

Phylogeny of *Lotus* (Leguminosae: Loteae): Partial incongruence between nrITS, nrETS and plastid markers and biogeographic implications

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Abstract *Lotus* comprises ca. 130 species of herbs, semishrubs and shrubs native to the Old World, including important pasture crops and a model legume, *L. japonicus*. Earlier nrITS-based phylogenies were incongruent with all taxonomic classifications of the genus. In particular, members of the former genus *Dorycnium* were unexpectedly placed near species of *L.* sect. *Lotus*. The primary goal of the present study is to explore whether the unexpected placement of members of sect. *Lotus* and the former genus *Dorycnium* in earlier phylogenetic studies resulted from (1) insufficient taxon sampling and/or (2) the use of only one DNA marker. The rooting of the *Lotus* phylogeny, its major clades and basic biogeographic patterns are also discussed. This is the first global phylogenetic study of *Lotus* that uses both plastid and nuclear markers. The nrITS region was analyzed in 155 ingroup specimens representing 98 species of *Lotus*. Sequences of nrITS, nrETS, *psbA-trnH* spacer and *rps16* intron were analyzed for 70 ingroup specimens representing 54 species. The placement of the segregate genera *Dorycnium* and *Tetragonolobus* in the synonymy of *Lotus* was confirmed. Analyses of plastid data strongly supported a basal split of *Lotus* into two clades, one comprising species of sect. *Lotus* plus those traditionally placed in *Dorycnium* and the other including the rest of the species. The former clade has a centre of species diversity in Europe and N Asia, and the latter in Macaronesia, Africa and S Asia. Only the “Southern” clade is resolved in analyses of nrITS and nrETS data. Trees inferred from plastid, nrITS and nrETS data shared the occurrence of several smaller clades corresponding to traditionally recognized infrageneric taxa or species groups as well as the occurrence of some well-supported clades that differ from traditional taxonomic concepts. Several instances of incongruence were documented between nuclear and plastid markers and between the two nuclear markers, possibly resulting from reticulate evolution. The extant geographic patterns of *Lotus* are likely biased by at least one round of area fragmentation followed by expansion coupled with extensive speciation associated with the complex history of the Mediterranean biome.

Keywords 5'ETS; biogeography; *Dorycnium*; ITS; Leguminosae; *Lotus*; molecular phylogeny; *psbA-trnH*; *rps16* Mediterranean

Supplementary Material Electronic Supplement (Figs. S1–S3) and DNA sequence alignments are available in the Supplementary Data section of the online version of this article at <http://ingentaconnect.com/content/iapt/tax>

■ INTRODUCTION

The genus *Lotus* L. comprises 120–130 species of annual or perennial herbs, semishrubs or rarely shrubs naturally distributed in Europe, Asia, Africa, Australia, some islands of the Atlantic and Pacific Oceans and the Socotra archipelago in the Indian Ocean. The centre of species diversity is in the Mediterranean, where about one-half of the *Lotus* species, representing

the majority of sections, occur. This is the largest genus of tribe Loteae, which belongs to the Robinioid clade of the large and mainly temperate Hologalegina clade of papilionoid legumes (Wojciechowski & al., 2004; Legume Phylogeny Working Group, 2013). The genus *Lotus* includes the model legume *L. japonicus* (Regel) K.Larsen, several perennial pasture legumes of agricultural importance, such as *L. corniculatus* L., as well as ornamental plants, such as *L. berthelotii* Masf.

There are various opinions regarding the generic limits of *Lotus* (Greene, 1890; Taubert, 1894; Brand, 1898; Ottley, 1944; Gillett, 1958; Callen, 1959; Hutchinson, 1964; Isely, 1981; Polhill, 1981, 1994; Lassen, 1986; Ali, 1994; Kirkbride, 1994, 1999; Kramina & Sokoloff, 1997, 2001; Sokoloff, 1999, 2000, 2003a, b; Talavera & Salgueiro, 1999). Both morphological and molecular phylogenetic data strongly support recognizing the monospecific Old World genera *Kebirita* Kramina & D.D.Sokoloff, *Podolotus* Royle ex Benth. and *Pseudolotus* Rech.f. as distinct from *Lotus* (Allan & al., 2003; Sokoloff, 2005; Degtjareva & al., 2006, 2010, 2012). Phylogenetic data also clearly support separation of the native American species from *Lotus* (Allan & al., 2003; Degtjareva & al., 2006, 2010), though whether they should be assigned to two (Brouillet, 2008) or four (Sokoloff & al., 2007) segregate genera is still a matter of discussion. Two Old World segregate genera, *Dorycnium* Mill. and *Tetragonolobus* Scop., used to be accepted by most European botanists (Taubert, 1894; Rikli, 1901; Dominguez & Galiano, 1979; Talavera & Salgueiro, 1999), but phylogenetic analyses based on sequences of the nuclear ribosomal ITS region suggested that they should not be separated from *Lotus* (Allan & al., 2003, 2004; Degtjareva & al., 2006, 2008; Sandral & al., 2010). With the adjustments outlined above, the genus *Lotus* was found to be monophyletic in analyses based

on nrITS sequences and morphology (Degtjareva & al., 2006; Sokoloff & al., 2007).

Subdivision of *Lotus* into infrageneric taxa, such as subgenera and sections, also underwent several revisions, summarized by Degtjareva & al. (2006), but none of the proposed systems was found to be fully congruent with the phylogenies inferred from analyses of nrITS sequences (Allan & al., 2003, 2004; Degtjareva & al., 2006, 2008; Sandral & al., 2010). The nrITS data tentatively suggested a possible close phylogenetic relationship between species traditionally placed in *Lotus* sect. *Lotus*, including the model legume *L. japonicus* and the pasture plant *L. corniculatus*, with species traditionally placed in the genus *Dorycnium*. Such a pattern was quite surprising for a morphologist because the two groups differ in several important characters (Fig. 1). Both groups are common in Europe where all Floras during the last 200 years have consistently recognized them as distinct genera. It was also intriguing that neither sect. *Lotus* nor the group of former members of *Dorycnium* were well supported as monophyletic lineages, and members of the two groups appeared as several unresolved branches (Allan & al., 2003; Degtjareva & al., 2006).

The primary goal of the present study is to explore whether the unexpected placement of members of sect. *Lotus* and the former genus *Dorycnium* in earlier phylogenetic studies resulted



Fig. 1. Differences in flower and inflorescence characters between members of *Lotus* sect. *Dorycnium* and *L.* sect. *Lotus*. **Left**, *Lotus graecus* L. (= *Dorycnium graecum* (L.) Ser.), sect. *Dorycnium*: partial inflorescence with numerous small flowers, a foliage leaf situated in the middle of the inflorescence peduncle, corollas white, wing petals distally adhering to each other, saccate. **Right**, *Lotus stepposus* Kramina, sect. *Lotus*: partial inflorescences with fewer but larger flowers, a foliage leaf (in this case trifoliolate) situated at the top of the peduncle, corollas yellow to orange, wing petals free, not saccate. — Images courtesy of Maxim Nuraliev (left) and Segrei Majorov (right).

from (1) insufficient taxon sampling and/or (2) use of only one DNA marker. To explore the first possibility, taxon sampling was greatly extended in the nrITS dataset, trying to include as many species as possible as well as several accessions of each species of particular interest. Approximately 80% of the currently recognized *Lotus* species were sampled. Most species not included belong to the /Pedrosia clade, see below. To explore the hypothesis of potential inadequateness of the single-region approach, a dataset with two nuclear (nrITS and nrETS regions) and two plastid (*psbA-trnH*, *rps16* intron) DNA regions, covering ca. 43% of *Lotus* species was generated. In addition to the question on relationships between sect. *Lotus* and former members of *Dorycnium*, this dataset allowed us to investigate the following problems: (1) the basal splits in the *Lotus* phylogeny that were left unresolved in earlier studies based only on nrITS sequences; (2) the accuracy of *Tetragonolobus* placement in the synonymy of *Lotus*; (3) the phylogeny-based sectional classification of *Lotus*; and, (4) biogeographic implications of molecular phylogenetic data.

The present work is the first global phylogenetic study of *Lotus* that used both plastid and nuclear markers. Earlier multi-gene phylogenetic studies in *Lotus* covered the /Pedrosia clade (i.e., *L.* sect. *Pedrosia* (Lowe) Christ and sect. *Rhyncholobus* (Monod) D.D.Sokoloff); its extensive speciation in Macaronesia being remarkable given the young evolutionary history of the clade (Schmidt, 2011; Ojeda & al., 2012, 2014). Due to the recent speciation in the /Pedrosia clade, species of this group are much less differentiated at the molecular level than those in other groups of the genus (e.g., Degtjareva & al., 2008). For example, different species belonging to the /Pedrosia clade sometimes have nearly identical nrITS sequences. This dictates use of different sets of phylogenetic markers for a global study of the genus and for studies of speciation in the Macaronesian group (Schmidt, 2011; Ojeda & al., 2012, 2014). For this reason, the Macaronesian endemics of *Lotus* are not extensively covered in the present study. Relationships among these taxa are out of the scope of this paper.

Our study shows that increased taxon and marker sampling resolved some important issues of the *Lotus* phylogeny. On the other hand, the present study documents several instances of remarkable incongruence among nrITS, nrETS and plastid datasets, possibly resulting from reticulate evolution.

■ MATERIALS AND METHODS

Taxon sampling. — The sequences of the internal transcribed spacer region of nrDNA (ITS1, 5.8S, ITS2) were analyzed in 155 ingroup specimens, representing 98 species of *Lotus*. The taxon sampling covers all traditionally recognized sections of *Lotus* (Degtjareva & al., 2006) except for the monospecific Moroccan *L.* sect. *Benedictella* (Maire) Kramina & D.D. Sokoloff (*Lotus benoistii* (Maire) Lassen). Recent collections of *L. benoistii* are absent, and extensive attempts by one of us (JHK) to recollect the species in Morocco were not successful. We extended the nrITS sampling of *Lotus* in previous studies (Allan & al., 2003, 2004; Degtjareva & al.,

2006, 2008) by an additional 21 species, covering the entire distribution range of the genus from the Azores to New Caledonia and from tropical Africa to northern European Russia. Previous molecular analyses covering all genera of the tribe Loteae (Degtjareva & al., 2008, 2010) revealed a clade comprising members of *Hammatolobium* Fenzl, *Tripodion* Medik. and *Cytisopsis* Jaub. & Spach. as sister to *Lotus* (including *Tetragonolobus* and *Dorycnium*), and these relationships as well as monophyly of *Lotus* were highly supported. Therefore, the following three species are used here as outgroups: *Hammatolobium kremerianum* (Coss.) Müll.Berol., *Hammatolobium lotoides* Fenzl and *Cytisopsis pseudocytisus* (Boiss.) Fertig.

The sequences of the external (5'ETS) transcribed spacer region of nrDNA and two regions of plastid DNA (*psbA-trnH* intergenic spacer, *rps16* intron) were analyzed in a dataset including 70 ingroup specimens, which represent 54 species and all sections of *Lotus* except sect. *Benedictella*. The outgroups were the same as in the large ITS dataset. For a closer comparison between topologies obtained using different markers, the ITS sequences were also analysed for the same taxon sampling as the three other markers (Electr. Suppl.: Fig. S1).

Samples for analysis were obtained from silica-gel dried leaves or herbarium specimens collected by the authors or provided by different herbaria (AA, B, BP, BR, E, G, GOET, H, LE, LISC, M, MHA, MW, NSW, P, S, WAG, Z). The taxonomic identity of all voucher specimens was verified by the authors. In addition, 19 sequences of the ITS region from GenBank were used. Voucher information and GenBank accession numbers for the investigated species are listed in Appendix 1.

DNA extraction, amplification and sequencing. — Total genomic DNA was extracted using a NucleoSpin Plant II genomic DNA extraction kit (Macherey-Nagel, Düren, Germany), following the manufacturer's protocol. Details of the protocols for PCR and sequencing of the ITS and *rps16* regions are provided elsewhere (ITS: Valiejo-Roman & al., 2002; *rps16*: Marazzi & al., 2006). The amplification and sequencing of the *psbA-trnH* spacer was conducted as for the ITS region at the same PCR conditions, except that it was amplified using primers trnH2 (Tate & Simpson, 2003) and psbAF (Sang & al., 1997).

The portion of the ETS locus sequenced in this study corresponds to the ETSf region of Bena & al. (1998). This region is simply referred to as "ETS". To amplify and sequence this region, we used a primer annealing in the highly conserved 18S gene (18S-ETS; 5'-ACTTACACATGCATGGCTTAATCT-3'; Baldwin & Markos, 1998) in combination with a reverse primer generated specifically for the tribe Loteae (Lot-ETS; 5'-GTATGAGTTGTGWTTGGGWTG-3'). Design of the Lot-ETS primer required amplification of the entire intergenic spacer (IGS) region using long PCR, with universal primers annealing at the 5' end of 18S (18S-ETS) and at the 3' end of 26S (26S-IGS; 5'-GGATTGTTACCCACCAATAGGGAACGTGAGCTG-3'). Such a reaction was applied to five species of Loteae (*Coronilla vaginalis* Lam., *Acmispon parviflorus* (Benth.) D.D.Sokoloff, *Scorpiurus muricatus* L., *Hosackia pinnata* (Hook.) Abrams, *Lotus preslii* Ten.). These sequences were aligned and used to develop the Lot-ETS primer. This

primer anneals approximately 450 bp upstream from the annealing site of the 18S primer. PCR for amplification of ETS was performed using the same program and reagents as for ITS.

The PCR products were purified using a Cleanup Mini kit (Evrogen, Moscow, Russia). Direct sequencing was performed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, U.S.A.) using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit for cycle sequencing reactions, following manufacturer instructions. Both forward and reverse strands were sequenced for all samples. The obtained sequences were examined for single nucleotide polymorphism by studying chromatograms by eye. No polymorphic sites were detected, so the sequences were used in phylogenetic analyses without cloning.

