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Two cryptic species of *Lotus* (Fabaceae) from the Iberian Peninsula

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Summary: The problem of cryptic species is well known in taxonomy of different groups of organisms, including plants, and their recognition can contribute to the assessment of global biodiversity and the development of conservation methods. Analyses of *Lotus glareosus* and related taxa from the Iberian Peninsula based on various types of data (i.e. sequences of nuclear ribosomal ITS-1-2, 5'ETS and cpDNA *trn*L-F, seven loci of nuclear microsatellites) revealed that the material earlier determined as '*L. glareosus*' is subdivided into two genetically distant groups: *L. carpetanus*, related to *L. conimbricensis*, and *L. glareosus,* included in the *L. corniculatus* complex. Though only slight morphological distinctions were found between them, significant genetic differences comparable to those between sections of the genus *Lotus* (p-distance 0.07–0.08 in ITS, 0.060–0.067 in ETS and 0.010–0.013 in *trn*L-F; substitution number 43–47 bp in ITS, 22–24 bp in ETS and 12–14 bp in *trn*L-F) and no evidence of genetic exchange suggest that these groups may represent two deeply diverged lineages that should be treated as two separate species. This example corresponds to the concept of cryptic species. Based on molecular study of type specimens of *L. carpetanus*, we propose to reestablish this name, previously synonymized with *L. glareosus*.

Keywords: ETS, ITS, Loteae, *Lotus carpetanus*, *Lotus glareosus*, morphological variability, nuclear microsatellites, *trn*L-F

The problem of cryptic species is well known in taxonomy of different groups of organisms (e.g. reviews Bickford et al. 2007; Fišer et al. 2018). Bickford et al. (2007) defined cryptic species as two or more species which are sufficiently distinct based on molecular or other evidence, but classified as a single nominal species, because they are at least superficially morphologically indistinguishable. With rapid development of molecular methods within the last two decades, the number of discovered cryptic species dramatically increased (Fišer et al. 2018). Their recognition and detailed description can contribute to the assessment of global biodiversity and to the development of evolutionary theory, biogeography and conservation planning (Bickford et al. 2007). Fišer et al. (2018) stressed the importance of formal naming of cryptic species, which will allow better integration in areas of research that use species as units of analysis. In the literature, there are much fewer examples of the detection of cryptic species in higher plants than among animals (Bickford et al. 2007), however, some cases are described and studied by molecular methods (e.g. Vigalondo et al. 2015; Sokoloff et al. 2019). This paper presents a new case of cryptic species discovered in flowering plants from legume family.

Genus *Lotus* L. (Linnaeus 1753) (Fabaceae, Loteae) is a taxonomically complicated group, which comprises ca 130 species of Old World herbs, semishrubs and shrubs, including important pasture crops and a model legume, *L. japonicus* (Regel) K. Larsen (Kramina et al. 2016). The boundaries and system of the genus have remained a subject of discussion for several centuries (see reviews Degtjareva et al. 2006; Kramina et al. 2016). The Iberian Peninsula is one of diversity centres of the genus, where members of most sections accepted by Degtjareva et al. (2006) occur.

One of the most taxonomically problematic groups of the genus is *Lotus corniculatus* L. (Linnaeus 1753) complex comprising from one to many species, according to different viewpoints (e.g. Linnaeus 1753; Brand 1898; Ball & Chrtková-Žertová 1968; Heyn 1970a; Chrtková-Žertová 1973, 1984; Greuter et al. 1989; Valdés 2000). Western Mediterranean region was supposed to be the distribution area of the most ancient cpDNA haplotypes of the *L. corniculatus* complex and related haplotypes of its sister species *L. conimbricensis* Brot. (Avellar Brotero 1805), which belongs to the same section, *Lotus* section *Lotus* L. (Kramina et al. 2018). In the Iberian Peninsula and adjacent areas, several species of the complex have been described such as *L. glareosus* Boiss. & Reut. (Boissier & Reuter 1852), *L. glacialis* (Boiss.) Pau (Pau 1922), *L. carpetanus* Lacaita (Lacaita 1928) and *L. delortii* Timb.-Lagr. in Schultz (Timbal-Lagrave 1852). Interspecific differences between them were not that clear, and their taxonomic rank has been reduced to subspecies within *L. corniculatus* later (Valdés 2000). However, recent phylogenetic studies put into question close relationships between some of western specimens collected in Spain and Portugal and *L. corniculatus* complex (Kramina et al. 2016, 2018). A necessity of additional study based on a representative sampling became obvious.

