank accessions of the sect. *Tetragonolobus* group together with very low support. A well-supported subclade of clade F includes members of section *Lotea* plus the rare endemic *L. simonae* from south Morocco, which was originally placed in section *Stipulati* (Maire et al. 1935) and subsequently transferred to section *Ononidium* (Sokoloff 2003b).

All sampled members of sections *Pedrosia* and *Rhyncholobus* plus a problematic species *Lotus creticus* (that has been placed in either sect. *Lotea* or *Pedrosia*) form a well-supported clade (clade G) with posterior probability of 1.00 and bootstrap support of 100%. Section *Pedrosia* is paraphyletic with section *Rhyncholobus* embedded within it. *Lotus creticus* is supported (posterior probability 1.00; bootstrap support 83%) as a member of a clade that includes members of section *Pedrosia* (*L. campylocladus*, *L. lancerottensis*, and *L. assakensis*).

Sister-group relationship between clades F and G is only supported in the tree inferred from the Bayesian analysis (posterior probability 0.85; not shown in Fig. 2). These two clades form a polytomy with clade E in the strict consensus.

The ITS1 sequence of *Lotus benoistii* (sect. *Benedictella*), according to our preliminary data (tree not shown), groups with sequences of *L. glinoides* and *L. schimperii*, but bootstrap support of this grouping is low.

Analyses of the combined matrix (nrITS sequences plus morphology) (Fig. 3)

In the maximum parsimony analysis, 34 000 shortest trees (1762 steps) were found, with a consistency index of 0.374 and a retention index of 0.720. A strict consensus of

all shortest trees is shown in Fig. 3. The Bayesian tree (not shown) is generally similar to the strict consensus. In both analyses, the genus *Lotus* is a well-supported clade sister to *Tripodion*, *Hammatolobium*, and *Cytisopsis*.

Only a few well-supported clades in the molecular analyses are unresolved in trees inferred from analyses of the combined matrix. For example, *Lotus maroccanus* and *L. eriosolen* group in the molecular analyses (bootstrap support 69%, posterior probability 0.86), but this is not supported in the combined analyses.

Some clades receiving low support in the molecular analyses are well supported in the combined analyses. For example, in the latter, the *Tetragonolobus* clade has a posterior probability of 0.91 and a bootstrap support of 78%; the *Rhyncholobus* clade has a posterior probability of 1.00 and a bootstrap support of 100%. These two groups are morphologically well defined by apomorphic character states.

In contrast to the analyses of nrITS data alone, analyses of the combined data set show clades A and B grouping together with bootstrap support of 57% and posterior probability of 0.97. In the combined analyses, the strict consensus of shortest trees shows a clade comprising all sampled members of sections *Dorycnium* and *Bonjeanea* (i.e., former genus *Dorycnium*). This clade is sister to clades A + B. However, the *Dorycnium*+*Bonjeanea* clade and its sister group relationship with clades A + B received very low bootstrap support and posterior probabilities.

Discussion

Monophyly of the genus *Lotus*

The present analyses support the segregation from *Lotus* of the Old World monospecific genera *Podolotus*, *Pseudolotus*, and *Kebirita*, as well as the New World genera *Hosackia* s.str., *Ottleya*, *Acmispon*, and *Syrmatium*. These genera were previously included in *Lotus* by various authors (e.g., Polhill 1981). In the trees obtained in this study, the genera *Hosackia*, *Ottleya*, *Acmispon*, and *Syrmatium* are represented by one species each. We have also performed analyses with more extensive sampling of these American genera. Each segregate genus is monophyletic in these analyses (data not included).

The present study supports monophyly of the genus *Lotus* within the limits suggested by Sokoloff (2003a, 2003b), that is, including the segregate genera *Tetragonolobus* and *Dorycnium*. The current circumscription of the genus *Lotus* is restricted only to Old World species. The monophyly of this group was also revealed in the molecular phylogenetic studies of Allan and Porter (2000) and Allan et al. (2003, 2004). Previous studies, however, did not include material for all genera of the tribe. Some problematic taxa within *Lotus* were also previously not sampled for DNA, for example, sections *Canaria* and *Ononidium*.

Morphological synapomorphies of major clades within *Lotus* are summarized in Table 2. Of 30 major clades recognized (Table 2), 14 clades have no obvious morphological synapomorphies. Six clades have a single synapomorphy each, four clades two synapomorphies each, two clades three synapomorphies, three clades four synapomorphies, and one clade (sect. *Tetragonolobus*) has five synapomorphies. Nine putative uniquely derived synapomorphies within *Lotus* are

Fig. 2. Strict consensus of 20004 trees (1461 steps) derived from a maximum parsimony analysis of ITS sequence data. Numbers above branches are bootstrap support values obtained by maximum parsimony analysis with bootstrap resampling and posterior probabilities found in Bayesian analysis. Only bootstrap values above 50% are shown. Terminal groups represented by new nrITS sequences are underlined. *Lotus* species are attributed to sections according to the classification of Kramina and Sokoloff (2003) and Sokoloff (2003a, 2003b).

found. There is no obvious correlation between number of morphological synapomorphies and node support in molecular phylogenetic analyses. For example, clade E2-1 (Fig. 3), with four morphological synapomorphies, including one uniquely derived synapomorphy, does not appear in a strict consensus of trees inferred from molecular analysis (Fig. 2). Alternatively, we found no obvious morphological synapomorphy for *Lotus corniculatus* group (clade B2, Fig. 3), although it has bootstrap support of 100% and posterior probability of 1.00 in molecular analyses (Fig. 2). A lot of authors have proposed the same limits of the *Lotus corniculatus* group solely on the basis of morphological data (see below). This shows that a search of synapomorphies does not represent a panacea in analyses of morphological data.

Most morphological characters show significant level of homoplasy in the genus *Lotus*. Nevertheless, adding of morphological data set to nrITS data allows the resolution of relationships for some critical nodes.