Phylogenetic analyses. — Sequences of each marker were aligned using the program MUSCLE v.3.6 (Edgar, 2004) and manually adjusted using the program BioEdit v.7.2.5 (Hall, 1999). Gap-rich columns and positions of ambiguous alignment were excluded from phylogenetic analyses. Maximum parsimony (MP) analyses were conducted in PAUP* v.4.0b8 (Swofford, 2003) using tree-bisection-reconnection (TBR) branch swapping and 1000 random addition replicates, with equally weighted and unordered parsimony. Bootstrap support values (Felsenstein, 1985) were calculated from 1000 replicate analyses with maximal number of saved trees per replicate set to 1000, and a single random addition replication per bootstrap resampling was performed. For Bayesian inference the program MrBayes v.3.2.2 (Huelsenbeck & Ronquist, 2001; Ronquist & al., 2012) was used. The GTR+ Γ DNA substitution model (i.e., the general time reversible model with among site rate variation) was selected in ModelTest v.3.7 (Posada & Crandall, 1998) using the Akaike information criterion, for both separate and combined datasets. Bayesian analyses were performed using two parallel runs with four chains (one cold and three heated under default heating values) in each run. The chains were started with a random tree and run for 20 million generations; trees were sampled every 1000 generations. The number of generations to be discarded was determined using the cold

chain log likelihood examination in Tracer v.1.5 (Rambaut & Drummond, 2007). From the remaining trees, majority-rule consensus trees were produced. In all analyses gaps were treated as missing data. Basic characterization of each dataset used here and statistics of maximum parsimony analyses are provided in the Table 1. The trees inferred from separate analyses of each marker were examined for congruency. Topology differences with support higher than 0.95 (posterior probability, PP) and 80% (bootstrap proportion, BP) were considered as hard incongruence preventing data concatenation; otherwise data were concatenated, and combined phylogenetic analyses were performed as described above.

Biogeographic analysis. — Analysis of potential ancestral distribution areas at internal nodes was conducted using RASP v.2.1b (Yu & al., 2010, 2013), which implements the S-DIVA (statistical dispersal-vicariance analysis) method accounting for uncertainty of both phylogenetic and ancestral area reconstructions (Nylander & al., 2008; Harris & Xiang, 2009; Yu & al., 2010, 2013). The input file for RASP consisted of the 39,400 post-burn-in trees from the ETS sequence analysis with MrBayes. Relative frequencies of ancestral areas reconstructed for each node were recorded and plotted onto the majority-rule consensus tree from the Bayesian analysis. Four areas of endemism were defined for the biogeographic analysis based on the extant distribution of the genus: A, Europe, Mediterranean islands, northern part of Asia, including Turkey, northern Syria, Lebanon, northern-most parts of Iraq, Iran and Pakistan, Afghanistan, China, Japan and countries located to the north of this list; B, Africa, Socotra, southern part of Asia (excluding areas mentioned above); C, Macaronesia; and, D, Australia, New Caledonia and Vanuatu. In terms of biogeography (Takhtajan, 1986), our subdivision of Asia can be summarized as following: A, Holarctic kingdom except the Saharo-Arabian region; and, B, Palaeotropical kingdom plus the Saharo-Arabian region.

Nomenclature. — Infrageneric taxa (sections) of *Lotus* followed Degtjareva & al. (2006). Species not included in the study by Degtjareva & al. (2006) are assigned to infrageneric taxa

Table 1. Statistics of maximum parsimony analyses.

Dataset	ITS large	ITS	ETS	<i>psbA-trnH+rps16</i> intron
Number of accessions	169	74	74	73
Total characters	711	696	589	1343
Variable characters	276	256	254	184
Ingroup variable characters	255	233	238	154
Maximum parsimony, informative characters	226	187	212	109
Ingroup maximum parsimony, informative characters	212	165	195	88
Number of most parsimonious trees	920,000	184,011	65,155	6,950
Score of best trees	875	755	837	266
Consistency index (CI)	0.4361	0.4887	0.4683	0.6825
Retention index (RI)	0.9009	0.7946	0.8185	0.9325
Rescaled consistency index (RC)	0.4242	0.3883	0.3833	0.7222

according to Kramina & Sokoloff (2003), Sokoloff (2003a, b) and Kirkbride (2010). In addition, informal clade names are used in *Lotus* further developing the approach introduced by Degtjareva & al. (2008). A forward slash is used before clade names to distinguish them from section names. Clades and sections with the same names are generally congruent, with some exceptions that are discussed in detail below.

■ RESULTS

Topologies of trees obtained from the maximum parsimony and Bayesian analyses of the plastid data were similar, differing in groups of low (less than 0.75 PP and 65% BP) and moderate (ranging from 0.75 to 0.95 PP and from 65% to 80% BP) support, so we classified it as soft incongruence and concatenated both plastid markers for combined analysis. The ETS and ITS data were not concatenated, because the resulting trees from nuclear markers contained highly supported groups incongruent to each other and to those in the plastid trees. These topological differences are discussed in detail below.

Combined analyses of the two cpDNA regions (Fig. 2).

— Two large, well-supported clades can be distinguished in Bayesian and maximum parsimony analyses of the concatenated dataset of the two cpDNA regions. Clade 1 includes members of sect. *Canaria* (Rikli) D.D.Sokoloff, sect. *Chamaelotus* Kramina & D.D.Sokoloff, sect. *Heinekenia* Webb & Berth., sect. *Krokeria* (Moench) Ser., sect. *Lotea* (Medik.) DC., sect. *Ononidium* Boiss., sect. *Pedrosia*, sect. *Rhyncholotus* and sect. *Tetragonolobus* (Scop.) Benth. & Hook.f. and as well as the recently described *L. alianus* J.H.Kirkbr. (sect. *Lotus*).

Within clade 1, the clades /*Pedrosia*, /*Chamaelotus*, and /*Lotea* are well supported. The /*Pedrosia* clade includes all sampled species of sect. *Pedrosia* and sect. *Rhyncholotus*. In addition, the Moroccan endemic *L. weilleri* Maire (sect. *Lotea*) is strongly supported as a member of the /*Pedrosia* clade. *Lotus simonae* Maire & al. (sect. *Ononidium*) is a member of the /*Lotea* clade. *Lotus alianus* (sect. *Lotus*) is a member of the /*Chamaelotus* clade (1.00 PP; 96% BP). *Lotus edulis* L. (monospecific sect. *Krokeria*) is sister to the /*Lotea* clade (1.00 PP; 76% BP). All sampled members of sect. *Heinekenia* (10 species) belong to clade 1 where their relationships are largely unresolved. All three sampled species from the northern part of the distribution range of the section group together (/*Heinekenia*-N clade, 0.979 PP; 85% BP).

Clade 2 contains species of sect. *Bonjeanea* (Rchb.) D.D. Sokoloff, sect. *Dorycnium* (Mill.) D.D.Sokoloff, sect. *Erythrolotus* Brand and sect. *Lotus* (except *L. alianus*). None of the sections forms a clade. Members of sect. *Lotus* fall into two clades. The /*Lotus castellanus* clade (0.998 PP; 83% BP) comprises species traditionally recognized as the “*L. angustissimus* group” (Kramina, 2006: mainly annuals and biennials) and the “*L. pedunculatus* group” (see Chrtková-Zertová, 1966; stoloniferous perennials). The well-supported /*Lotus corniculatus* clade includes mainly perennial, usually yellow-flowered species without stolons traditionally recognized as the “*L. corniculatus* group” (Kramina, 1999b, 2000). The red-flowered

annual *L. conimbricensis* Brot. (sect. *Erythrolotus*) is strongly supported as sister to the /*Lotus corniculatus* clade, though on a long branch.

Species of sect. *Bonjeanea* and sect. *Dorycnium* fall into highly supported clades that do not correspond to the previously accepted sectional division (Kramina & Sokoloff, 2003; Sokoloff, 2003a, b). The /*Lotus dorycnium* clade (1.00 PP; 100% BP) includes four species of sect. *Dorycnium* plus *L. hirsutus* L. (sect. *Bonjeanea*). Out of the five members of the /*Lotus dorycnium* clade, four are represented by more than one accession in the present study. Surprisingly, accessions of these four species did not group together in the trees inferred from analyses of the plastid markers. Accessions of the same species failed to group together not merely due to the unresolved tree topology. Phylogenetic relationships within the /*Lotus dorycnium* clade are at least partially resolved and some branches are relatively long, yet different accessions of *L. dorycnium* L. and *L. hirsutus* are scattered among different subclades within the /*Lotus dorycnium* clade. The /*Lotus graecus* clade (1.00 PP; 99% BP) comprises a fraction of the species in sect. *Dorycnium*. Two species of sect. *Bonjeanea* (*L. rectus* L., *L. strictus* Fisch. & C.A.Mey.) appear in an unresolved position within the clade 2.

Nuclear ribosomal ETS and ITS trees compared to plastid phylogenies (Figs. 3–5).

— The overall tree topologies obtained in analyses of nrETS and nrITS are somewhat different (see next sections), and the ETS tree is more congruent with the plastid tree than the ITS tree. The main common feature of the plastid (Fig. 2) and the ETS trees (Fig. 3) is the presence of the large clade 1 of the same composition. Its members possess a papillose style and a base chromosome number of $x = 7$. Clades 2A and 2B revealed in the ETS trees (Fig. 3) comprise members of clade 2 from analyses of the plastid data (Fig. 2). Clade 2A (0.992 PP; 74% BP) comprises species with a papillose style and a base chromosome number of $x = 6$. Clade 2B (0.983 PP; <50% BP) comprises species with a smooth style and a base chromosome number of $x = 7$ and corresponds to the genus *Dorycnium* as defined by Lassen (1986). In the classification followed here, clade 2B includes members of *Lotus* sect. *Dorycnium* and sect. *Bonjeanea*. In the ETS trees (Fig. 3), clades 1 and 2A form a sister pair with low support (0.724 PP; 62% BP). This large group corresponds to the genus *Lotus* as defined by Lassen (1986). All the species of the group possess a papillose style. This feature should be considered as a synapomorphy because the outgroups possess a smooth style. In the ITS trees (Figs. 4, 5), only the core groups of clades 1 and 2A are revealed, not the complete clades, and clade 2B cannot be recognized. The absence of clades 1, 2A and 2B in the ITS trees is due to a lack of resolution at the base of the *Lotus* phylogeny rather than to strongly supported alternative topologies that violate monophyly of these clades.

Common features of nrITS and 5'ETS tree topologies (Figs. 3–5).

— Despite the differences in overall topology, the trees constructed using ETS and ITS have many common features. The large core group of clade 1 (called here the /CHZ clade) is well-supported in the Bayesian nrETS tree, and the same clade is found in the Bayesian nrITS tree (see also Electr. Suppl. Fig. S1). The group is named the /CHZ clade because

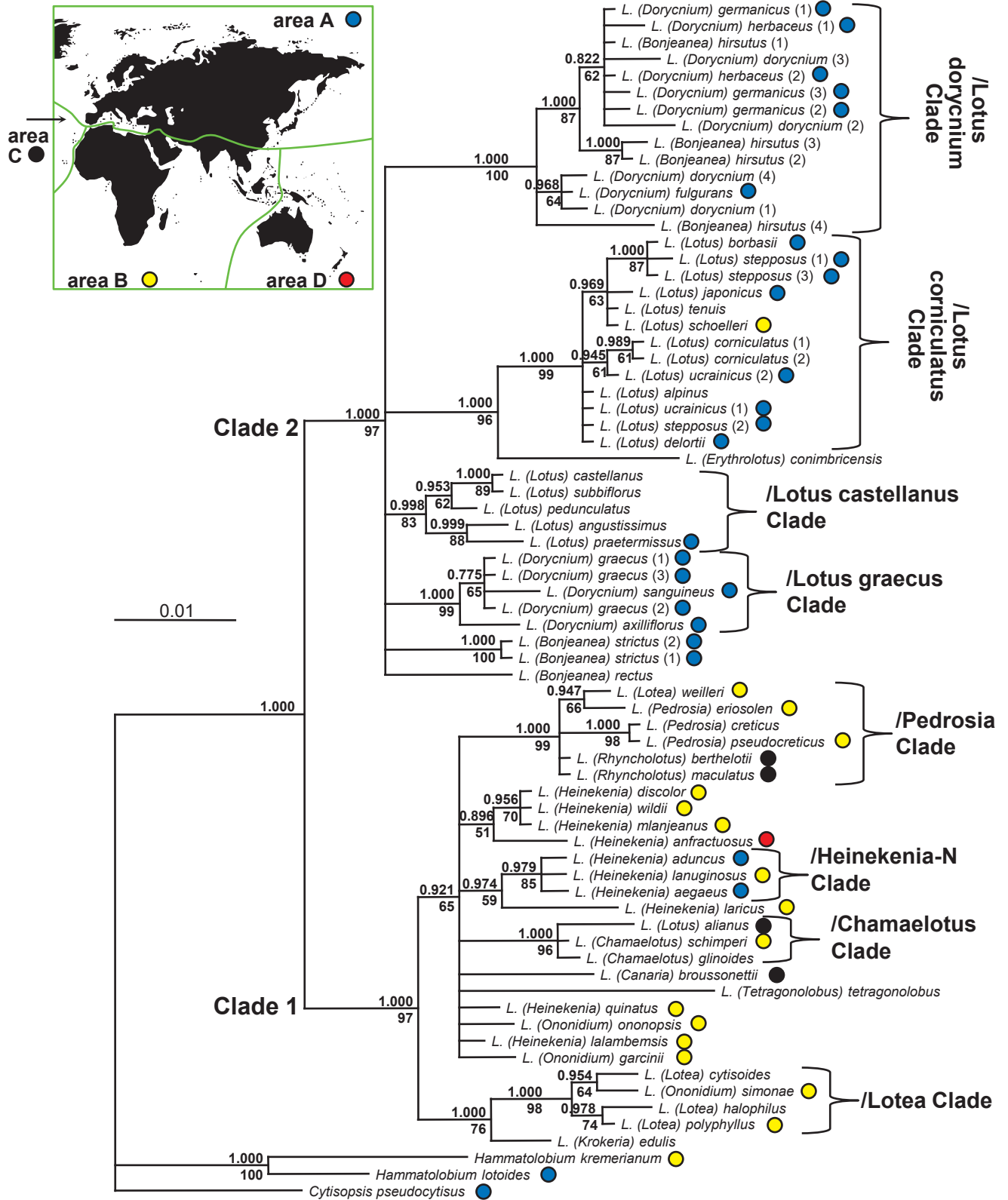


Fig. 2. Phylogenetic relationships in *Lotus* inferred from Bayesian analysis of the plastid dataset. Branch lengths are proportional to the number of expected nucleotide substitutions. Major clades of *Lotus* are indicated. Numbers above branches are posterior probabilities. Numbers below branches are bootstrap values found in the maximum parsimony analysis of the same dataset. Sectional names are indicated in parentheses before species names. See text for the concept of sections used here. Figures in parentheses after species names refer to numbers of particular accessions of the same species. See Appendix 1 for voucher information. Colour circle after species name indicate that a species is endemic to one of the four geographic areas recognized here. See inset map defining the areas and the colours of the circles. Names without circles are species that occur in more than one area.