Species related to *L. corniculatus*, described from the Iberian Peninsula and adjacent areas. Boissier & Reuter (1852) described *L. glareosus* as perennial plant with more or less elongated shoots with hard base, calyx teeth slightly extended at the base and with long linear-subulate upper part. They designated three varieties within this species: var. α *glabrescens* Boiss. & Reut. (Boissier & Reuter 1852) with appressed sparse indumentum, ascending long shoots, large leaves and 5–6-flowered heads, located in the vicinity of Granada, near the river Darro; var. β *villosus* Boiss. & Reut. (Boissier & Reuter 1852) with denser, short hispid indumentum and suberect shoots, from middle altitudes in Sierra Nevada; near the river Monachil, and var. γ *glacialis* Boiss. & Reut. (Boissier & Reuter 1852) with appressed silvery indumentum, abbreviated shoots, small leaves and 1–2-flowered heads, from high altitudes in Sierra Nevada. The last variety, previously described within the species *L. corniculatus* (Boissier 1840), was transferred into the new species *L. glareosus* by Boissier & Reuter (1852). Later, Pau (1922) detached a separate species, *Lotus glacialis*, corresponding to the var. γ *glacialis*.

Timbal-Lagrave (1852) described *Lotus delortii* from south France, Low Occitanie, occurring in La Clape near Narbonne, Saint-Gely near Montpellier and Sisteron, partially corresponding to *L. corniculatus* var. *villosus* Ser. (Seringe 1825). In the diagnosis, Timbal-Lagrave (1852) mentioned large flowers, ca 15 mm long, some degree of calyx zygomorphy, which appears in unequal calyx teeth, two upper sharply and three lower gradually elongated; corolla turning green after desiccation; whole plant glaucous, densely pubescent with simple long white trichomes.

Lacaita (1928) described *Lotus carpetanus*, a member of *Lotus corniculatus* group. He mentioned that the new taxon differs from forms occurring in Gallia and Central Europe by habitus and leaf shape and considered it as a geographical subspecies, not as a variety. The author listed Castilla, Salmantica province, piedmont of Sierra de Guadarrama and west Salamanca as geographical localities of *L. carpetanus*. Lacaita noticed that the plant is totally white pubescent, with hard rhizomes, almost suffrutescent, with many slender shoots 5–15 cm long, prostrate or ascending, with small basal leaves, almost roundish, with leaflets ca 2 mm long, and somewhat larger middle stem leaves with ovate lateral leaflets and an obovate terminal one, all leaflets obtuse, sessile; peduncles normally 4-flowered, calyx teeth widely lanceolate at the base, shorter than calyx tube;

petals yellow, not turning green after desiccation; usually ca 1 cm long, outside often orange or with red stripes; pods straight, ca 2.5 cm long.

Rivas Martínez (1964) made a combination *Lotus corniculatus* ssp. *carpetanus* (Lacaita) Rivas Mart. He mentioned that this taxon occurs as shrub in disturbed oak forests on southern slopes of Guadarrama, in association *Cistion laurifolii* and reaches La Pedriza at altitudes of 1700 m. Neither Lacaita nor Rivas Martínez chose the type for this taxon. In Herbarium BM, two authentical specimens collected by Lacaita are kept: 28783 - Peña Gudina prope Veguillas (Salamanca), 5.VI.1925, Lacaita [BM]; 25710 - Cercedilla at the foot of Sierra de Guadarrama in rocky grass (Castile), 10.VI.1923, Lacaita [BM]. All six individuals from both herbarium sheets correspond to the diagnosis of *Lotus carpetanus*. This material can be used for its lectotypification.