Section *Chamaelotus* and section *Benedictella*

The section *Chamaelotus* was described by Kramina and Sokoloff (2003) to segregate three closely related species of desert annuals having sessile umbels and very small flowers. In the majority of *Lotus* species, the umbels are pedunculate. Molecular data support segregation of section *Chamaelotus* (although only two of three species have so far been sampled). Members of section *Chamaelotus* were traditionally associated with *Lotus arabicus* (sect. *Heinekenia*). Our data on ITS1 of *L. benoistii* (sect. *Benedictella*) clearly shows that this species belongs to the genus *Lotus*. Lassen (1986) suggested that *L. benoistii* should be placed in the same section with species that we classified as section *Chamaelotus*. The ITS1 sequence in *L. benoistii* does not allow testing of this hypothesis.

Section *Lotus* and section *Erythrolotus*

Section *Lotus* is not revealed as monophyletic in all cladistic analyses since *L. conimbricensis* (sect. *Erythrolotus*) is resolved together with members of section *Lotus* (Arambarri 2000b; Allan et al. 2003; Sokoloff 2006; this study).

Brand (1898) accepted two species-rich sections of *Lotus*, sect. *Erythrolotus* Brand and sect. *Xantholotus* Brand. These sections were considered to share such characters as the styloidium lacking a tooth, leaves with five leaflets, and fruit dehiscent by two valves. According to Brand, members of the section *Erythrolotus* have red (or pink) flowers while members of section *Xantholotus* have yellow (or white) flowers. The name *Xantholotus* is illegitimate because the lectotype of the genus, *L. corniculatus*, belongs here, and thus the section should be called sect. *Lotus* (although Brand's section *Xantholotus* also includes many species that are now excluded from section *Lotus*). Chrtková-Žertová (1984) selected *L. conimbricensis* as a lectotype of sect. *Erythrolotus*, and Kramina and Sokoloff (2003) postulated that this species alone should be included in sect. *Erythrolotus*.

The most important difference between *L. conimbricensis* and section *Lotus* is petal color (red vs. yellow), although this character is much more variable in the genus than was considered by Brand (1898). In particular, some species that undoubtedly belong to section *Lotus* such as *L. krylovii* and *L. schoelleri* often have red petals (e.g., Schweinfurth 1896; Schischkin and Sergievskaja 1932). Given the phylogenetic data, it is clear that *L. conimbricensis* should be placed in the section *Lotus*.

Although it is clear that section *Lotus* is not monophyletic if *L. conimbricensis* is excluded, it remains to be ascertained whether it is monophyletic even with *L. conimbricensis* included. The present study splits this group into two clades (clade A and clade B). Each clade is strongly supported in all analyses, but their sister relationship is not supported in the analyses of molecular data alone and has high support only in the Bayesian analysis of the combined data set. It is important that all members of section *Lotus* (including *L. conimbricensis*) studied so far share basic chromosome number $x = 6$, and this may represent a uniquely derived synapomorphy within the genus *Lotus*. The number $x = 6$ has been reported for some species of other lineages (e.g., *L. aegaeus*, *L. arabicus*, *L. polyphyllus*). However, $x = 7$ was also reported for these species (Grant 1995), and they merit future cytological studies.

We hesitate to further subdivide section *Lotus* (e.g., into two sections corresponding to clades A and B) until strong phylogenetic evidence for doing so can be demonstrated, for example, by using different DNA markers.

The *Lotus corniculatus* group (sect. *Lotus*)

The present phylogenetic data allow discussion of the limits of the *Lotus corniculatus* species group. There are two principal questions regarding the limits of this group.

(1) *Lotus palustris* is either included in the *L. corniculatus* group (Ball and Chrtková-Žertová 1968) or allied with *L. angustissimus* (Brand 1898; Heyn 1970a) by different taxonomic authorities. *Lotus palustris* is similar to the species of the *L. angustissimus* group by the indumentum type, leaf rachis usually prolonged above the insertion of upper lateral leaflets, comparatively small flowers (ca. 6–10 mm), keel shape (similar to that in *L. castellanus*), but it differs from them by predominantly perennial life form and larger dimensions of vegetative organs. Both nrITS and the combined molecular and morphological data, however, show that *L. palustris* belongs to the *L. corniculatus* species group. Allan et al. (2003) when they first published the ITS sequence of *L. palustris* also revealed its grouping with *L. corniculatus* and its allies, but with bootstrap support less than 50%. However, a marked seasonal polymorphism in *L. palustris* noted by Heyn (1970a) and Zohary (1987) as well as varying chromosome numbers in this species ($2n = 12, 14, \text{ and } 24$; Grant 1965, 1995) may bear evidence to a considerable variability of this taxon. Its limits and relationships