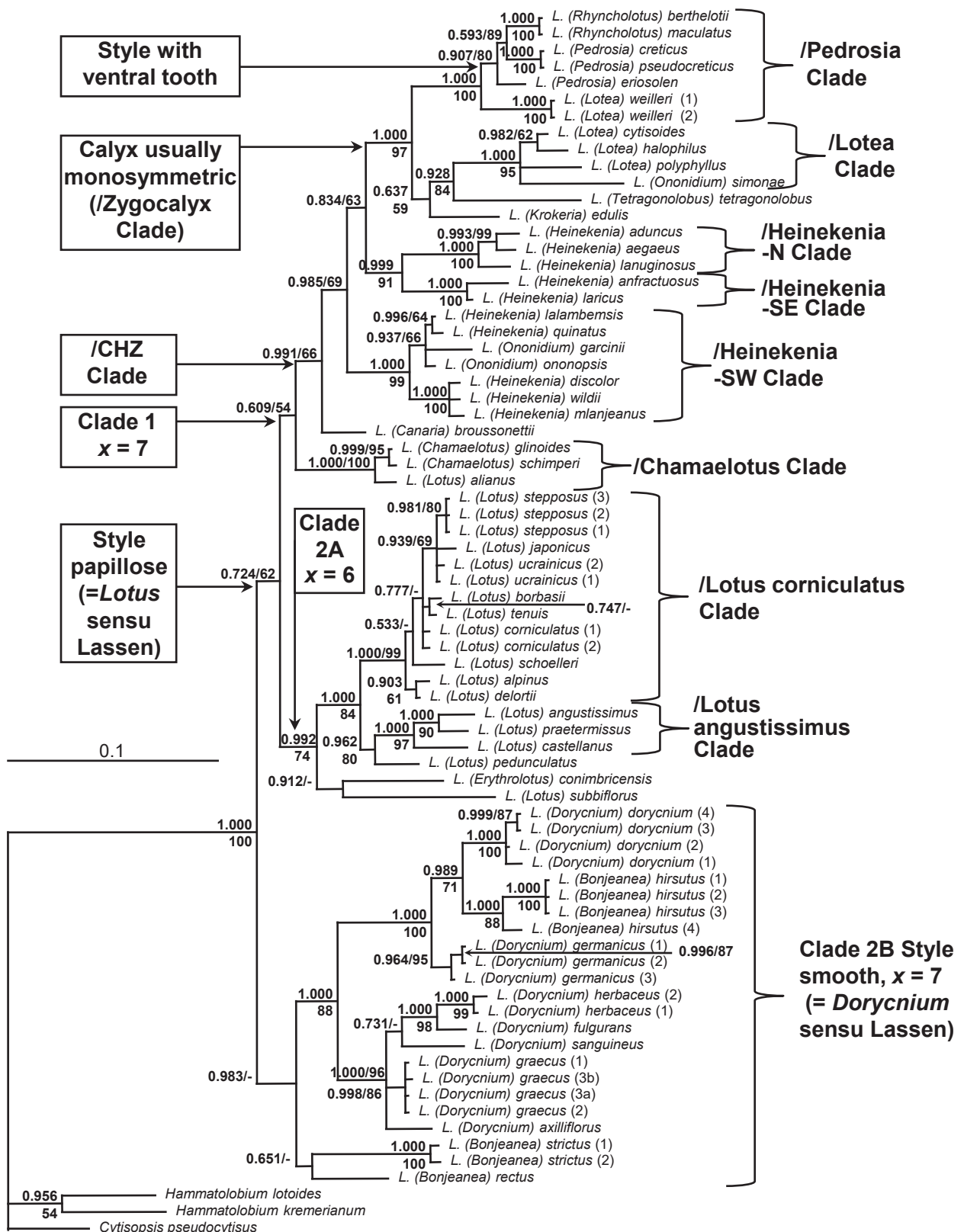


Fig. 3. Phylogenetic relationships in *Lotus* inferred from Bayesian analysis of the nrETS dataset. Branch lengths are proportional to the number of expected nucleotide substitutions. Major clades of *Lotus* and occurrence of some morphological features are indicated. Numbers above branches are posterior probabilities. Numbers below branches or after slashes are bootstrap values found in the maximum parsimony analysis of the same dataset. Sectional names are indicated in parentheses before species names. See text for the concept of sections used here. Figures in parentheses after species names refer to numbers of particular accessions of the same species. See Appendix 1 for voucher information.

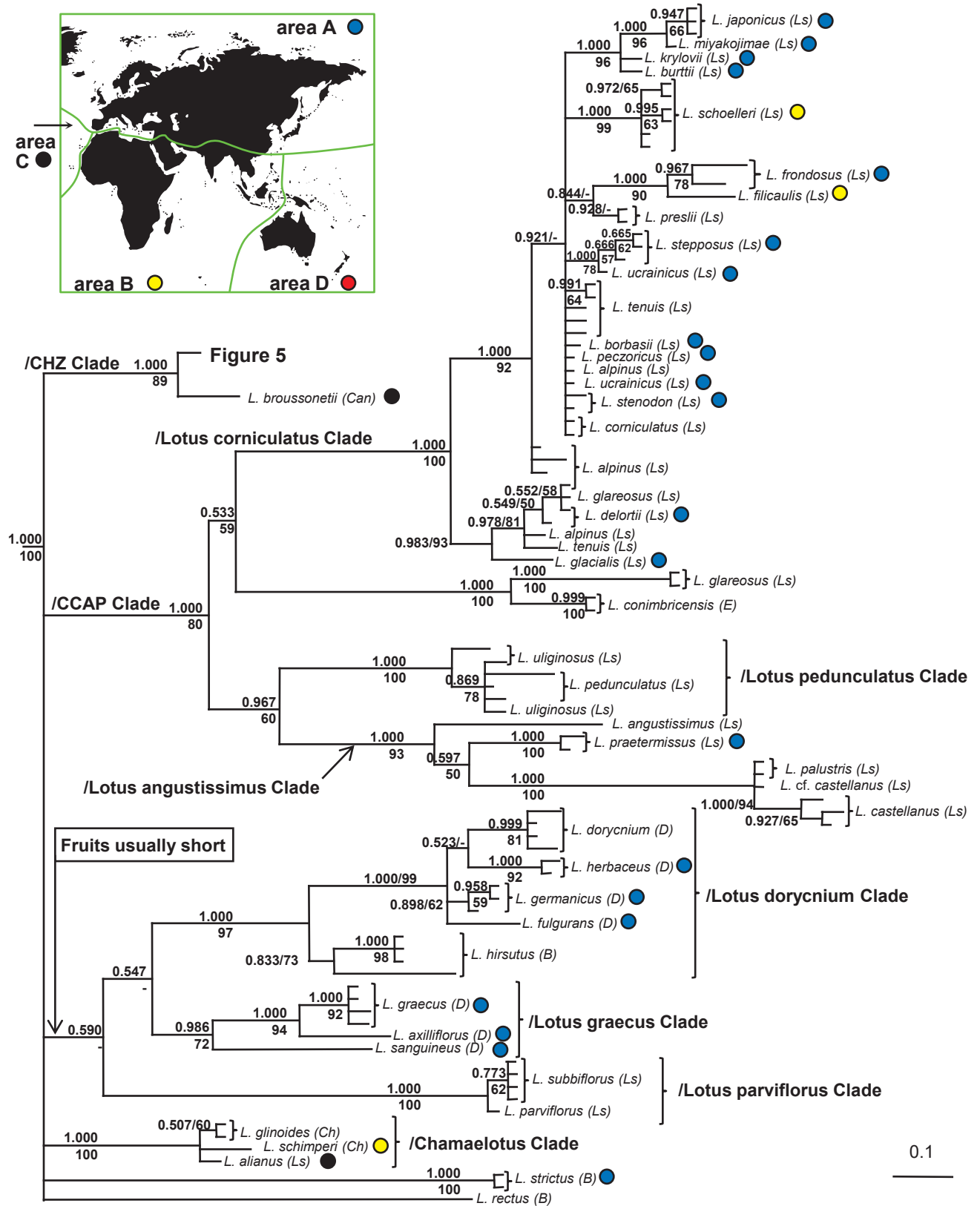


Fig. 4. Phylogenetic relationships in *Lotus* inferred from Bayesian analysis of the nrITS dataset, outgroups (not shown) are the same as in Figs. 2 and 3. Branch lengths are proportional to the number of expected nucleotide substitutions. Major clades of *Lotus* and occurrence of some morphological features are indicated. Numbers above branches are posterior probabilities. Numbers below branches or after slashes are bootstrap values found in ▶

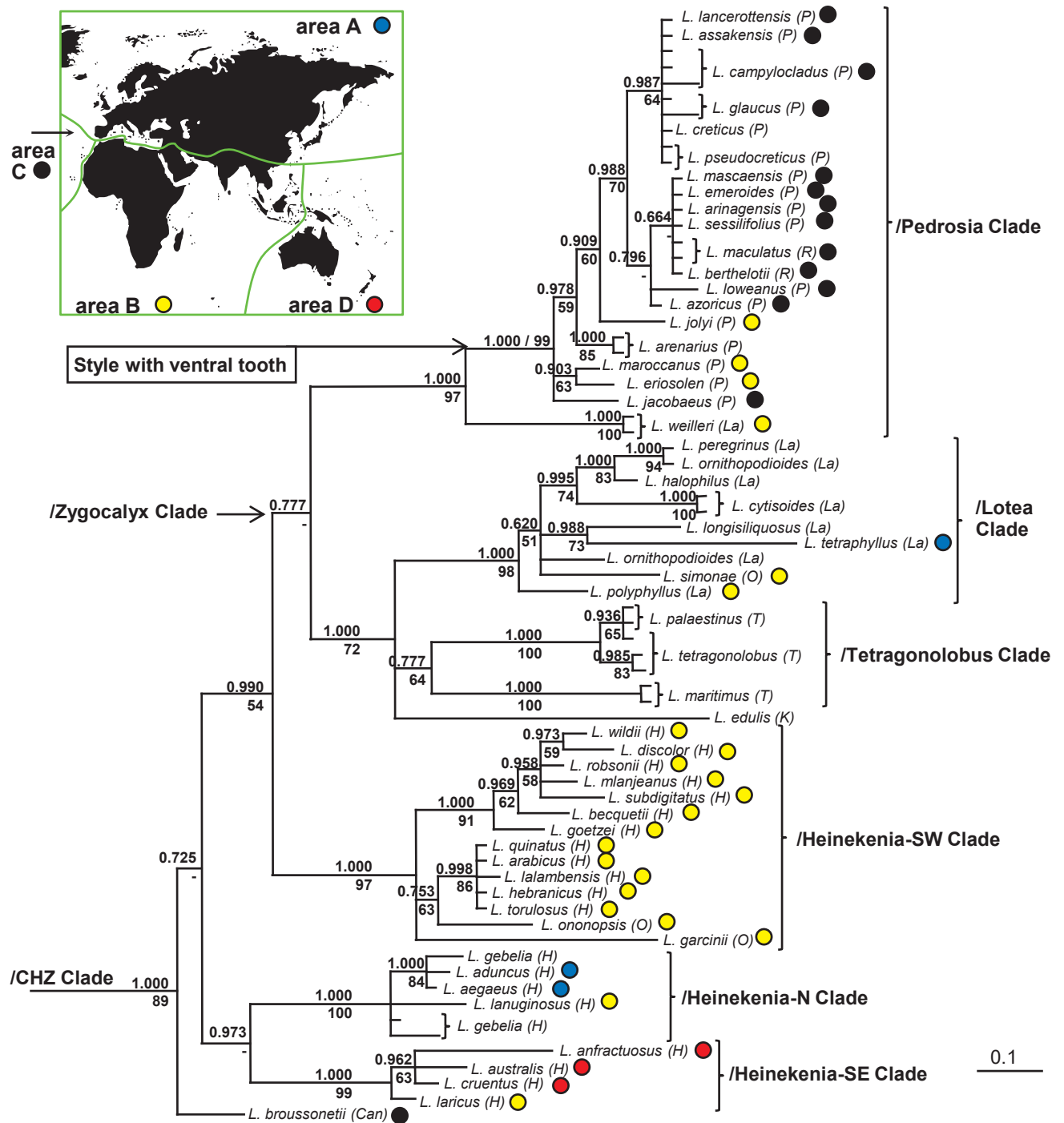


Fig. 5. Phylogenetic relationships in *Lotus* inferred from Bayesian analysis of the large nrITS dataset, continued from Fig. 4.

► the maximum parsimony analysis of the same dataset. Abbreviated sectional names are indicated in parentheses after species names (*B* = *Bonjeanea*, *Can* = *Canaria*, *Ch* = *Chamaelotus*, *D* = *Dorycnium*, *E* = *Erythrolotus*, *H* = *Heinekenia*, *K* = *Krokeria*, *La* = *Lotea*, *Ls* = *Lotus*, *O* = *Ononidium*, *P* = *Pedrosia*, *R* = *Rhyncholotus*, *T* = *Tetragonolobus*). See text for the concept of sections used here. When more than one accession of a species was included, the arrangement of the accessions in Appendix 1 follows their arrangement in this figure. Colour circle after species name indicate that a species is endemic to one of the four geographic areas recognized here. See inset map defining the areas and the colours of the circles. Names without circles are species that occur in more than one area. The tree is continued on Fig. 5.

it comprises members of sect. *Canaria* with sect. *Heinekenia* and the */Zygocalyx* clade. The */Zygocalyx* clade, further separated as a core group of the */CHZ* clade in trees inferred from both markers, though it received high support in the ETS trees only (Figs. 3, 5; Electr. Suppl.: Fig. S1). The clade comprises members of sect. *Krokeria*, sect. *Lotea*, sect. *Pedrosia*, sect. *Rhyncholotus*, sect. *Tetragonolobus* and *L. simonae* (sect. *Ononidium*). The species placed in this clade, except *L. edulis* (sect. *Krokeria*), have a pronounced monosymmetric calyx, hence the name */Zygocalyx* (Degtjareva & al., 2006).

Both nuclear markers revealed the clades */Pedrosia*, */Lotea* and */Chamaelotus* with high support. The composition of the clades is the same as that obtained in analyses of plastid data. Within the */Pedrosia* clade, all species except *L. weilleri* form a well-supported monophyletic group defined by the occurrence of a tooth on the ventral side of the style, a uniquely derived synapomorphy. The placement of *L. alianus* in the */Chamaelotus* clade agrees with the plastid trees.

In addition, many smaller clades corresponding to parts of sections or species groups are similarly distinct in Bayesian trees constructed from both nuclear markers (Electr. Suppl.: Fig. S1). Each of the three geographical groups of sect. *Heinekenia* (i.e., */Heinekenia*-SW, -N and -SE clades) is highly supported, however, they do not form a joint clade (Figs. 3, 5). Support of these three clades is also high in maximum parsimony ETS trees (Fig. 3), but varies slightly in maximum parsimony ITS tree (Fig. 5). The */Lotus corniculatus* and */Lotus angustissimus* clades are highly supported in both ETS and ITS trees.