In Flora Europaea, Ball & Chrtková-Žertová (1968) regarded *L. glareosus* (incl. *L. glacialis*) and *L. delortii* as separate, but closely related species included in the *L. corniculatus* species complex, which contains twelve species in Europe. Fernandes (1981) conducted a revision of this complex on the Iberian Peninsula and the Balearic Islands. He assigned seven species to the complex, i.e. *L. tenuis* Waldst. et Kit. ex Willd., *L. preslii* Ten., *L. alpinus* (Ser.) Schleich. ex Ramond, *L. corniculatus*, *L. pedunculatus* Cav., *L. boissieri* A. Fern., *L. glareosus* and *L. delortii*. Fernandes (1981) described a new species *L. boissieri* A. Fern. and proposed to use this name instead of *L. glacialis* (Boiss.) Pau for the species corresponding to the var. γ *glacialis*. However, the name *L. boissieri* is illegitimate and superfluous (Valdés 2000). Within *L. glareosus*, Fernandes (1981) retained two of three varieties, designated by Boissier and Reuter, i.e. var. *glareosus* (corresponding to var. *glabrescens*) and var. *villosus*. According to Fernandes (1981), var. *glareosus* differs from var. *villosus* by plant colour: green in the former and greyish in the latter. However, a transition row is observed between these two colour forms and the same can be attributed to pubescence.

Greuter et al. (1989) treated *L. glareosus*, *L. glacialis* and *L. delortii* as members of *L. corniculatus* aggr. and considered *L. corniculatus* L. ssp. *carpetanus* as a synonym of *L. glareosus*.

Valdés (2000) synonymized *L. glareosus*, *L. glacialis* and *L. delortii* with broadly treated species *Lotus corniculatus* and reduced their taxonomic rank to subspecies (Valdés Castrillón 1999; Valdés 2000). He accepted *L. corniculatus* L. ssp. *carpetanus* (syn. *L. glareosus* and *L. carpetanus*), ssp. *glacialis* (Boiss.) Valdés (Valdés Castrillón 1999) and ssp. *delortii* (Timb.-Lagr.) O.Bolòs & Vigo (de Bolòs & Vigo 1984). The main morphological feature of these three subspecies, distinguishing them from other subspecies of the *Lotus corniculatus* complex, is a bilabiate (or zygomorphic) calyx (Valdés 2000), which appears in a greater width and partly also in the length of the upper teeth compared to the lower, however, the manifestation degree of this character varies, which was noted by many authors (Ball & Chrtková-Žertová 1968; Fernandes 1981; Valdés 2000).

Chromosome numbers. *Lotus* section *Lotus* is the only section with the basic chromosome numbers x = 6 and rarely x = 5, while other sections of *Lotus* have x = 7 (Grant 1995). *Lotus corniculatus* complex from *Lotus* section *Lotus* includes diploids (2n = 12), tetraploids (2n = 24) and rarely hexaploids (2n = 36), except for *L. glareosus* which has 2n = 10 (Grant 1995). The chromosome number 2n = 10 was first reported in a specimen from Sierra de Guadarrama, Madrid, Spain (Angulo & Real 1977), determined as *L. castellanus* Boiss. & Reut. in Boissier (Boissier 1849). This was a discovery of new basic chromosome number x = 5 in the genus *Lotus*,

which was earlier known as having basic chromosome numbers 6 and 7 (Grant 1965). Fernandes defined this specimen as *L. glareosus* (Fernandes 1981) and made several more chromosome countings for this species and obtained the same number (i.e. 2n = 10, x = 5) in all of them (Fernandes & Santos 1975; Fernandes et al. 1977; Fernandes 1981). Forde & De Latour (1977) counted chromosome number 2n = 10 in an introduced specimen of *Lotus* originated from Coimbra, Portugal. Later, the specimen was determined by Chrtková-Žertová as *L. glareosus*. Lago Canzobre & Castroviejo (1992) determined chromosome number 2n = 10 in several specimens of *L. glareosus* var. *glareosus* collected by Ferreiro, Lago and Paz in Spain, A Coruña [MA 459769, MA 459766] and Lugo [MA 459768]. So far, the number 2n = 10 found in *L. glareosus* remains the only case of this chromosome number in *Lotus* (Goldblatt & Johnson 1979; Grant 1995). Valdés (2000) also cited chromosome numbers 2n = 12 and 24 for *L. corniculatus* L. ssp. *carpetanus,* which he considered identical to *L. glareosus* and *L. carpetanus*. However, he provided no information on the origin of material used for chromosome number counting. For *L. glacialis*, the chromosome number 2n = 12 and for *L. delortii*, the number 2n = 24 were reported (Grant 1995).