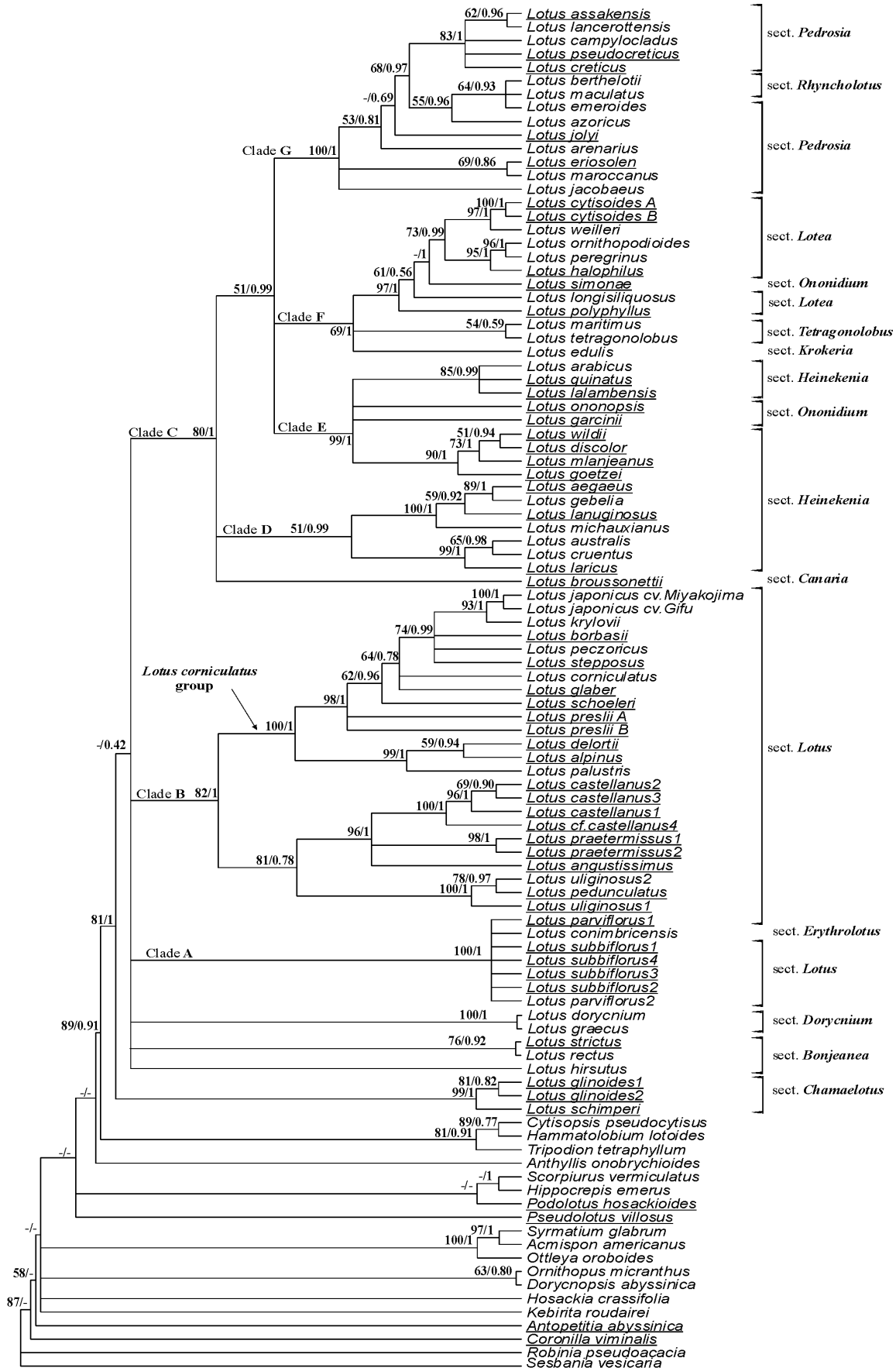


Fig. 3. Strict consensus of 34 000 trees (1762 steps) derived from a maximum parsimony analysis of the combined matrix (ITS sequence data plus morphology). Numbers above branches are bootstrap support values obtained by maximum parsimony analysis with bootstrap resampling and posterior probabilities found in Bayesian analysis. Only bootstrap values above 50% are shown. Terminal groups represented by new nrITS sequences are underlined. *Lotus* species are attributed to sections according to the classification of Kramina and Sokoloff (2003) and Sokoloff (2003a, 2003b).

with other species need to be tested by additional analyses using different DNA markers.

(2) *Lotus pedunculatus* and its allies (*L. uliginosus* and *L. granadensis* Žertová) are either treated as members of the *L. corniculatus* group (Ball and Chrtková-Žertová 1968) or as more isolated members of section *Lotus* (see Grant and Zandstra 1968; Raelson and Grant 1988, 1989). *Lotus pedunculatus* and allies share a perennial life form with the *L. corniculatus* group but differ in the presence of stolons and other characters. *Lotus pedunculatus* resembles members of *L. angustissimus* group in the type of hairs on the stems and leaves and leaf rachis often prolonged above the insertion of upper lateral leaflets (e.g., Chrtková-Žertová 1966). The present data show that *L. pedunculatus* and *L. uliginosus* should not be included in the *L. corniculatus* species group (*L. granatensis* has not been studied to date).

In the above-defined limits (i.e., including *L. palustris* and excluding *L. pedunculatus* s.l.), the *Lotus corniculatus* group is revealed as a monophyletic group. Relationships within the group are relatively well resolved. Among the species included here, diploids *L. schoelleri*, *L. glaber*, *L. stepposus*, *L. peccoricus*, *L. borbassii*, *L. krylovii*, and *L. japonicus* are closest to tetraploid *L. corniculatus*. These species should be taken into account when discussing the allotetraploid origin of *L. corniculatus* (for a review, see Grant and Small 1996). Two GenBank nrITS accessions of *L. japonicus* are closest to each other despite of obvious morphological (Kawaguchi et al. 2001; Barykina and Kramina 2005) and genomic (Hayashi et al. 2001) differences between these plants (Barykina and Kramina (2005) even suggest that *L. japonicus* 'Miyakojima' could be accepted as a distinct species, *Lotus miyakojimae* Kramina nom. nov. provis.).

The *Lotus angustissimus* group (sect. *Lotus*)

This group, as traditionally circumscribed, is clearly not monophyletic in the analyses presented here. It is subdivided into two subgroups.

The first subgroup includes *L. castellanus*, *L. praetermissus*, and *L. angustissimus*. The studied accessions of *L. angustissimus* and *L. praetermissus* do not form a clade. *Lotus praetermissus* was segregated by Kuprijanova (1937) on the basis of complex of characters including an indumentum of long but sparse patent hairs, solitary erect stems with spreading branches, wider and shorter legumes (16–20 mm long, not 20–30 mm as in *L. angustissimus*), and dark brown seeds. However, many authors consider these characters as not decisive and prefer to treat *L. praetermissus* as a synonym of *L. angustissimus*. The present data suggest that further studies should be conducted prior to accepting the synonymy of these two names.