Differences in ITS and 5'ETS tree topologies. — Apart from Figs. 3–5, differences in tree topologies revealed in analyses of individual datasets of nuclear ribosomal ITS and 5'ETS DNA regions can be seen in Fig. S1 (Electr. Suppl.), where a Bayesian ITS tree includes only those taxa that are present in the Bayesian ETS tree. Removing part of the sequences from the full ITS dataset only affected some nodes with low support, see Figs. 4, 5 and Electr. Suppl.: Fig. S1.

The clade comprising all species of *Lotus* sensu Lassen (1986) is found only in the ETS trees. The clade comprising all species of *Dorycnium* sensu Lassen (1986) is found only in the Bayesian analysis of the ETS data (0.983 PP); it is absent in the maximum parsimony ETS tree and in the ITS trees.

The two major clades of *Lotus* sensu Lassen (clade 1 and clade 2A) were found in the analyses of the ETS sequences, but not supported in the ITS trees due to the unresolved relationships of their peripheral elements, i.e., the */Chamaelotus* clade and *L. subbiflorus* Lag. In the */CHZ* clade, phylogenetic relations among the three geographic lineages of sect. *Heinekenia*, i.e., */Heinekenia*-SW, -SE and -N clades, differ in the ITS and ETS trees. In the Bayesian ITS tree topologies (Figs. 4, 5; Electr. Suppl.: Fig. S1), a group consisting of */Heinekenia*-N+*/Heinekenia*-SE occupies a nearly basal position within the */CHZ* clade 1, and the */Heinekenia* SW clade is sister to the */Zygocalyx* clade. In the corresponding ETS tree (Fig. 3; Electr. Suppl.: Fig. S1), the */Heinekenia* SW clade is placed more basally, and the group consisting of */Heinekenia* N+*/Heinekenia* SE is sister to the */Zygocalyx* clade. Some aspects of these alternative topologies are only moderately supported, so there is no strong conflict.

While clade 2A is not present in the ITS tree, a near-identical */CCAP* clade, differing in the absence of *L. subbiflorus*, is revealed. The name */CCAP* is given because the clade includes *L. conimbricensis*, the */Lotus corniculatus* clade, the */Lotus angustissimus* clade and the */L. pedunculatus* clade (Fig. 4). In the Bayesian analysis of the ETS data, *L. conimbricensis* groups with *L. subbiflorus* (0.912 PP), and this grouping questions the monophyly of the */CCAP* clade (Fig. 3).

There is significant incongruence in relationships among members of sect. *Dorycnium* and sect. *Bonjeanea* (i.e., *Dorycnium*, sensu Lassen) inferred from analyses of the ETS and ITS datasets (Figs. 3, 4; Electr. Suppl.: Fig. S1). Both ETS and ITS topologies are also incongruent with the plastid topology (Electr. Suppl.: Fig. S2). In some instances, conflicting placements of species are well supported in both ETS and ITS trees.

Analyses of the ITS data strongly support monophyly of a species group with similar flower and fruit morphologies including *L. dorycnium* s.str., *L. fulgurans* (Porta) D.D.Sokoloff, *L. germanicus* (Gremli) Peruzzi, and *L. herbaceus* (Vill.) Jauzein (Fig. 4). A sister relationship of this clade with *L. hirsutus* is well-supported in the ITS trees (Fig. 4; Electr. Suppl.: Figs. S1, S2), in contrast to the ETS trees (Fig. 3; Electr. Suppl.: Figs. S1, S2), which show the monophyly of a clade comprising *L. dorycnium* s.str., *L. germanicus*, and *L. hirsutus* (1.00 PP; 100% BP). Also, the ETS dataset strongly supports placement of *L. fulgurans* and *L. herbaceus* with *L. axilliflorus* (Hub.-Mor.) D.D. Sokoloff, *L. graecus*, and *L. sanguineus* (Vural) D.D.Sokoloff (Fig. 3; Electr. Suppl.: Figs. S1, S2). This is not congruent with the */Lotus dorycnium* and */Lotus graecus* clades found in the analyses of the plastid markers (Fig. 2) and nrITS sequences (Fig. 4; Electr. Suppl.: Fig. S1).

Biogeography. — The ETS phylogeny is generally more resolved than the ITS and plastid phylogenies. When more than one accession of a species was analyzed, the accessions formed a clade or fell into the same polytomy in the ETS trees, in contrast some species were not monophyletic in plastid and ITS trees. These features indicate that the ETS trees are more suitable for formal biogeographic analyses than the other two sets of trees. A dataset containing one ETS accession per species was therefore used for producing individual Bayesian trees used for S-DIVA analysis. The resulting Bayesian consensus tree (Fig. 6) was highly similar to the ETS tree inferred from the analysis of multiple accessions (Fig. 3) in terms of topology as well as posterior probabilities of particular nodes. The S-DIVA analysis (Fig. 6) highlighted strong links between species distribution patterns and the three major clades of the ETS tree. Most species of clade 1 are endemic to areas B, C and D (i.e., Macaronesia, Africa, S Asia and Australasia), and analyses of all individual Bayesian trees revealed area B (Africa, S Asia) as an ancestral area for the clade. In contrast, clades 2A and 2B are rich in species restricted to area A (Europe plus N Asia), and the vast majority of individual Bayesian trees suggested that area A was ancestral for each of these two clades. Analyses of the ITS dataset added more species to the core groups of clades 1, 2A and 2B, and this expanded taxonomic sampling highlights the same geographical patterns (Figs. 4, 5). As the analyses of the plastid dataset showed that clade 2 contains members

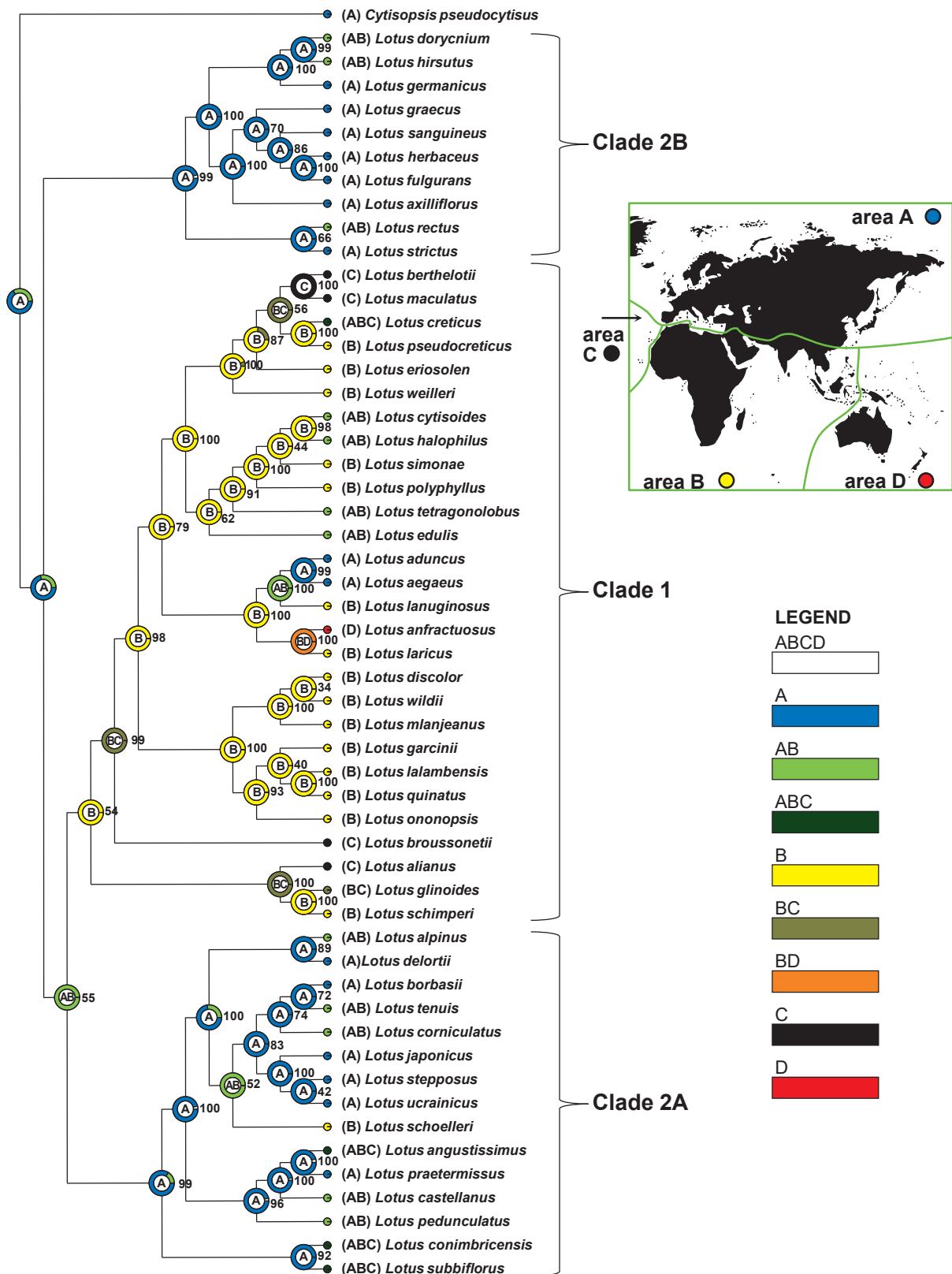


Fig. 6. Results of S-DIVA analysis of the nrETS dataset. Tree topology is inferred from the Bayesian analysis of the nrETS dataset. Numbers to the right of nodes are posterior probabilities (given as percents). Major clades are indicated. Colours indicate species distribution in the four major areas, as outlined in the inset map. Colour legend is provided. Possible geographic distribution is reconstructed for all internal nodes. Relative widths of sectors of different colours in rings situated at internal nodes show the proportion of individual Bayesian trees in which a particular geographical area is found to be ancestral for a given clade.

of clades 2A and 2B of the ETS tree, the overall correlation between major clades and geography is even stronger. The plastid tree splits *Lotus* into two clades, one with most species endemic to areas B, C and D and the other with more than half of the species endemic to area A (Fig. 2).

■ DISCUSSION

Overall tree topologies and (in)congruence of trees. — Our work represents the first world-wide molecular phylogenetic study of *Lotus* based on plastid as well as nuclear DNA markers. The study revealed many shared aspects of the trees obtained in analyses of different markers, both at the level of overall tree topology and smaller scale levels, such as groups of species or particular clades. These aspects of congruency may serve as a basis for a future taxonomic revision of the genus. At the same time, a number of incongruences both between nuclear and plastid trees as well as between nuclear ETS and ITS trees have been discovered.

The main features of the Bayesian and maximum parsimony trees based on nrITS sequences are predictably similar to those obtained in previous studies (Allan & al., 2003, 2004; Degtjareva & al., 2006, 2008), but our ITS tree (Figs. 4, 5) contains a larger representative set of species and accessions than all earlier studies. The monophyly of the genus *Lotus* within the limits suggested by Sokoloff (2003a, b) and supported by previous ITS-based phylogenetic studies (Allan & al., 2003; Degtjareva & al. 2006, 2008, 2010) is confirmed based on nuclear and plastid DNA markers and on improved sampling of *Lotus* species.

The following similarities were found in our trees based on plastid and nuclear ETS and ITS markers.

(1) Occurrence of clade 1 or a core group of it, i.e., the /CHZ clade comprising members of sect. *Canaria*, sect. *Heinekenia*, sect. *Krokeia*, sect. *Lotea*, sect. *Ononidium*, sect. *Pedrosia*, sect. *Rhyncholotus* and sect. *Tetragonolobus*. The /CHZ clade differs from clade 1 found in analyses of plastid and ETS sequences in the absence of a small unit, the /Chamaelotus clade (Degtjareva & al., 2006, 2008; this study). However, alternative placements of the /Chamaelotus clade are poorly supported, and thus the ITS data weakly contradict the recognition of clade 1. A group corresponding to clade 1 was never proposed in morphological classifications. It can be precisely defined by a combination of two morphological features (Fig. 3), a papillose style and a base chromosome number of $x = 7$. These features cannot be regarded as potential unequivocal synapomorphies. Indeed, the base chromosome number $x = 7$ is widespread in the tribe Loteae and most likely represents a plesiomorphy in *Lotus* and Loteae (Degtjareva & al., 2004) while the papillose style occurs in both, clade 1 and clade 2A. Clade 1 has major diversity centres outside Europe and northern Asia, and this geographical tendency further supports the naturalness of the group.

(2) Occurrence of several identical clades that are close, but not always identical, to taxonomic circumscriptions of traditionally recognized infrageneric taxa or species groups. These are the /Chamaelotus, /Lotea, /*Lotus* corniculatus and /*Pedrosia* clades.

(3) Shared occurrence of some well-supported clades that differ from traditional taxonomic concepts, namely the /*Lotus* dorycnium and /*Lotus* graecus clades.

The plastid tree (Fig. 2) shows a well-supported basal split in *Lotus*, into clades 1 and 2, but basal relationships within these major clades are not well resolved. In contrast, the ETS and ITS trees (Figs. 3–5) generally provide poor support for each of the major clades (1, 2A, 2B) and especially for their possible interrelationships, but give much better resolution for basic relationships within the major clades. The /CHZ and /*Zygocalyx* clades are well-supported by nuclear ETS and partially by ITS data, but not by the plastid data, though the alternative topologies of the plastid trees are not well resolved. The /*Zygocalyx* clade was found in earlier ITS-based analyses with only low support, but its support increased when morphological characters were analysed together with the ITS dataset (Degtjareva & al., 2006). In the present study ETS data strongly supported the /*Zygocalyx* clade.

Several instances of strongly supported topological differences between nrITS, nrETS and plastid trees and between nrETS and nrITS trees (Figs. 3–5; Electr. Suppl.: Figs. S1, S2) are documented in the present study.

Conflicting phylogenetic signals between datasets were reported for various plant taxa. Many hypotheses were proposed to explain such incongruences (e.g., Rieseberg & al., 1996), and lineage sorting and reticulate evolution (including ancient and/or recent hybridization) are often named as main reasons (e.g., Rieseberg & al., 1996; Xiang & al., 1998; Okuyama & al., 2005). To elucidate the precise cause of incongruencies, data from several nuclear gene regions are needed (Záveská & al., 2012).

The combination of hybridization and complete or incomplete homogenization of the multiple-copy ITS region could result in contradictory phylogenetic reconstructions, and lineage sorting might influence the abundance of ITS alleles so that their patterns differ from those of cpDNA haplotypes (Záveská & al., 2012). These authors reported a high degree of homoplasy in the ITS dataset of *Curcuma* L. (Zingiberaceae), confirmed by low consistency index (CI) values, as evidence for reticulate evolution, connected to hybridization and/or polyploidization (Záveská & al., 2012). CI values in our ITS dataset are lowest among all datasets studied (Table 1). Reticulate evolution likely occurred in some lineages of *Lotus*, especially in sect. *Dorycnium* (Conesa & al., 2010), sect. *Lotus* (e.g., Gauthier & al., 1998; Kramina & Schanzer, 2010) and sect. *Pedrosia* (Sandral & al., 2006). For these sections, as well as for sect. *Lotea*, polyploidy was reported, while in other sections of *Lotus* polyploid species have not been found so far (e.g., Grant, 1995). Most cases of incongruency between datasets in the present study are related to placement of some members of sect. *Dorycnium* and sect. *Lotus*.