The second subgroup of the traditional *L. angustissimus* group includes *L. parviflorus* and *L. subbiflorus*. These species are closely associated with *L. conimbricensis*. Differen-

ces between nrITS sequences of *L. parviflorus*, *L. subbiflorus*, and *L. conimbricensis* are surprisingly low (morphologically, the three species are clearly distinguishable by several characters, especially by fruit shape and size). In contrast, we found significant differences between nrITS sequences of *L. subbiflorus* and *L. castellanus*. Morphologically, *L. subbiflorus* and *L. castellanus* are rather closely related species differing from each other mainly by keel shape (with a long straight-tipped beak in *L. subbiflorus* Lag., syn. *L. suaveolens* Pers.; long-beaked with incurved tip in *L. castellanus* Boiss. & Reut., syn. *L. subbiflorus* sensu Heyn, non Lag.) (Heyn 1970a). However, this character is variable to some extent. Some authors accepted *L. castellanus* as a subspecies of *L. subbiflorus* (Ball and Chrtková-Žertová 1968). Kramina (in preparation) found other morphological differences justifying the specific rank of *L. castellanus*. One of the most important characters is the presence of hairs along the ventral suture of the ovary and fruit in *L. castellanus* and absence of such hairs in *L. subbiflorus*. Except for *L. castellanus*, and some specimens of *L. palustris* all other studied members of section *Lotus* have glabrous pods. *Lotus castellanus* is mostly restricted to Western Mediterranean (Kramina, in preparation). A specimen from Turkey (listed in Table 1 as *L. cf. castellanus*) fits traditionally used morphological features of *L. castellanus*. However, it has completely glabrous fruits. This specimen may represent an undescribed species.

The non-monophyletic nature of the *L. angustissimus* group is an unexpected finding of the present study. Morphologically members of this group are alike. The unexpected tree topology in this region is unlikely to result from low species sampling. We have sampled all members of the *L. angustissimus* group (as accepted by Heyn 1970a), with exception of the rare endemic of Turkey, *L. macrotrichus* Boiss. It is also unlikely that members of section *Lotus* exhibit high infraspecific polymorphism in nrITS sequences. To test this hypothesis, we have studied several accessions of *L. castellanus* and several accessions of *L. subbiflorus*. We have revealed only very low infraspecific variation in each species.

Former members of the genus *Dorycnium*

Rikli (1901) accepted three sections of the genus *Dorycnium*, namely *Canaria*, *Bonjeanea*, and (*Eu*)*Dorycnium*. This study analysed members of all three sections. Section *Canaria* includes three closely related species endemic to the Canary Islands. It is represented by *L. broussonettii* in our analyses. Our phylogenetic data clearly show that section *Canaria* is not closely related to sections *Bonjeanea* and *Dorycnium*. This supports previous findings by Gillett (1959). Morphologically, section *Canaria* differs from sections *Bonjeanea* and *Dorycnium* by large leaves, long petal claws, pronouncedly rostrate keel, and by presence of some papillae on stylodium. In addition, the geographical distribu-

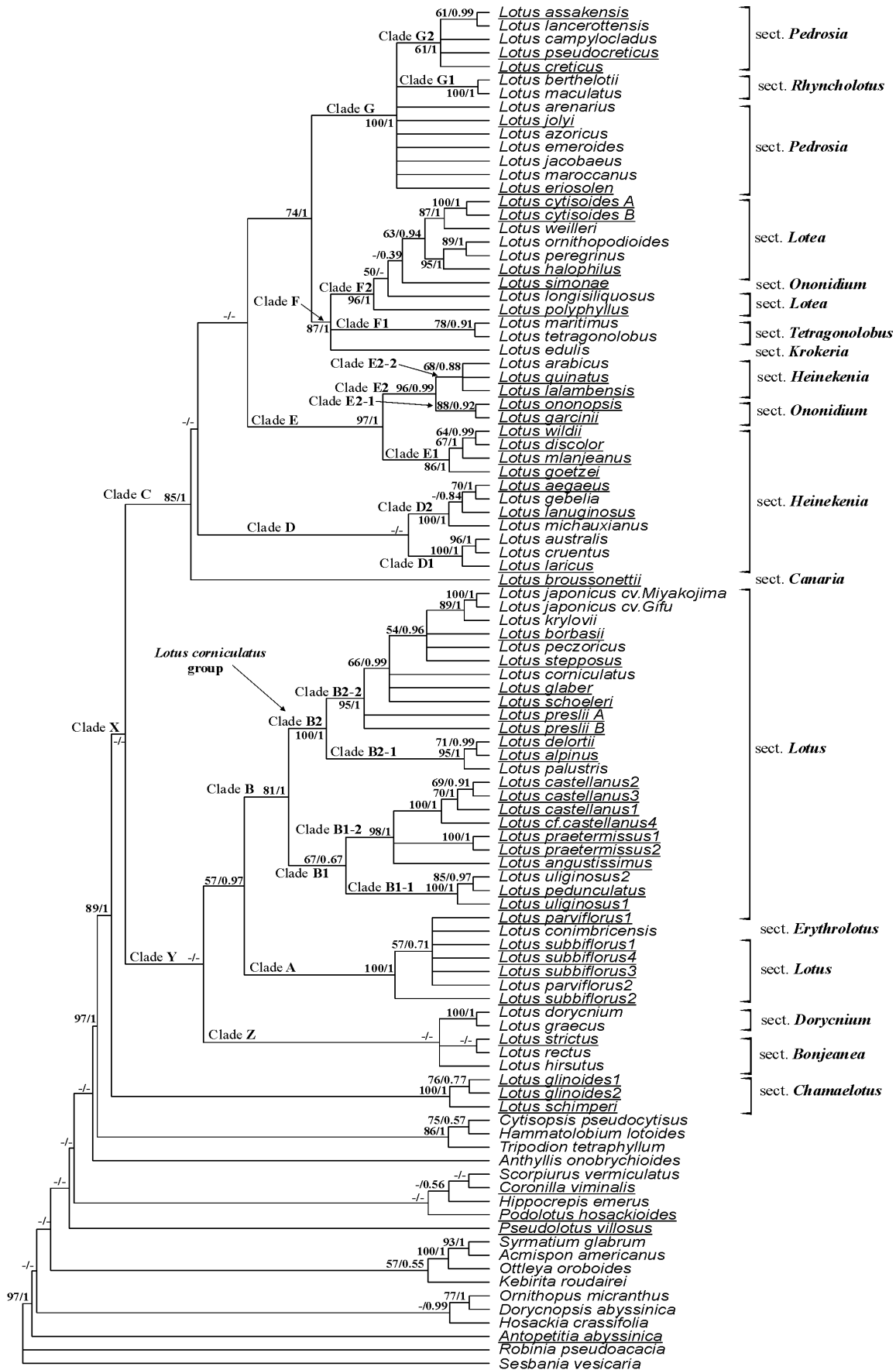


Table 2. Putative morphological synapomorphies of major clades in the genus *Lotus*.

Clade name and corresponding sectional names ^a	Morphological synapomorphies	Apomorphy status and comments
<i>L. glinoides</i> + <i>L. schimperi</i> (=sect. <i>Chamaelotus</i>)	Habit: annuals Peduncle shortened Flowers less than 7 mm long	Also in clades A, B1–2, in some members of DEFG clade, and in some outgroups Also in F2–1 and in some outgroups Also in <i>L. garcinii</i> , <i>L. rectus</i> , <i>L. dorycnium</i> , <i>L. graecus</i> , and in some outgroups
Clade X	Basal leaflets with maximum width near the base	Almost unique, 8 reversals within X clade
Clade Y	None	
Clade Z (=sect. <i>Dorycnium</i> + sect. <i>Bonjeanea</i>)	Elongate internode between the sterile bract and umbel Umbels typically with more than 8 flowers Stylodium smooth (not papillose)	Almost unique within <i>Lotus</i> (present as an unstable feature in few <i>Lotus</i> taxa, e.g., sect. <i>Canaria</i>), occurs also in some distantly related outgroups; absent in <i>L. strictus</i> (reversal) Homoplastic A uniquely derived synapomorphy within <i>Lotus</i> but present in many outgroups (including those closest to <i>Lotus</i>)
<i>L. dorycnium</i> + <i>L. graecus</i> (=sect. <i>Dorycnium</i>)	Rachis shortened (leaves palmate) Flowers less than 7 mm long Keel obtuse (not rostrate) Fruit twice as long as the calyx or shorter	Also in clade E2–1 and some species of clade G, <i>L. simonae</i> , <i>L. polyphyllus</i> , and some outgroups Homoplastic, see above Also in <i>L. rectus</i> and in some outgroups Also in <i>L. garcinii</i> , <i>L. polyphyllus</i> , <i>L. parviflorus</i> , and some outgroups
Clade A+B (=sect. <i>Lotus</i> + sect. <i>Erythrolotus</i>)	Flowers yellow Basic chromosome number $x = 6$	With a reversal in <i>L. conimbricensis</i> , also in many other clades Possibly a uniquely derived synapomorphy within <i>Lotus</i> but present also in some outgroups
Clade A	Habit: annuals (biennials)	Homoplastic, see above
Clade B	None	
Clade B1	None	
Clade B1–1	Flowers more than 10 mm long	Also in other clades
Clade B1–2	Habit: annuals (biennials)	Homoplastic, see above
Clade B2 (= <i>L. corniculatus</i> complex)	None	We found no synapomorphies also for B2–1 and B2–2
Clade C	Flowers more than 10 mm long	Very homoplastic
Clade D+E+F+G	None	
Clade D (=part of sect. <i>Heinekenia</i>)	None	We found no synapomorphies also for D1 and D2
Clade E+F+G	None	
Clade E	Flowers less than 10 mm long	Also in other clades; not always so in two species of E
Clade E1 (=part of sect. <i>Heinekenia</i>)	None	
Clade E2	Leaflet number variable Basal leaflets of a leaf with maximum width near the middle or at the apex of the leaflet	Also in most outgroups, in clade G1, in <i>L. graecus</i> , <i>L. australis</i> , <i>L. cruentus</i> ; not so in <i>L. arabicus</i> and <i>L. ononopsis</i> Homoplastic, see under clade X.
Clade E2-1 (=part of sect. <i>Ononidium</i>)	Leaf rachis shortened Peduncle shortened Sterile bract scale-like	Homoplastic, see above Not always so in <i>L. ononopsis</i> ; see also above Almost unique within <i>Lotus</i> (present as an unstable feature in sect. <i>Canaria</i>); present in some outgroups
Clade E2-2 (=part of sect. <i>Heinekenia</i>)	Umbels always one-flowered None	Homoplastic
Clade F+G (“Zygocalyx clade”)	Flowers yellow Calyx monosymmetric	With a lot of reversals; present also in other clades With a reversal in <i>L. edulis</i> ; present also in few other <i>Lotus</i> spp. and in some outgroups.
Clade F	None	

Table 2 (concluded).

Clade name and corresponding sectional names ^a	Morphological synapomorphies	Apomorphy status and comments
Clade F1 (=sect. <i>Tetragonolobus</i>)	Foliage leaves encircle their nodes	A uniquely derived synapomorphy within <i>Lotus</i> ; present in some outgroups
	Basal leaflets of a leaf fused to rachis	A uniquely derived synapomorphy
	Flowers more than 15 mm long	Homoplastic
	Dorsal styloidium outgrowth present	A uniquely derived synapomorphy
	Paired fruit wings present	A uniquely derived synapomorphy within <i>Lotus</i>
Clade F2 (=sect. <i>Lotea</i> + <i>L. simonae</i> of sect. <i>Ononidium</i>)	None	
Clade G (=sect. <i>Pedrosia</i> + sect. <i>Rhyncholotus</i>)	Ventral styloidium tooth present	A uniquely derived synapomorphy
	Hairs along the ventral slit on ovary and fruit present	Also in <i>Lotus castellanus</i> and some outgroups (plus in <i>L. hebecarpus</i> of sect. <i>Heinekenia</i> that is not covered by our study)
Clade G1 (=sect. <i>Rhyncholotus</i>)	Leaflet number variable	Also in some other <i>Lotus</i> (outside clade F) and in most outgroups
	Flowers more than 25 mm long	Also in very few species (<i>L. maritimus</i> in our matrix)
	Standard indumentum present	Also in very few <i>Lotus</i> spp. not included in our matrix and in few outgroups
	Standard strongly bent backward	Autapomorphy
Clade G2 (=part of sect. <i>Pedrosia</i>)	None	

Note: For clade names, see Fig. 3.