Conflicts between two nuclear DNA regions (i.e., ITS and ETS) may appear as a result of different evolutionary rates of the two markers or of recombination. Bailey & al. (2003) noted that complex molecular evolutionary processes usual for the nrITS and ETS regions might confound their utility in phylogenetic reconstruction. Kovarik & al. (2005) and Mavrodiev & al. (2013) discussed several cases in which “recombinant” clones

of rDNA regions were detected, particularly in ITS. ITS and ETS sequences may have different evolutionary histories, which are possibly caused by different patterns of concerted evolution within the rDNA arrays (e.g., Okuyama & al., 2005). The region with slower concerted evolution may show evidence of ancient and/or recent introgression events (Sang & al., 1995). In a review of nuclear ribosomal spacer regions in plants, Poczai & Hyvönen (2010) noted faster evolutionary rates of ETS compared to ITS as a main tendency. They also pointed out that ETS usually contains more phylogenetically informative characters than ITS. In consequence, when the two rDNA regions are analyzed separately, the phylogenetic signal provided by each region may be variable. This variability might depend on phylogenetic histories and be displayed differently at different taxonomic levels.

With respect to all datasets explored, Bayesian analyses usually produced trees with better resolution than parsimony. Topological discordance between the two types of trees is comparatively low, and usually concerns branches with low support. However, even if we use a clade support criterion of $\geq 80\%$ BP for parsimony trees and ≥ 0.95 PP for Bayesian consensus trees (Harris & al. 2009), some conflicts can be observed between the trees based on the same datasets. As a rule, these conflicts involve relationships among parts of sect. *Heinekenia* (ITS, ETS, and plastid dataset analyses) and sect. *Lotus* (ITS and ETS analyses).

Species traditionally placed in *Dorycnium*. — Since the early 19th century, most European authors accepted *Lotus* and *Dorycnium* as two closely related, but distinct genera. In non-Mediterranean Central Europe, differences between *Lotus* s.str. and *Dorycnium* are clear and sharp, and this was probably a major factor in the recognition of the two genera as distinct. All members of *Dorycnium* occurring in Central Europe belong to the *D. pentaphyllum* Scop. s.l. species complex; *Dorycnium pentaphyllum* is the nomenclatural type of the genus. They differ from members of the *L. corniculatus* group occurring in Central Europe in an impressive set of characters, such as usually suffrutescent habit (rather than herbaceous), absence of a leaf rachis, presence (rather than absence) of an elongate portion of the peduncle between the sterile foliage leaf and partial inflorescence, very small flowers in many-flowered partial inflorescences, white to pink petals (rather than yellow), wing petals distally adhering to each other and conspicuously saccate, keel petal shorter than the wings and obtuse (rather than rostrate), carpel style smooth (rather than papillose) and fruits short, one-seeded, with valves not contorting at dehiscence. However, some species occurring in the Canary Islands and Mediterranean region (some as far eastwards as Central Asia) possess a mosaic of features characteristic of typical members of *Lotus* and *Dorycnium*. It is therefore not surprising that the precise taxonomic boundaries between these genera have been the subject of discussions (Electr. Suppl.: Fig. S3).

Earlier molecular phylogenetic studies, all based only on nrITS sequences, clearly showed that all species of *Lotus* s.str. and several segregate genera, including *Dorycnium*, collectively form a well-supported clade. However, with the exception of the first publication that considered only very few species (Allan & Porter, 2000), these studies (Allan & al., 2003; Degtjareva

& al., 2006, 2008) failed to resolve the precise phylogenetic relationships at the base of this large clade, and this uncertainty affected placement of species included by various authors in *Dorycnium*. Ambiguities found in both molecular and morphological studies led to the conclusion that *Dorycnium* should not be segregated from *Lotus* as a distinct genus (Sokoloff, 2003a, b; Degtjareva & al., 2006). This view follows Linnaeus (1753) and Polhill (1981). According to Sokoloff (2003a, b), species of *Dorycnium* sensu Lassen should be classified in two sections, *Lotus* sect. *Dorycnium* and *L.* sect. *Bonjeanea*.

The present molecular study includes the largest sampling of taxa placed by various authors in *Dorycnium*. Even with increased taxon sampling, the nrITS tree (Fig. 4), like previous studies, does not resolve all relationships of these taxa. In contrast, when the nrETS marker is considered (Fig. 3), a clade is found that precisely corresponds to the taxonomic circumscription of *Dorycnium* proposed in one of the recent accounts (Lassen, 1986: fig. 8). Our analyses of the plastid dataset did not reveal this clade but did not contradict its existence (Fig. 2). According to Lassen (1986), *L. strictus* that was earlier consistently placed in *Lotus* is a member of *Dorycnium*. Lassen (1986) highlighted morphological similarities between *L. strictus* and *Dorycnium hirsutum* (L.) Ser. (= *L. hirsutus*), but our ETS tree places *L. strictus* together with another species, *L. rectus* (= *D. rectum* (L.) Ser.). Lassen's circumscription of *Dorycnium* is corroborated by morphological characters such as the smooth carpel style and presence of brown cells in the fruit endocarp. Bayesian analysis of the ETS data revealed members of *Dorycnium* sensu Lassen as sister to the remainder of *Lotus* s.l. as found with poor taxon sampling in the nrITS tree of Allan & Porter (2000) and in the morphological tree of Sokoloff (2005). We do not advocate re-erecting *Dorycnium* as a distinct genus because branch support of the clade comprising the remainder of *Lotus* s.l., i.e., putative *Lotus*, s.str., is rather low (0.724) in the Bayesian ETS tree, and 62% in the MP analysis. The nrITS analysis did not reveal this grouping at all. Also, analyses of the plastid dataset revealed a different, well-supported rooting of the *Lotus* s.l. clade.

Section *Dorycnium*, as defined by Sokoloff (2003a, b), is not recovered by any of the studied DNA markers. Instead, a highly supported clade combining sect. *Dorycnium* and *L. hirsutus* (sect. *Bonjeanea*) appears in ETS trees and with poor support in Bayesian ITS tree (Fig. 4). This clade is absent in cpDNA trees, but plastid data also show close relationships between *L. hirsutus* and members of sect. *Dorycnium* (Fig. 2). The inferred relationships of *L. hirsutus* can hardly be explained by morphology because the accessions of *L. hirsutus* appear in two parts of the traditionally recognized *L. dorycnium* (= *Dorycnium pentaphyllum*) complex, i.e., *L. dorycnium* and *L. germanicus*, in the nrETS trees (Fig. 3), and especially because *L. hirsutus* is not monophyletic in the plastid trees (Fig. 2). However, a group containing members of sect. *Dorycnium* and *L. hirsutus* is characterized by a set of morphological characters related to fruit structure. They all possess short pods, not exceeding 10 mm in length, which are oblong or oval to globose, 1- or 2-seeded (up to 4-seeded in *L. hirsutus*), and with thick, and not or only slightly contorting valves.

Section *Bonjeanea* (as defined by Sokoloff, 2003a, b; i.e., including *L. hirsutus*, *L. rectus* and *L. strictus*) does not appear to be monophyletic in our molecular phylogenetic analyses. *Lotus hirsutus* never groups with *L. strictus* and *L. rectus*, however, the two last species form a weakly supported clade in the Bayesian analysis of nrETS.

Relationships of sect. *Canaria*. — Section *Canaria* includes three rare and morphologically similar species endemic to the Canary islands. They are shrubs with large leaves and long petal claws, exceeding the calyx. Rikli (1901) placed this section in *Dorycnium*, together with two other sections, *Dorycnium* and *Bonjeanea*. Members of the *Canaria* group were also placed in *Dorycnium* by a few other authors, including Taubert (1894; see Electr. Suppl.: Fig. S3). Many recent authors, however, followed Gillett (1958) and Lassen (1986) and placed these three species in *Lotus* (Electr. Suppl.: Fig. S3).

All molecular analyses of the present and earlier studies included only one member of sect. *Canaria*, *L. broussonetii* Choisy ex Ser. The association of *L. broussonetii* with any members of sections *Dorycnium* and *Bonjeanea* was not observed. In our analyses, *L. broussonetii* was placed in the large clade 1 (plastid and nrETS markers) or in its core group, the /CHZ clade (nrITS marker), together with representatives of sect. *Heinekenia*, sect. *Krokeria*, sect. *Lotea*, sect. *Pedrosia* and sect. *Tetragonolobus*.

Section *Lotus*. — This is the taxonomically most problematic section of the genus, which contains agriculturally important pasture plants as well as the model legume *L. japonicus*. The section includes annual and perennial herbs with five-leaflet leaves and yellow flowers with a polysymmetric calyx and glabrous pods, the last two characters with a few exceptions. Members of the section are native to the Circumboreal, East-Asian, Macaronesian, Mediterranean, Saharo-Arabian, Irano-Turanian, and Sudano-Zambesian floristic regions of Takhtajan (1986). This is the most widespread section of *Lotus*, and the only section occurring in large areas of temperate northern Eurasia.

According to our earlier morphological studies, sect. *Lotus* includes species arranged in several species complexes, i.e., the *L. corniculatus* group (including smaller subgroups, such as the *L. delortii*, *L. glareosus*, and *L. stenodon* subgroups), *L. pedunculatus* group, and *L. angustissimus* group (Kramina, 1999a, b, 2006). However, studies of the nrITS region (Degtjareva & al., 2006, 2008; this paper) revealed that the *L. angustissimus* group, as defined by Heyn (1970) and Kramina (2006), is not monophyletic, and its members form two unrelated clades, the /*Lotus parviflorus* clade (*L. parviflorus* Desf., *L. subbiflorus*) and the /*Lotus angustissimus* clade (remaining species). Our ETS trees show a similar split of the *L. angustissimus* group, although only one species of the /*Lotus parviflorus* clade, *L. subbiflorus*, was studied (Fig. 3).

In accordance with earlier ITS-based studies (Allan & al., 2003; Degtjareva & al., 2006, 2008), the present study discovered close relationships between *L. conimbricensis* (monospecific sect. *Erythrolotus*, Kramina & Sokoloff, 2003) and members of sect. *Lotus* with nuclear markers (clade 2A, Figs. 3, 4), or its part, i.e., the /*Lotus corniculatus* clade, with plastid markers (Fig. 2).

The *L. corniculatus* group. — The *L. corniculatus* group is taxonomically complicated, and its exact number of species cannot be determined due to extreme morphological variation. We prefer distinguishing diploid forms (or races) as separate species when they differ in any morphological character from other diploid forms, as well as from tetraploid species, especially from the main and widely distributed tetraploid species, *L. corniculatus* (Kramina, 1999a, b, 2000, 2006; Barykina & Kramina, 2006). Intraspecific variability of nrITS sequences has been studied in only a small number of species (Kramina & al. 2012; present study). Some differentiation among nrITS sequences of different accessions of the *L. corniculatus* group are apparent, but sequences of the same species do not always group together (Fig. 4). Monospecific clades were revealed for *L. schoelleri* Schweinf. from East Africa and *L. stepposus* from Ukraine and adjacent regions of Russia only. For several species in the *L. corniculatus* group a tendency towards grouping according to geographical distribution in the nrITS tree can be marked (Fig. 4). Thus, accessions from the Western Mediterranean (Spain) occupy early branching positions, with one exception. These are *L. alpinus*, *L. delortii* Timb.-Lagr. ex F.W.Schultz, *L. glacialis* (Boiss.) Pau and *L. glareosus*. An accession of *L. tenuis* from Luxembourg is associated with them. However, two other accessions of *L. glareosus* from Spain and Portugal are united with *L. conimbricensis* and form a well-supported clade that tends to group with the /*Lotus corniculatus* clade. The basal position of the accessions of *L. alpinus* and *L. delortii* from Spain in the /*Lotus corniculatus* clade is also observed in analyses of nrETS (Fig. 3). On the other hand, the Asian *L. burttii* Borsos (Pakistan), *L. japonicus* (mainly Japan), *L. krylovii* Schischk & Serg. (Central Asia) and *L. miyakojimae* Kramina form a well-supported unit within the core of the /*Lotus corniculatus* clade on the ITS tree (Fig. 4).

The position of *L. conimbricensis*. — *Lotus conimbricensis* is a pink-flowered annual with one-flowered umbels and peduncles shorter than the leaves, widely distributed in the Mediterranean floristic region and also occurring on Madeira. After the revision of Brand's large sect. *Erythrolotus* (Brand, 1898) by Kramina & Sokoloff (2003), the section became monospecific with the single species *L. conimbricensis* (Kramina & Sokoloff, 2003; Degtjareva & al., 2006). However, molecular phylogenetic studies of *Lotus* based on nrITS sequences revealed close relationships between *L. conimbricensis* and sect. *Lotus* (Allan & al., 2003; Degtjareva & al., 2006). Section *Lotus* could be recognized as a monophyletic unit only if it includes *L. conimbricensis* (Degtjareva & al. 2008; this study). However, the exact relationships between *L. conimbricensis* and various members of sect. *Lotus* have been the subject of discussions.

Two main ideas concerning the phylogenetic relationships of *L. conimbricensis* have been suggested so far: (1) *L. conimbricensis* is related to *L. parviflorus* (Allan & al., 2003; Degtjareva & al. 2006); and, (2) *L. conimbricensis* is allied to the *L. corniculatus* complex (Degtjareva & al., 2008). The first concept is based on a single accession, #AF450186, and needs checking concerning the correctness of the identification of the specimen. The results of the present study obtained using both ITS and plastid DNA markers and several accessions clearly

confirm the second suggestion (Figs. 2, 4). The evidence based on an ITS phylogeny by Faria & al. (2012) does not contradict this idea. However, our Bayesian ETS tree (Fig. 3) showed a grouping of *L. conimbricensis* and *L. subbiflorus* that disagrees with results obtained by other markers and partially supports the first concept.

To summarize, all studied DNA markers provided evidence that *L. conimbricensis* is a member of sect. *Lotus*. However, the position of the species within the section varies depending on marker. The main common feature (synapomorphy, see Fig. 3) of *L. conimbricensis* and other members of sect. *Lotus* is the basic chromosome number of $x = 6$, differing from $x = 7$, which is typical for other sections of *Lotus* (Grant, 1995).