^aAccording to classification by Kramina and Sokoloff (2003) and Sokoloff (2003a, 2003b).

tion of *Canaria* is distinctive, because the sections *Bonjeanea* and *Dorycnium* are absent from the Canary Islands.

Section *Dorycnium* is represented here by two species, *L. dorycnium* (= *D. pentaphyllum*) and *L. graecus* (= *D. graecum*). The latter species was previously placed in section *Bonjeanea* (e.g., Rikli 1901; Demiriz 1970) but Sokoloff (2003a, 2003b) recently suggested its placement in section *Dorycnium*. A clade comprising *L. dorycnium* and *L. graecus* has been found by Allan and Porter (2000). This finding was repeated in the present analysis. Other members of section *Dorycnium* are morphologically very close to *L. dorycnium* (see Demiriz 1970; Lassen 1979; Diaz Lifante 2000; Sokoloff 2003a).

According to Sokoloff (2003a, 2003b), section *Bonjeanea* includes three species, *L. strictus* (*D. strictum*), *L. hirsutus* (*D. hirsutum*), and *L. rectus* (*D. rectum*). All species were included in the present analyses. The combined analysis suggests that section *Bonjeanea* is paraphyletic but that *Bonjeanea*+*Dorycnium* may be monophyletic. The paraphyly of section *Bonjeanea* was found earlier by Sokoloff (2003b) in a parsimony analysis of morphological data, the two sections differing only by plesiomorphic characters. It may be reasonable to combine sections *Bonjeanea* and *Dorycnium*. However, prior to making any taxonomic decisions, it is important to produce a well-supported molecular phylogeny for this group.

Section *Heinekenia* and section *Ononidium*

According to Kramina and Sokoloff (2003), section *Heinekenia* includes most species that were placed by Brand (1898) into his broadly defined section *Erythrolotus*. Brand characterized this section by 5-foliolate leaves and red (pink) flowers. The lectotype of the section *Erythrolotus* is *L. conimbricensis*. Since morphologically and phylogeneti-

cally it is not close to other members of Brand's section (see above), another name, *Heinekenia*, must be used for the rest of the section (see also Lassen 1986). In addition to *L. conimbricensis*, we have excluded from this section small-flowered desert annuals (section *Chamaelotus*, see above). Finally, Kramina and Sokoloff (2003) placed in section *Heinekenia* some species that Brand included in his section *Xantholotus* (*L. discolor*, *L. namulensis* Brand, *L. aegaeus*). Section *Heinekenia* is unusual among sections of *Lotus* (except sect. *Ononidium*) in having its diversity centers outside the Mediterranean region. Kramina and Sokoloff (2003, see also Sokoloff 2001, Kramina and Sokoloff 2004) accepted four informal groups within section *Heinekenia* (Table 1). The present study does not support the monophyly of section *Heinekenia*. Also, of the four informal groups, only two are monophyletic (*L. australis* group and *L. discolor* group). Members of section *Heinekenia* fall in two clades (one of them includes also two species of section *Ononidium*, *L. garcinii* and *L. ononopsis*). These two clades (clade D and clade E) are close to each other in our phylogenetic trees, but never group together. It is almost impossible to distinguish clades D and E by using of morphological characters. However these clades show a good correspondence with geographic distribution.

Species of clade E occur in Africa plus in western and southwestern parts of the Arabian Peninsula, and in Socotra. The only exception is *L. garcinii*, which has a wide distribution extending from Somalia eastwards to Pakistan and west India. Two subclades of clade E are also well defined in terms of ecology and geography. The first subclade includes *L. wildii*, *L. discolor*, *L. mlanjeanus*, and *L. goetzei*, and corresponds to the *L. discolor* species group that occurs in mountains of tropical Africa and is morphologically well defined. Its sister group (*L. lalambensis*, *L. quinatus*,

L. arabicus, *L. ononopsis*, and *L. garcinii*) has a center of diversity in Ethiopia, around the Red Sea and in Socotra.

Clade D includes Asian and Australian species. Those species that are present in the Arabian peninsula occur in its northern (*L. lanuginosus*) or eastern (*L. laricus*) part. *Lotus gebelia* is the only member of clade D that was recorded from Africa (north Libya); however, most of its wide distribution area lies in Asia, and Libya is far away from centers of diversity of clade E in Africa. The Australian species *L. australis* and *L. cruentus* are sister to *L. laricus*, which has the most eastern distribution among species included in clade D except to the Australian species (eastwards to Pakistan). Morphologically, *L. laricus* has no obvious synapomorphies with Australian *Lotus* species.

Relationships between *L. gebelia*, *L. michauxianus*, and *L. aegaeus* are of particular interest. *Lotus aegaeus* is the only species of section *Heinekenia* that has yellow petals. Other species of the section are red-, pink- or white-flowered. Our results support earlier conclusions by Heyn (1970b) and Chrtková-Žertová (1967) that *L. aegaeus* is closest to *L. gebelia*. In some cases it is difficult to distinguish between *L. aegaeus* and *L. gebelia* if petal color is lost on herbarium material. The position of *L. michauxianus* on some distance from *L. gebelia* is intriguing. Morphologically, these two species are closest to each other. The main difference is flower size (Chrtková-Žertová 1984). Some authors consider *L. michauxianus* as a synonym of *L. gebelia* (e.g., Heyn 1970b). More material should be studied to understand if we have indeed an obvious conflict between morphological and nrITS data in this case.

Section *Ononidium* was traditionally circumscribed as a small group of species occurring in southwestern Asia and east Africa. These species differ from most representatives of the genus in having leaves with typically three (not five) leaflets. *Lotus simonae* also has leaves with three leaflets but occurs in Morocco, that is, far away from members of sect. *Ononidium*. Sokoloff (2003b) suggested including *L. simonae* in sect. *Ononidium*. Present data does not support this idea. It is not clear if two other species of section *Ononidium* studied here form a single clade. Grouping between these species (*L. ononopsis* and *L. garcinii*) is well supported only in the combined analysis. The only species of section *Ononopsis* not studied here is *L. mollis* Balf.f., a rare endemic of Socotra. Morphologically it is similar to *L. ononopsis*.

It is obvious, on the basis of present phylogenetic data, that trifoliolate leaves represent a derived condition that appeared many times in the evolution of *Lotus*. This condition is also characteristic for *L. robsonii* E.S. Martins & D.D. Sokoloff that is morphologically close to *L. goetzei* (Martins and Sokoloff 2003). Leaflet number is variable in some *Lotus* species (Sokoloff 2003b; Kramina and Sokoloff 2004). It seems that this character cannot be used to segregate taxa of sectional rank. It may be possible to include *L. garcinii* and *L. ononopsis* (plus *L. mollis*) into section *Heinekenia*.

Paraphyletic nature of section *Heinekenia* creates a very difficult taxonomic problem because of a lack of obvious morphological differences between clades D and E. Even if paraphyletic nature of the section will be confirmed by future studies, it might be possible to keep it as paraphyletic

one at least until morphological evidence will be found to characterize segregated sections.

“Zygocalyx” clade (=clade F+G)

Members of this clade usually have a zygomorphic (monosymmetric) calyx while most other *Lotus* species have an actinomorphic (polysymmetric) calyx. Thus we could also call this lineage “Zygocalyx” clade. This moderately supported clade includes members of sections *Lotea*, *Tetragonolobus*, *Krokeria*, *Pedrosia*, and *Rhyncholotus*. A similar clade, although with lower taxon sampling, was earlier found by Allan et al. (2003, 2004). Although the tendency to have a monosymmetric calyx is very prominent and characteristic for this clade, this feature occurs rarely also in some other species of *Lotus*, for example in *L. borbassii*, *L. delortii* (Ujhelyi 1960), and *L. peczoricus* (Miniaev and Ulle 1977). Besides, a few members of the “Zygocalyx” clade have polysymmetric calyx, for example, *L. edulis* (monospecific section *Krokeria*).

The present phylogenetic data support the presence of two major clades (F and G) within the “Zygocalyx” clade. Clade F includes sections *Tetragonolobus*, *Krokeria*, and *Lotea*. Section *Tetragonolobus* is well defined by four uniquely derived synapomorphies (Table 2). As mentioned above, it is not reasonable to accept generic rank for this taxon. Our phylogenetic data suggest that *L. simonae* should be re-classified as a member of section *Lotea*. Although this species differs from other members of the section in trifoliolate leaves, it has a monosymmetric calyx, a key morphological character of this group. Morphologically, this species differs from members of other sections of the “Zygocalyx” clade in fruit and styloidium morphology. Clade G includes sections *Pedrosia* and *Rhyncholotus*. Our data support previous findings by Allan et al. (2004) on the paraphyly of section *Pedrosia*. It is paraphyletic because members of the section *Rhyncholotus* are embedded within it. It may be possible to combine both sections under the name *Pedrosia*, although more extensive morphological and molecular data should be collected to make formal taxonomic decisions. A clear morphological synapomorphy of clade G is presence of a ventral tooth on the styloidium. In clade F, the ventral styloidium tooth is always absent, although species of *Tetragonolobus* have a dorsal tooth or outgrowth. The presence of dorsal styloidium structures in *Tetragonolobus* and similar ventral structures in *Pedrosia/Rhyncholotus* is an obvious example of evolutionary parallelism. Similar structures are rare in the family Leguminosae. They are definitely absent among other members of Loteae and their closest relatives, Robinieae and Sesbanieae (Mönch 1910; Lavin and Delgado 1990; Lavin and Sousa 1995; Kramina and Sokoloff 1999).

There is an apparent functional correlation between styloidium and keel evolution in *Lotus*. In sections *Dorycnium* and *Bonjeanea*, the keel is often obtuse and the styloidium is always smooth, while in other members of *Lotus* the keel is beaked and the styloidium is papillose. The beak is especially long in some members of the “Zygocalyx” clade. An elaborated styloidium surface has a functional significance for secondary pollen presentation. In Loteae, during the visit of a pollinator, the pollen is pushed through an opening at the top of the keel aided by the dilated stamen filaments (Faegri and van der Pijl 1979). The styloidium may also act

in this process. A papillose styloidium surface may help to push the pollen, and the ventral or dorsal tooth may be even more effective mean for achieving this. We could speculate that, in *Lotus*, the longer the keel beak the more important is the contribution of the styloidium to secondary pollen presentation. All *Lotus* species with obtuse keel have a least elaborated styloidium. Species with an exceedingly long keel beak have the ventral tooth on the styloidium.

Phylogenetic relationships of *Lotus creticus* are of special interest because this species has a very small ventral styloidium tooth (Kramina and Sokoloff 1999; Valdés 2000). The tooth in *L. creticus* is smaller than in most members of *Pedrosia* and *Rhyncholotus*. Sometimes, the tooth is almost absent. Traditionally, *L. creticus* was placed in section *Lotea* (Ball and Chrtková-Žertová 1968; Valdés 2000). Kramina and Sokoloff (1999) have suggested moving *L. creticus* to section *Pedrosia*. Apart from the presence of the styloidium tooth, they noted similarity between this species and *L. pseudocreticus* (sect. *Pedrosia*) in general habit, certain floral features, and ecology. However, Allan et al. (2003, 2004), on the basis of nrITS molecular phylogenetic data suggested placement of *L. creticus* in section *Lotea*. That result implies a double origin of the ventral styloidium tooth in the genus *Lotus*. We have produced a new ITS sequence based on another voucher specimen. Our data strongly support placement of *L. creticus* in the section *Pedrosia*, close to *L. pseudocreticus* and *L. campylocladus*. Our results suggest a single origin of the ventral styloidium tooth in the genus *Lotus*. Detailed studies should be undertaken to determine if the ITS region is variable in *L. creticus*.