Placement of the accessions of *L. glareosus* in the nrITS tree (Fig. 4) is intriguing. One of the three accessions examined is a member of the /*Lotus corniculatus* clade, which agrees with traditional views of this taxon (Ball & Chrtková-Žertová, 1968; Fernandes, 1982). The other two accessions are related to *L. conimbricensis*, which cannot be explained morphologically. Additional studies involving more accessions and other DNA markers are needed.

The *L. angustissimus* group. — According to the nrITS phylogeny (Fig. 4), the /*Lotus pedunculatus* and /*Lotus angustissimus* clades are closely related to each other, but are separate groups belonging to the large /CCAP clade together with the /*Lotus corniculatus* clade. In the phylogenetic trees constructed from nrETS (Fig. 3) they belong to clade 2A. In analyses using plastid markers, members of the *L. pedunculatus* and *L. angustissimus* groups are also closely related and intermingle forming a /*Lotus castellanus* clade (Fig. 2), but separate from the *L. corniculatus* group. This indicates that maternal lineages of members of the /*Lotus castellanus* and /*Lotus corniculatus* clades evolved independently for a long time, and that *L. pedunculatus* is not a close relative of the *L. corniculatus* group.

Six species of the *L. angustissimus* group (Heyn, 1970; Kramina, 2006) were studied (*L. angustissimus*, *L. castellanus* Boiss. & Reut., *L. palustris* Willd., *L. parviflorus*, *L. praetermissus* Kuprian., *L. subbiflorus*). Two species, *L. angustissimus* and *L. praetermissus*, may be one polymorphic species (see Kramina, 2006). Two other species, *L. castellanus* and *L. palustris*, are morphologically rather similar, but can be distinguished as different species (Kramina, 2006). In previous molecular phylogenetic studies of *Lotus*, an ITS sequence AF450195 of a cultivated plant #PI311427 identified as *L. palustris* was placed in the /*Lotus corniculatus* clade (Allan & al., 2003; Degtjareva & al., 2006, 2008). This result was in agreement with Ball & Chrtková-Žertová (1968) who considered *L. palustris* to be a member of the *L. corniculatus* species complex. However, nrITS sequences of two other accessions of *L. palustris* from Greece and Israel studied here were very similar to each other and the sequences of *L. castellanus*. The present result coincides with the ideas of Heyn (1970) and Kramina (2006) who considered *L. palustris* to be a part of the *L. angustissimus* species complex. These contradictions may be due either to high intraspecific polymorphism in *L. palustris*, mistakes in identification or the molecular analysis of the first accession. Further investigations with other DNA markers could confirm one of these hypotheses.

All species of the *L. angustissimus* group occur in countries bordering the Mediterranean Sea (Heyn, 1970; Greuter & al., 1989; Kramina, 2006). *Lotus angustissimus* (including *L. praetermissus*) has the largest distribution area reaching further north and east than all other members of the group. It reaches the Ukraine, and the European and to a lesser extent the Asian parts of Russia and Kazakhstan.

Nuclear DNA markers suggest that two species forming the /*Lotus parviflorus* clade, i.e., *L. parviflorus* and *L. subbiflorus*, are not close to other members of the *L. angustissimus* group as was supposed earlier (Heyn, 1970; Kramina, 2006). However, in our study of plastid DNA markers, *L. subbiflorus* is close to other members of the *L. angustissimus* group with high support (*L. parviflorus* was not included). In general, our plastid, nrETS and nrITS data are strongly incongruent with respect to the members of the *L. angustissimus* group (Figs. 2–4).

The *L. pedunculatus* group. — The *L. pedunculatus* group includes three closely related species, *L. granadensis* Chrtková, *L. pedunculatus* Cav. and *L. uliginosus* Schkuhr (Chrtková-Zertová, 1966), which are weakly differentiated morphologically and can be considered as members of one polymorphic species. This idea is supported by our ITS data for *L. pedunculatus* and *L. uliginosus*. A common feature of these species is the presence of stolons, while other species of sect. *Lotus* are characterized by a well-developed taproot, or even a caudex, and lack stolons. Rarely, stolons or rhizomes were observed in material from Morocco (Kallenbach & al., 1989; Beuselinck & al., 1996) and Spain (Escaray & al., 2014) identified as *L. corniculatus*. Their morphology and molecular phylogenetic placement should be carefully studied to clarify their relationships with other members of sect. *Lotus*. Observed discrepancies between nuclear ETS and ITS and plastid trees regarding placement of *L. pedunculatus* (Figs. 2–4) may reflect hybridization which took place in early evolution of sect. *Lotus* and its lineages.

Section *Heinekenia*. — *Lotus* sect. *Heinekenia* includes 23 species occurring in the Circumboreal, East-Asian, Mediterranean, Saharo-Arabian, Irano-Turanian, Sudano-Zambesian, Malesian, Fijean, New-Caledonian, North-East-Australian, South-West-Australian and Central-Australian floristic regions (Takhtajan, 1986). The section contains the largest number of *Lotus* species distributed in the tropics. It includes annuals and perennials with variable leaves (pinnate or palmate, with three to about eight leaflets) and usually white, pink or red flowers, with the exception of *L. aegaeus* Boiss. with yellow flowers (Kramina & Sokoloff, 2003). ITS sequences have been studied for 20 species of sect. *Heinekenia*, while sequences of four DNA markers have been obtained for 12 species.

Morphology does not provide any potential synapomorphy or single clear diagnostic character for the section (Kramina & Sokoloff, 2003; Degtjareva & al., 2006). None of the molecular analyses conducted for this study or earlier studies supported its monophyly.

Degtjareva & al. (2008), using ITS sequences, recognized three clades with species of sect. *Heinekenia* that differ in the geographic distribution of their members with very limited overlapping of ranges between the clades. The /*Heinekenia*-N

and /Heinekenia-SE clades comprise only members of sect. *Heinekenia*, whereas the /Heinekenia-SW clade contains members of sect. *Heinekenia* plus two species of the polyphyletic sect. *Ononidium*. The present analysis of the ITS dataset confirmed the monophyly of the clades and their geographic specificity. Moreover, two subclades of the /Heinekenia-SW clade (Fig. 5) also have clear eco-geographical differences. *Lotus becquetii* Boutique, *L. discolor* E.Mey., *L. goetzei* Harms, *L. mlanjeanus* J.B.Gillett, *L. robsonii* E.S.Martins & D.D. Sokoloff, *L. subdigitatus* Boutique and *L. wildii* J.B.Gillett are a group of tropical African species occurring in mountainous regions from Ethiopia to South Africa at elevations of (1200) 1400 to 3200 m. The other subclade has a centre of species diversity around the Red Sea, with one species, *L. arabicus* L., extending to other parts of tropical Africa where it grows at low elevations. Our ETS data are congruent with recognition of three major /Heinekenia clades. In the majority of our nuclear trees (ETS—both trees, ITS—Bayesian tree), /Heinekenia-N and /Heinekenia-SE are sister clades.

Relationships found in analyses of the nuclear markers can be interpreted as paraphyly of sect. *Heinekenia* relative to the /Zygocalyx clade. However, as our data do not provide robust and stable placement for the /Heinekenia-SW and /Heinekenia-N+/Heinekenia-SE clades (Figs. 3, 5; Electr. Suppl.: Fig. S1), there is not enough evidence to reject a group of all three /Heinekenia clades.

Morphological data do not provide any good features characterizing the /Heinekenia clades, although some aspects of geographical grouping of species were predicted in earlier morphological studies (Sokoloff, 2001; Kramina & Sokoloff, 2003).

Section *Chamaelotus* and *L. alianus*. — Section *Chamaelotus*, like sect. *Heinekenia*, was segregated from Brand's sect. *Erythrolotus* (Kramina & Sokoloff, 2003), and in its first circumscription included three species distributed in the Macaronesian, Saharo-Arabian, and Sudano-Zambesian floristic regions. The included species are characterized by umbels of red or pink flowers on very short peduncles. Two of the species initially included in this section were studied using both nuclear and plastid DNA markers. All analyses conducted for this study revealed a highly supported and phylogenetically isolated /*Chamaelotus* clade that included the two studied species of the section plus *L. alianus*.

Lotus alianus was recently described from Cape Verde (Kirkbride, 2010) using two herbarium specimens from two of the northwesternmost islands of the archipelago, Ilhas de Santo Antão and São Vicente. These are perennial subshrubs with trifoliolate leaves (i.e., the basal leaflet pair absent), single-flowered umbels on short axillary peduncles not or slightly exceeding the subtending leaves, small flowers (5–5.5 mm long), calyx with subequal lobes, corolla yellow, style without a tooth, pod straight, linear, glabrous, multi-seeded, dehiscent, with contorting valves. Such a combination of morphological characters does not allow unambiguous placement of *L. alianus* in any of the existing sections and made it difficult to discover its true taxonomic relationships. Kirkbride (2010) tentatively classified this species as a member of sect. *Lotus*. Chromosome number, a key character of sect. *Lotus*, is unknown for *L. alianus*. The

present molecular data on the close relationships between sect. *Chamaelotus* and *L. alianus* are in good agreement with their biogeography. Common morphological characters are small flowers and short peduncles in *L. alianus* or very short peduncles in members of sect. *Chamaelotus*. The most important difference between *L. alianus* and the other species of sect. *Chamaelotus* is its yellow corolla.

Sections *Lotea* and *Pedrosia*. — Members of sect. *Lotea* and many species of sect. *Pedrosia* are herbs mainly with leaves with five leaflets and yellow flowers. One of the most prominent features of these two sections is a monosymmetric calyx. The main difference between the two sections is the presence of a ventral tooth on the style of the species of sect. *Pedrosia*, and also those of the small sect. *Rhyncholotus*, which also falls in the /*Pedrosia* clade, and the absence of such a tooth in all members of sect. *Lotea*, and in all other legumes. In addition, species of sections *Pedrosia* and *Rhyncholotus* usually have hairy ovaries and fruits, while they are invariably glabrous in sect. *Lotea* and in most other members of *Lotus*.

Species of sect. *Pedrosia* and sect. *Lotea* occur in the Macaronesian, Mediterranean and Saharo-Arabian floristic regions (Takhtajan, 1986), and one species of sect. *Lotea* also exists in the Sudano-Zambesian floristic region. However, the sections demonstrate divergent geographical tendencies. *Lotea* species prefer the continental parts of Africa and Eurasia and Mediterranean islands, and *L. ornithopodioides* L. is the only member of the section occurring in the Macaronesian region on the island of Madeira. Most species of *Pedrosia*, and all members of *Rhyncholotus*, are endemic to the Macaronesian islands. Several species occur in NW Africa, especially in Morocco, two species occur in the Iberian Peninsula, and only one, *L. creticus* L., extends into the central and eastern Mediterranean region.

The widely distributed Mediterranean and Macaronesian *L. creticus* was traditionally placed in sect. *Lotea* (e.g., Ball & Chrtková-Žertová, 1968; Valdés, 2000). The discovery of a small rudimentary tooth on the ventral side of the style (Kramina & Sokoloff, 1999; Valdés, 2000) led to the transfer of the species into sect. *Pedrosia*. This idea was subsequently supported by analyses of nrITS sequences (Degtjareva & al., 2006, 2008; Sandral & al., 2010). In this study, placement of *L. creticus* as sister to an accession of *L. pseudocreticus* Maire & al. (sect. *Pedrosia*) was fully congruent and well-supported in the analyses of all nuclear and plastid markers. Two hypotheses were proposed regarding the nature of *L. pseudocreticus*. In the first (Sandral & al., 2006), *L. pseudocreticus* was hypothesized to be an interspecific hybrid between *L. creticus* and *L. assakensis* Brand. Alternatively, Kirkbride (2010) hypothesized that the names *L. pseudocreticus* and *L. creticus* are synonymous. The data from nrITS sequences (Sandral & al., 2010) supported the first idea.

Earlier studies based on nrITS sequences (Degtjareva & al., 2006, 2008) demonstrated that a member of sect. *Ononidium*, the Moroccan *L. simonae*, is nested among members of sect. *Lotea*, in the /*Lotea* clade. This result was reproduced in this study using nrETS sequences and plastid markers. Section *Ononidium* differs from other sections of *Lotus* mainly by leaf type, i.e., having three rather than five leaflets. The present

study supports the polyphyly of *Ononidium* and its taxonomic dismemberment (Degtjareva & al., 2006). *Lotus simonae* possesses all key diagnostic characters of sect. *Lotea*, including a monosymmetric calyx, style without a tooth, yellow flowers and glabrous fruits dehiscent by two valves, and should be transferred into this section (Degtjareva & al., 2006).

Our analyses of nuclear and plastid markers strongly support placement of the Moroccan endemic *L. weilleri* in the /Pedrosia clade. Sokoloff (2003b) and Degtjareva & al. (2006) placed *L. weilleri* in sect. *Lotea* mainly based on earlier analyses of nrITS sequences in the accession #PI368910 (Allan & al., 2003; Degtjareva & al., 2006, 2008; Sandral & al., 2010). Two new accessions of *L. weilleri* with herbarium vouchers were included in the present study. We suppose that the earlier sequence based on #PI368910 does not belong to *L. weilleri*. There are three main arguments in favour of this idea. (1) Herbarium vouchers for the new accessions were compared with type material of *L. weilleri* and its protologue, and the taxonomic identification of specimens used here were confirmed. There is no preserved voucher for #PI368910. (2) The two new accessions group together in all analyses. (3) Sandral & al. (2010) studied multiple accessions of *L. cytisoides* (sect. *Lotea*) and found several haplotypes of nrITS. The nrITS sequence of #PI368910 (Allan & al., 2003) is identical to one of the haplotypes found in *L. cytisoides*. The published sequence of #PI368910 probably belongs to *L. cytisoides*.

Lotus weilleri differs from other members of the /Pedrosia clade, as well as from members of the /Lotea clade, in its more or less polysymmetric calyx. It has no tooth on the style and glabrous fruits. As our nrETS and nrITS data strongly support the placement of *L. weilleri* as sister to the remainder of the /Pedrosia clade, the occurrence of a ventral tooth and hairy fruits are, therefore, synapomorphies of its sister lineage that comprises members of sect. *Pedrosia* and sect. *Rhyncholotus*. The position of *L. weilleri* as sister to *L. eriosolen* (Maire) Mader & Podlech, a typical member of sect. *Pedrosia*, as found in plastid trees, is only moderately supported, and this is not in strong phylogenetic conflict with the nuclear data.