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Appendix A. Morphological characters used in the analysis

The full morphological data matrix is available as supplementary data.²

- Habit. 0, tree or shrub; 1, perennial herb or suffrutescent; 2, annual or biennial herb. (*Nonadditive*).
- Phyllotaxis on vegetative shoots. 0, spiral; 1, distichous.
- Pulvinus at the leaf base. 0, present; 1, absent.
- Leaf base width of lower foliage leaves. 0, leaf base does not (or does not completely) encircle the node; 1, leaf base completely encircles the node.
- Stipule morphology. 0, stipules entirely membranous or with membranous part; 1, membranous part of stipules absent.
- Stipels. 0, present; 1, absent.
- Petioles of foliage leaves. 0, always present and distinct; 1, variable (present and distinct or absent); 2, always absent or extremely short (up to 1 mm). (*Nonadditive*).
- Rachis of upper foliage leaves. 0, elongated; 1, shortened. (If leaflet number is more than three, then 0 means leaves pinnate and 1 means leaves palmate.)
- Leaflet number in foliage leaves. 0, variable; 1, five; 2, three. (*Nonadditive*).
- Shape of basal leaflets of a leaf. 0, maximum width

- near the middle or at the apex of the leaflet; 1, maximum width at the leaflet base.
11. Terminal leaflet shape. 0, obovate to oblanceolate or elliptical (length to width ratio less than or equal to 3); 1, linear or narrowly-oblanceolate (length to width ratio more than 3).
 12. Basal leaflet fusion. 0, basal leaflets of a leaf free; 1, basal leaflets fused to the rachis.
 13. Peduncle length. 0, elongated (the peduncle, i.e., umbel stalk, is much longer than its width); 1, shortened (the peduncle is almost as long as wide).
 14. Sterile bract (pseudobract – see Degtjareva et al. 2003) presence. 0, absent; 1, present.
 15. Sterile bract position. 0, typically at the base of the partial inflorescence; 1, typically separated from the partial inflorescence by an elongated internode.
 16. Sterile bract morphology. 0, foliage leaf; 1, scale-like leaf.
 17. Partial inflorescence type. 0, raceme; 1, head or umbel.
 18. Flower number per partial inflorescence. 0, one; 1, two or three; 2, four to seven; 3, eight and more. (*Additive*).
 19. Floral bud position. 0, bent backwards; 1, not bent backwards.
 20. Bract fusion. 0, always free; 1, (often) fused to each other.
 21. Bracteoles. 0, always present; 1, always or often absent.
 22. Flower size. 0, not exceeding 7 mm; 1, 7–10 mm; 2, 10–15 mm; 3, 15–25 mm; 4, more than 25 mm. (*Additive*). Univariate analysis was made to analyse this character. It helped to determine character states. More than 2000 individual measurements of flower length was made (Kramina and Tikhomirov 1991; Kramina 1992, 1999 and T.E. Kramina, unpublished data).
 23. Calyx symmetry (terminology after Endress 1994). 0, polysymmetric (with five symmetry planes), 1, monosymmetric (with single symmetry plane).
 24. Lower calyx teeth length. 0, shorter than the tube (plus hypanthium) or as long as the tube; 1 longer than the tube (plus hypanthium).
 25. Yellow color of petals. 0, petals never yellow; 1, petals at least sometimes yellow.
 26. Red, pink or dark color of wings and standard. 0, wings and standard never red, pink or dark (sometimes except veins); 1, wings and standard at least sometimes or partially red, pink or dark.
 27. Petal claws. 0, not or slightly exceeding calyx tube; 1, considerably exceeding calyx tube.
 28. Standard indumentum. 0, absent; 1, present.
 29. Keel shape. 0, rostrate (as in Figs. 171–185 in Valdés 2000); 1, obtuse (as in Figs. 187–188 in Diaz Lifante 2000).
 30. Keel tip shape. 0, straight; 1, incurved.
 31. Styloidium surface. 0, smooth; 1, papillose.
 32. Stamen filaments. 0, distally not dilated; 1, distally dilated.
 33. Hairs along the ventral slit on ovary and fruit. 0, absent; 1, present.
 34. Hairs at lateral ovary and fruit surface. 0, absent; 1, present.
 35. Ventral tooth on the styloidium. 0, absent; 1, present.
 36. Dorsal tooth or outgrowth on the styloidium. 0, absent; 1, present.
 37. Ovule orientation pattern (terminology after Tikhomirov and Sokoloff 1997). 0, micropylae superae; 1, micropylae alternantes; 2, micropylae inferae. (*Nonadditive*).
 38. Fruit length. 0, more than 2 times as long as the calyx; 1, shorter than or up to 1–2 times as long as calyx.
 39. Fruit shape. 0, straight or almost straight; 1, incurved toward ventral side; 2, incurved toward dorsal side. (*Nonadditive*).
 40. Dorsal fruit dehiscence. 0, present; 1, absent.
 41. Ventral fruit dehiscence. 0, present; 1, absent.
 42. Transversal fruit breaking. 0, absent (i.e., fruits not lomentaceous); 1, present (i.e., fruits lomentaceous).
 43. Structure of pericarp parchment layer (see Sokoloff 1997 and Tikhomirov and Sokoloff 1997, for details and illustrations). 0, fibres form single stratum in each fruit valve or parchment layer absent; 1, fibres form two strata in each fruit valve.
 44. Seed shape. 0, rounded or slightly elongated; 1, considerably elongated or linear.
 45. Seed surface. 0, with large conspicuous papillae; 1, without large papillae.
 46. Basic chromosome number. 0, $x = 10$; 1, $x = 8$; 2, $x = 7$; 3, $x = 6$. (*Additive*).