Our ITS tree (Fig. 5) shows a basal grade within the /Pedrosia clade that comprises species occurring in mainland Africa, one species extending to mainland Europe and Cape Verde, and a monophyletic core group comprising species from the Canary Islands, Madeira and the Azores plus some mainland taxa. The basal position of Cape Verde species was demonstrated by Schmidt (2011) in his analysis of Macaronesian *Lotus* species based on a combined dataset including nrITS, seven regions of cpDNA and two low-copy nuclear genes. However, his study did not include any members of sect. *Pedrosia* from mainland Africa. Ojeda & al. (2012, 2014), using several nuclear and plastid regions, revealed the basal position of species from mainland Africa and Cape Verde in the phylogeny of *Pedrosia*. In general, our study agrees with earlier data on the phylogeny of the /Pedrosia clade, but provides the first evidence of its rooting through *L. weilleri*. In spite of the absence of the key character of sect. *Pedrosia*, i.e., a ventral tooth on the style, in *L. weilleri*, and its distribution in Morocco, it can be expected as the basal member of the /Pedrosia clade.

Details of relationships among the species of the core group of the /Pedrosia clade are outside the scope of the present study because they were explored in detail by Schmidt (2011) and Ojeda & al. (2012, 2014). This is a recently evolved group with many local island endemics and some taxa presumably of hybrid origin (Sandral & al., 2010; Schmidt, 2011; Ojeda & al., 2012, 2014).

Sections *Tetragonolobus* and *Krokeria*. — The monospecific sect. *Krokeria* includes only the annual *L. edulis*. Our nrETS and nrITS data suggested a close relationship of *L. edulis* to the /Lotea and /Tetragonolobus clades, but do not provide clear evidence for sister relationships with any other clade, which agrees with earlier studies (Allan & al., 2003; Degtjareva & al., 2006, 2008). The plastid data also indicated its position sister to the /Lotea clade. The Mediterranean range of *L. edulis* is shared with sect. *Lotea*. *Lotus edulis* possesses some remarkable autapomorphies, such as inflated fruits dehiscent along the ventral suture only, and papillose seeds. Together with its unresolved phylogenetic placement in the nuclear ETS and ITS trees, this supports maintaining *L. edulis* in a monospecific section.

Section *Tetragonolobus* contains five species, three of which were included in phylogenetic studies based on nrITS, and form the /Tetragonolobus clade, and only one was studied using four markers. Many researchers (e.g., Dominguez & Galiano, 1979) considered *Tetragonolobus* a distinct genus due to the presence of specific morphological traits, uniquely derived apomorphies at the level of *Lotus* or higher, such as leaflets of the lower pair attached to the leaf rachis, winged pods and a dorsal outgrowth on the style, which is nonhomologous to the tooth of *Pedrosia* species. Earlier studies based on nrITS sequences showed that the /Tetragonolobus clade is nested in *Lotus* (Allan & Porter, 2000; Allan & al., 2003; Degtjareva & al., 2003, 2006, 2008). The plastid markers showed the same result, thus making the segregation of *Tetragonolobus* as a separate genus unsupported.

The relatively low support of the /Tetragonolobus clade in the nrITS tree (Fig. 5) is intriguing in light of its remarkable and unambiguous morphological synapomorphies. *Lotus maritimus* L., the only species of the section with yellow corollas, has a high level of molecular divergence from the two other species sampled here, *L. palaestinus* (Boiss. & Blanche) Blatter and *L. tetragonolobus* L.

Biogeography. — Traditionally, *Lotus* has been viewed as a Mediterranean group because the genus is most diverse in Mediterranean countries. The centre of extant taxonomic diversity of *Lotus* is Morocco. Members of nine sections and about a quarter of all known species of *Lotus* occur in this country. Also, all three genera closely related to *Lotus*, *Hammatolobium*, *Cytisopsis* and *Tripodion*, occur in Morocco. Only three sections are absent from Morocco, sect. *Canaria*, sect. *Heinekenia* and sect. *Rhyncholotus*. However, sect. *Rhyncholotus* is clearly nested among members of sect. *Pedrosia* (Degtjareva & al., 2006, 2008; Ojeda & al., 2012, 2014; this study) and should not be segregated as a distinct taxon. The present study identified the Moroccan endemic species *L. weilleri* as the basal extant member of the /Pedrosia clade. However, Morocco

may represent a secondary centre of *Lotus* diversification. The available phylogenetic data suggest a relatively complex biogeographic history for *Lotus* and related genera.

The present study revealed strong distribution patterns of members of the primary clades of *Lotus*. Despite all topological incongruences, the two or three largest clades of all trees clearly have centres of species diversity in either area A (Europe, N Asia) or areas B, C and D (Africa, S Asia, Macaronesia, Australasia) recognized here. S-DIVA analysis of the ETS dataset strongly suggests that area B was ancestral for clade 1 and area A for clades 2A and 2B. One may speculate that the Mediterranean region is a secondary diversity centre and that members of a “northern” (clade 2 or clades 2A and 2B) and a “southern” (clade 1) group migrated there, with subsequent radiations, after establishment of the current climatic conditions in this region. This scenario can be tested using time-calibrated phylogenies. Another possibility, that we tentatively consider more plausible, implies that initial diversification of *Lotus* took place close to or in the Mediterranean region, but that the extant geographic patterns are biased by at least one round of area fragmentation and following expansion coupled with extensive speciation associated with the complex history of the Mediterranean region. The impact of Late Quaternary climate changes on the evolution of several species is well documented (e.g., Comes & Kadereit, 1998; Kropf & al., 2002; Pfenninger & Posada, 2002), and it is possible that an earlier climate-based fragmentation (e.g., Linares, 2011) was responsible for the evolution of the primary clades of *Lotus*.

All three genera closest to *Lotus* (*Cytisopsis*, *Hammatolobium*, *Tripodion*) are Mediterranean elements. While the monospecific *Tripodion* occurs throughout the Mediterranean region, two other genera are bispecific, with similar disjunct distributions. One species of each genus is endemic to NW Africa while the other one is restricted to the NE Mediterranean region. In each genus, morphological differences between these species pairs are so impressive that two monospecific subgenera were recognized (Tikhomirov & Sokoloff, 1996; Sokoloff, 2003a). The patterns of disjunction in *Cytisopsis* and *Hammatolobium* could be caused by the same factors as the geographical patterns of the major *Lotus* clades. Future work will concentrate on producing a time-calibrated phylogeny of *Lotus* and its analysis in the historical context of the Mediterranean region.

Our ETS- and ITS-based phylogenies do not support monophyly of the “northern” clade found in analyses of plastid data. One of several possible explanations of these topological differences is reticulate evolution in the ancient diversification of the genus. In this scenario, clades 2A and 2B found in analyses of nrETS data were two independent lineages with centres of diversification in the northern range of *Lotus*, possibly in Europe and/or Asia Minor. Hybridization between early members of these two lineages migrating to the north was responsible for similarities in plastid DNA sequences observed in extant members of clade 2 in our plastid trees. Testing this hypothesis requires more direct evidence for reticulate evolution in *Lotus*, including use of low-copy nuclear markers.

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Appendix 1. Species names and authorities, geographical provenience, and voucher specimens for the material included in the phylogenetic analyses. GenBank accession numbers are given for the four markers sequenced, ITS, ETS, *psbA-trnH*, and *rps16* intron (new sequences indicated by an asterisk); for accessions taken from GenBank, voucher information is not presented; an n-dash denotes a missing marker.

INGROUP: Lotus L.: *L. aduncus* (Griseb.) Nyman, Greece: *Oberprieler 3109* (G), KT250843*, KT262719*, KT262793*, KT262864*; *L. aegaeus* Boiss., Turkey: *Khokhryakov & Mazurenko 1135* (MHA), DQ160276 (Degtjareva & al., 2006), KT262720*, KT262794*, KT262865*; *L. alianus* J.H.Kirkbr., Cabo Verde: *Grandvaux Barbosa 6977* (LISC), KT250844*, KT262721*, KT262795*, –; *L. alpinus* (Ser.) Schleich. ex Ramond (1), Turkey: *Uotila 2722* (H), KT250845*, –, –, –; *L. alpinus* (2, 4), Switzerland: *Ochsmann 94898* (GOET), KT250846* & KT250847*, –, –, –; *L. alpinus* (3), Switzerland: *Zogg 78/054* (H), KT250848*, –, –, –; *L. alpinus* (5), Spain: *Segura Zubizarreta 43694* (MHA), DQ160274 (Degtjareva & al., 2006), KT262722*, KT262796*, KT262866*; *L. alpinus* (6), Spain: *Haeggström 6519* (H), KT250849*, –, –, –; *L. anfractuoso* (Baker f.) Kramina & D.D.Sokoloff, New Caledonia: 18 Jul 1951, *Baumann-Bodenheim s.n.* (Z), FJ411111 (Degtjareva & al., 2008), KT262723*, KT262797*, KT262867*; *L. angustissimus* L., Australia: Norfolk Island, introduced, *Waterhouse 5510* (NSW), DQ166243 (Degtjareva & al., 2006), KT262724*, KT262798*, KT262868*; *L. arabicus* L., –; AF450176 (Allan & al., 2003), –, –, –; *L. arenarius* Brot. (1), –; AF218528 (Allan & Porter, 2000), –, –, –; *L. arenarius* (2), –; FJ938295 (Sandral & al., 2010), –, –, –; *L. arinagensis* Bramwell, Canary Is.: *Royl 778* (B), KT250850*, –, –, –; *L. assakensis* Brand, –; DQ160277 (Degtjareva & al., 2006), –, –, –; *L. australis* Andrews, Australia: *Haegi 3450 & Moore* (MHA), KT250851*, –, –, –; *L. axilliflorus* (Hub.-Mor.) D.D.Sokoloff, Turkey: *Duman & al. 5089* (E), KT250852*, KT262725*, KT262799*, KT262869*; *L. azoricus* P.W.Ball, –; AY294293 (Allan & al., 2004), –, –, –; *L. bequetii* Boutique, Burundi: *Reekmans 6042* (BR), KT250853*, –, –, –; *L. berthelotii* Masf., Cultivated material: originally from Canary Is., KT250854*, KT262726*, KT262800*, KT262870*, *L. borbasii* Ujhelyi, Czech Republic: *Smejkal 1441* (MHA), DQ166226 (Degtjareva & al., 2006), KT262727*, KT262801*, KT262870*; *L. brossonnetii* Choisy ex Ser., Cultivated at Royal Botanic Gardens, Kew, introduced from Canary Is., DQ160278 (Degtjareva & al., 2006), KT262728*, KT262802*, KT262872*; *L. burttii* Borsos, –; FJ411113 (Degtjareva & al., 2008), –, –, –; *L. campylocladus* Webb & Berthel. (1, 2), Canary Is.: *Väre 10656 & Kaipainen* (H), KT250855* & KT250856*, –, –, –; *L. campylocladus* (3), –; AF450196 (Allan & al., 2003), –, –, –; *L. castellanus* Boiss. & Reut. (1), Spain: *Segura Zubizarreta 38111* (MHA), DQ166238 (Degtjareva & al., 2006), KT262729*, KT262803*, KT262873*; *L. castellanus* (2), –; DQ166223 (Degtjareva & al., 2006), –, –, –; *L. castellanus* (3), –; DQ160272 (Degtjareva & al., 2006), –, –, –; *L. cf. castellanus* (4), –; DQ160275 (Degtjareva & al., 2006), –, –, –; *L. conimbricensis* Brot. (1), Spain: *Segura Zubizarreta 960* (Z), FJ411114 (Degtjareva & al., 2008), KT262730*, KT262804*, KT262874*; *L. conimbricensis* (2), –; FJ411115 (Degtjareva & al., 2008), –, –, –; *L. corniculatus* L. (1), European Russia: *Kramina 74-1* (MW), JF784198 & JF784199 (Kramina & al., 2012), KT262731*, KT262805*, KT262875*; *L. corniculatus* (2), European Russia: *Kramina 74-7* (MW), JF784200 & JF784201 (Kramina & al., 2012), KT262732*, KT262806*, KT262876*; *L. creticus* L., Cultivated in Australia: from seeds collected in Azores Is., *Sandral SA39213* (MW), FJ938296 (Sandral & al., 2010), KT262733*, KT262807*, KT262877*; *L. cruentus* Court, Australia: *Badman 905* (MHA), KT250857*, –, –, –; *L. cytoides* L. (1, 2), Cyprus: *Seregin & Sokoloff 280* (MW), DQ166241 & DQ160280 (Degtjareva & al., 2006), KT262734*, KT262808*, KT262878*; *L. delortii* Timb.-Lagr. ex F.W.Schultz (1), Spain: *Sandwith 4772* (LE), DQ166228 (Degtjareva & al., 2006), KT262735*, KT262809*, KT262879*; *L. delortii* (2), Spain: *Scholz & Hiepkol 1098* (B), KT250858*, –, –, –; *L. discolor* E.Mey., Cameroon: *Lisowski S. B-3330* (BR), DQ160288 (Degtjareva & al., 2006), KT262736*, KT262810*, KT262880*; *L. dorycnium* L. (1) (“*Dorycnium pentaphyllum* ssp. *gracile*”), France: *Lambinon 81/287* (H), KT250859*, KT262737*, KT262811*, KT262881*; *L. dorycnium* (2) (“*Dorycnium pentaphyllum* ssp. *transmontanum*”), Portugal: *Auriault 14166* (H), KT250860*, KT262738*, KT262812*, KT262882*; *L. dorycnium* (3) (“*Dorycnium pentaphyllum* ssp. *suffruticosum*”), France: *Charpin 9350* (H), KT250861*, KT262739*, KT262813*, KT262883*; *L. dorycnium* (4) (“*Dorycnium pentaphyllum* ssp. *pentaphyllum*”), Spain: *Reira 17073* (H), KT250862*, KT262740*, KT262814*, KT262884*; *L. edulis* L., –; AF450184 (Allan & al., 2003), –, –, –; *L. edulis*, Cyprus: *Seregin & Sokoloff A-280* (MW), KT250863*, KT262741*, KT262815*, KT262885*; *L. emeroideus* R.P.Murray, –; AY294295 (Allan & al., 2004), –, –, –; *L. eriosolen* (Maire) Mader & Podlech, Morocco: *Podlech 52619* (M), DQ160281 (Degtjareva & al., 2006), KT262742*, KT262816*, KT262886*; *L. filicaulis* Durieu, –; FJ411116 (Degtjareva & al., 2008), –, –, –; *L. frondosus* (Frey) Kuprian. (1), –; DQ166224 (Degtjareva & al., 2006) as *L. schoelleri* Schweinf., –, –, –; *L. frondosus* (2), European Russia: *Kramina DO-78* (MW),

Appendix 1. Continued.

KT250864*, –, –, –, *L. fulgurans* (Porta) D.D.Sokoloff, Cultivated at Royal Botanic Gardens, Kew: origin Spain, Balearic Is., KT250865*, KT262743*, KT262817*, KT262887*; *L. garcinii* DC., Iran: Leonard 5899 (LE), DQ166234 (Degtjareva & al., 2006), KT262744*, KT262818*, KT262888*; *L. gebelia* Vent. (1), –, AF450188 (Allan & al., 2003), –, –, –, *L. gebelia* (2) var. *anthylloides* Boiss., Turkey: Alava 6939 (MHA), KT250866*, –, –, –, *L. gebelia* (3) (“*L. michauxianus* Ser.”), Iran: Rechinger & Renz 49648 (B), KT250867*, –, –, –, *L. germanicus* (Gremli) Peruzzi (1), Slovenia: Trpin 9852/3 (H), KT250868*, KT262745*, KT262819*, KT262889*; *L. germanicus* (2), Germany: Kalheber 91-0625 (H), KT250869*, KT262746*, KT262820*, KT262890*; *L. germanicus* (3), Montenegro: Uotila 10652 (H), KT250870*, KT262747*, KT262821*, KT262891*; *L. glacialis* (Boiss.) Pau, Spain: 12 Jul 1978, Roivainen s.n. (H), KT250871*, –, –, –, *L. glareosus* Boiss. & Reut. (1), Spain: Valdés & al. 2959/88 (B), KT250872*, –, –, –, *L. glareosus* (2), Spain: Pons-Sorolla & Susanna 270 (B), KT250873*, –, –, –, *L. glareosus* (3), Portugal: Jalas 1768 (H), KT250874*, –, –, –, *L. glaucus* (1) var. *erythrorhizus* (Bolte) Brand, –, AY294296 (Allan & al., 2004), –, –, –, *L. glaucus* Ait. (2), Madeira: Alanko 95009 (H), KT250875*, –, –, –, *L. glinoides* Del. (1), Egypt: 7 May 1962, Bochantsev s.n. (LE), DQ160282 (Degtjareva & al., 2006), KT262748*, KT262822*, KT262892*; *L. glinoides* (2), –, DQ166220 (Degtjareva & al., 2006), –, –, –, *L. goetzei* Harms, –, DQ166235 (Degtjareva & al., 2006), –, –, –, *L. graecus* L. (1), Turkey: Lampinen 7871 (H), KT250876*, KT262749*, KT262823*, KT262893*; *L. graecus* (2), Greece: 18 May 1986, Raitthalme s.n. (H), KT250877*, KT262750*, KT262824*, KT262894*; *L. graecus* (3), The Crimea, Jul 1996, Sokoloff s.n. (MW), KT250878*, KT262751* & KT262752*, KT262825*, KT262895*; *L. graecus* (4), –, AF218500 (Allan & Porter, 2000) as *Dorycnium graecum*, –, –, –, *L. halophilus* Boiss. & Spruner, Greece: Raus 9307 (MHA), KT250879*, KT262753*, KT262826*, KT262896*; *L. hebranicus* Brand, Egypt: Leonard 3759 (P), KT250880*, –, –, –, *L. herbaceus* (Vill.) Jauzein (1), The Crimea: 17 Jul 1996, D.D. Sokoloff s.n. (MW), KT250881*, KT262754*, KT262827*, KT262897*; *L. herbaceus* (2), Austria: 7 Jul 1976, Mayrhofer & Teppner s.n. (H), KT250882*, KT262755*, KT262828*, KT262898*; *L. hirsutus* L. (1), Turkey: Lampinen 7355 (H), KT250883*, KT262756*, KT262829*, KT262899*; *L. hirsutus* (2), Croatia: Hämet-Ahti 2225 (H), KT250884*, KT262757*, KT262830*, KT262900*; *L. hirsutus* (3), Greece: 19 May 1986, Raitthalme s.n. (H), KT250885*, KT262758*, KT262831*, KT262901*; *L. hirsutus* (4), Spain: Jul 2006, Beer & Beer s.n. (MW), KT250886*, KT262759*, KT262832*, KT262902*; *L. jacobaeus* L., –, AY294299 (Allan & al., 2004), –, –, –, *L. japonicus* (Regel) Larsen (1, 2), Japan: Endo 371 (H), KT250887*, KT262760*, KT262833*, KT262903*; *L. japonicus* (3), Japan: Koponen 16097 (H), KT250888*, –, –, –, *L. jolyi* Battand., –, DQ166240 (Degtjareva & al., 2006), –, –, –, *L. krylovii* Schischk. & Serg., China: Dickoré 1339 (GOET), KT250889*, –, –, –, *L. lalambensis* Schweinf., Saudi Arabia: Collette 7908 (E), DQ166216 (Degtjareva & al., 2006), KT262761*, KT262834*, KT262904*; *L. lancerottensis* Webb & Berthel., –, AY294300 (Allan & al., 2004), –, –, –, *L. lanuginosus* Vent., Jordan: Townsend 65/22 (LE), DQ166221 (Degtjareva & al., 2006), KT262762*, KT262835*, KT262905*; *L. laricus* Rech.f., Aellen & Esfand., Abu Dhabi: Western 275 (E), DQ166233 (Degtjareva & al., 2006), KT262763*, KT262836*, KT262906*; *L. longisiliquosus* R. Roem., –, AF218526 (Allan & Porter 2000), –, –, –, *L. loweanus* Webb & Berthel., –, FJ411117 (Degtjareva & al., 2008), –, –, –, *L. maculatus* Breitf. (1), Canary Is. (cult.): Väre 10894 & Kaipainen (H), KT250890*, KT262764*, KT262837*, KT262907*; *L. maculatus* (2), –, AY294308 (Allan & al., 2004), –, –, –, *L. maritimus* L. (1), –, AF218505 (Allan & Porter, 2000), –, –, –, *L. maritimus* (2), Estonia: R. Lampinen 18056 & T. Lampinen (H), KT250891*, –, –, –, *L. maroccanus* Ball, –, AF450181 (Allan & al., 2003), –, –, –, *L. mascaënsis* Burchard., –, FJ411118 (Degtjareva & al., 2008), –, –, –, *L. miyakojimae* Kramina, Cultivated in Russia: origin Japan, 17 Jun 1999, Kramina s.n. (MW), KT250892*, –, –, –, *L. mlanjeanus* J.B.Gillett, Malawi: Chapman & Chapman 8807 (E), DQ166232 (Degtjareva & al., 2006), KT262765*, KT262838*, KT262908*; *L. ononopsis* Balf.f., Yemen: Miller & al. 10097 (E), DQ166219 (Degtjareva & al., 2006), KT262766*, KT262839*, KT262909*; *L. ornithopodioides* L. (1), –, AF450205 (Allan & al., 2003), –, –, –, *L. ornithopodioides* (2), Spain: Mejias & Valdés 12270 (H), KT250893*, –, –, –, *L. palaestinus* (Boiss. & Blanche) Blatter (1, 2), Israel, 8 Mar 1961, Kvist s.n. (H), KT250894* & KT250895*, –, –, –, *L. palustris* Willd. (1), Greece: Böhling 582 (B), KT250896*, –, –, –, *L. palustris* (2), Israel, Zohary & Amdursky 645 (B), KT250897*, –, –, –, *L. parviflorus* Desf., –, DQ166230 (Degtjareva & al., 2006), –, –, –, *L. pecoriscus* Miniaev & Z.G.Ulle, European Russia: Ulle 98 (H), KT250898*, –, –, –, *L. pedunculatus* Cav. (1, 2), Spain, 18 Jul 1972, Segura-Zubizarreta s.n. (H), KT250899* & KT250900*, –, –, –, *L. pedunculatus* (3), Spain, 18 Jul 1972, Segura-Zubizarreta s.n. (LE), DQ166222 (Degtjareva & al., 2006), KT262767*, KT262840*, KT262910*; *L. peregrinus* L., –, AF450177 (Allan & al., 2003), –, –, –, *L. polyphyllus* Clarke, –, DQ160289 (Degtjareva & al., 2006), KT262768*, KT262841*, KT262911*; *L. praetermissus* Kuprian. (1), –, DQ166227 (Degtjareva & al., 2006), KT262769*, KT262842*, KT262912*; *L. praetermissus* (2), –, DQ168370 (Degtjareva & al., 2006), –, –, –, *L. preslii* Ten. (1, 2), –, DQ166229 & DQ166236 (Degtjareva & al., 2006), –, –, –, *L. pseudocreticus* Maire & al. (1), Morocco: 31 Aug 2007, Nuraliev s.n. (MW), KT250901*, –, –, –, *L. pseudocreticus* (2), Morocco: Podlech 52358 (M), DQ160284 (Degtjareva & al., 2006), KT262770*, KT262843*, KT262913*; *L. quinatus* (Forssk.) J.B.Gillett, Yemen: Thulin & al. 9374 (E), DQ166217 (Degtjareva & al., 2006), KT262771*, KT262844*, KT262914*; *L. rectus* L., Crete: Aug 2012, Sokoloff s.n. (MW), KT250902*, KT262772*, KT262845*, KT262915*; *L. robsonii* E.S.Martins & D.D.Sokoloff, Malawi: Robson & Angus 430 (MW), KT250903*, –, –, –, *L. sanguineus* (Vural) D.D.Sokoloff, Turkey: Vural 1976 (E), KT250904*, KT262773*, KT262846*, KT262916*; *L. schimperii* Steud. ex Boiss., Oman: McLeish 3458 (E), DQ166218 (Degtjareva & al., 2006), KT262774*, KT262847*, KT262917*; *L. schoelleri* Schweinf. (1), Tanzania: Richards 23352 (WAG), KT250905*, –, –, –, *L. schoelleri* (2), Tanzania: de Boer-Kool 31 (WAG), KT250906*, KT262775*, KT262848*, KT262918*; *L. schoelleri* (3, 4), Ethiopia: de Wilde 5874 (WAG), KT250907* & KT250908*, –, –, –, *L. schoelleri* (5), Ethiopia: de Wilde 10869 (WAG), KT250909*, –, –, –, *L. schoelleri* (6), Ethiopia: de Wilde 8645 (WAG), KT250910*, –, –, –, *L. sessilifolius* DC., Canary Is.: Alanko 91396 (H), KT250911*, –, –, –, *L. simonae* Maire & al., Morocco: Podlech 49444 (M), DQ160285 (Degtjareva & al., 2006), KT262776*, KT262849*, KT262919*; *L. stenodon* Heldr. ex Nyman (1, 2), Montenegro: Uotila 10624 (H), KT250912* & KT250913*, –, –, –, *L. stepposus* Kramina (1), European Russia, Kramina 63-3 (MW), JF784205 (Kramina & al., 2012), KT262777*, KT262850*, KT262920*; *L. stepposus* (2), European Russia: Kramina 60-7 (MW), JF784206 (Kramina & al., 2012), KT262778*, KT262851*, KT262921*; *L. stepposus* (3), Ukraine, Kramina 14-4 (MW), JF784207 (Kramina & al., 2012), KT262779*, KT262852*, KT262922*; *L. strictus* Fisch. & C.A.Mey. (1), Asiatic Russia: 18 Sep 2003, Korozyuk s.n. (MW), DQ160286 (Degtjareva & al., 2006), KT262780*, KT262853*, KT262923*; *L. strictus* (2), Kazakhstan: 1956, Povalyaeva s.n. (MW), KT250914*, KT262781*, KT262854*, KT262924*; *L. subbiflorus* Lag. (1), Italy: Iberite 15222 (MHA), DQ166231 (Degtjareva & al., 2006), KT262782*, KT262855*, KT262925*; *L. subbiflorus* (2), –, DQ166237 (Degtjareva & al., 2006), –, –, –, *L. subbiflorus* (3), –, DQ166239 (Degtjareva & al., 2006), –, –, –, *L. subbiflorus* (4), –, DQ168369 (Degtjareva & al., 2006), –, –, –, *L. subdigitatus* Boutique, Congo: Lisowski & al. 10586 (BR), KT250915*, –, –, –, *L. tenuis* Waldst. & Kit. ex Willd. (1, 2), San Marino: Lampinen & Lampinen 12064 (H), KT250916* & KT250917*, –, –, –, *L. tenuis* (3), Czech Republic: Deyl 164 (H), KT250918*, –, –, –, *L. tenuis* (4), Slovakia: 16 Jul 1974, Chrték & Křisa s.n. (LE), DQ166225 (Degtjareva & al., 2006) as *L. glaber* Mill., KT262783*, KT262856*, KT262926*; *L. tenuis* (5), Spain: Segura-Zubizarreta 22.705 (MHA), KT250919*, –, –, –, *L. tenuis* (6), Luxembourg: Lambion 89/188 (H), KT250920*, –, –, –, *L. tetragonolobus* L. (1), Cyprus: Seregin & al. A-110 (MW), HM468334 (Degtjareva & al., 2012), KT262784*, KT262857*, KT262927*; *L. tetragonolobus* (2), Greece: Alanko 85940 (H), KT250921*, –, –, –, *L. tetragonolobus* (3), –, AF450225 (Allan & al., unpub.), –, –, –, *L. tetraphyllus* Murr., –, FJ411119 (Degtjareva & al., 2008), –, –, –, *L. torulosus* (Chiov.) Fiori, –, FJ411120 (Degtjareva & al., 2008), –, –, –, *L. ucrainicus* Klokov (1), European Russia: Kramina 59-3 (MW), JF784203 & JF784204 (Kramina & al., 2012), KT262785*, KT262858*, KT262928*; *L. ucrainicus* (2), European Russia: Kramina 62-4 (MW), JF784202 (Kramina & al., 2012), KT262786*, KT262859*, KT262929*; *L. uliginosus* Schkuhr (1), Germany: Larsen & Larsen 40490 (H), KT250922*, –, –, –, *L. uliginosus* (2), Spain: 13 Jun 1952, Roivainen s.n. (H), KT250923*, –, –, –, *L. uliginosus* (3), –, DQ160273 (Degtjareva & al., 2006), –, –, –, *L. weillieri* Maire (1), Cultivated in Canada: origin Morocco, Grant B-114 (BP 800231), KT250924*, KT262787*, KT262860*, KT262930*; *L. weillieri* (2), Morocco, J.H. Kirkbride 5851 (AA), KT250925*, KT262788*, KT262861*, KT262931*; *L. wildii* J.B.Gillett, Zimbabwe: Bayliss 110166 (E), DQ160287 (Degtjareva & al., 2006), KT262789*, KT262862*, KT262932*; **OUTGROUPS:** *Cytisopsis pseudocytisus* (Boiss.) Fertig, Turkey: 29–31 May 1995, Khokhryakov & Mazurenko s.n. (MHA), AY325282 (Degtjareva & al., 2003), KT262790*, HM468259 (Degtjareva & al., 2012), HM468299 (Degtjareva & al., 2012); *Hammatolobium kremerianum* (Coss.) C.Muell., Morocco: Podlech 51378 (MHA), KT250926*, KT262791*, KT262863*, KT262933*; *H. lotoides* Fenzl, Greece: 8 Jun 1996, Emanuelsson 1932 (S), AY325279 (Degtjareva & al., 2003), KT262792*, HM468262 (Degtjareva & al., 2012), HM468302 (Degtjareva & al., 2012).