Molecular phylogenetic studies. Phylogeny of the genus *Lotus* L. was recently studied using nrITS1-2 (Allan et al. 2003; Degtjareva et al. 2006, 2008; Kramina et al. 2016), nrETS and plastid markers (Kramina et al. 2016). Analysis of *Lotus* plastid data revealed early split into 'northern' and 'southern' lineages in the evolution of the genus (Kramina et al. 2016). The 'northern' clade included species of the typical *Lotus* section and species traditionally placed in *Dorycnium* Mill. (Miller 1754), whereas 'southern' clade was formed by all remaining *Lotus* species (Kramina et al. 2016). *Lotus corniculatus* complex from the typical *Lotus* section *Lotus* appeared to be monophyletic in analyses of nrITS1-2 and plastid data (Degtjareva et al. 2006, 2008; Kramina et al. 2016, 2018), except for several specimens of *L. glareosus*.

According to nrITS and plastid data, *L. corniculatus* complex and *L. conimbricensis* are sister taxa (Degtjareva et al. 2006, 2008; Kramina et al. 2016, 2018). *Lotus conimbricensis* is a species rather distant from members of *L. corniculatus* complex in morphology. This is a white- or pink-flowered annual with one-flowered peduncles shorter than leaves and indehiscent curved pods, distributed in the Mediterranean region and on Madeira (Brand 1898; Ball & Chrtková-Žertová 1968; Greuter et al. 1989). Previously, it was placed in *Lotus* section *Erythrolotus* (Brand 1898). However, all studied DNA markers provided evidence that *L. conimbricensis* is a member of section *Lotus* (Kramina et al. 2016). The main common synapomorphy of *L. conimbricensis* and other members of section *Lotus* is the basic chromosome number x = 6 (Grant 1995).

Kramina et al. (2016, 2018) demonstrated that one (for cpDNA *trn*L-F) or two (for nrITS) specimens determined as *L. glareosus* form a common clade with *L. conimbricensis*. Another specimen of *L. glareosus* in all mentioned analyses fell into *L. corniculatus* clade, as well as the studied specimens of *L. glacialis* and *L. delortii*. It is worth mentioning that nrETS showed more distant phylogenetic relationships between *L. corniculatus* complex and *L. conimbricensis* (Kramina et al. 2016). However, this marker was not studied in *L. glareosus*, so it will not be discussed here. Hereby, studied specimens of *L. glareosus* split into two groups, which are dissimilar by nuclear and plastid DNA markers, i.e. a group close to *L. conimbricensis* and another one, inserted into the *L. corniculatus* clade. The specimens SPAIN, Burgos: Santibáñez del Val, Barriosuso, 11.VII.1979 *Pons-Sorolla & Susanna 270* [B] and PORTUGAL, Viseu District: 25 km

W of Viseu, Cambarinho, Reserve do Cambarinho, 30.V.1972 *Jalas 1768* [H] are members of the first group, and a specimen SPAIN, Jaén: Sierra del Pozo, 30.06.1988 *Valdés et al. 2959/88* [B] is a member of the second one (Kramina et al. 2016, 2018). Differences between studied DNA sequences in these two groups of *L. glareosus* were impressive: more than 40 mononucleotide substitutions in nrITS1-2 and nine substitutions, one mononucleotide insertion and three longer indels in cpDNA *trn*L-F region, although the plants of both groups were very similar to each other in morphology. The need of additional research based on a representative sampling has become apparent.

Thus, the aims of the present study were to investigate genetic and morphological variability of *L. glareosus* and related species from the Iberian Peninsula and to conduct a taxonomic identification of the samples, which are very distant by molecular markers. To achieve this purpose, we put the following tasks: to collect a representative set of samples of *L. glareosus* and related species from the Iberian Peninsula and adjacent areas; to obtain sequences of nrITS, nrETS and cpDNA *trn*L-F, as well as nuclear microsatellites, for the samples and to conduct phylogenetic analyses using the material collected for *L. glareosus* and a comprehensive set of *Lotus* species; if the split of '*L. glareosus*' into two different groups is confirmed, to try finding the morphological characters to discriminate these groups; to identify the valid taxonomic names of the studied individuals of '*L. glareosus*'.

Materials and methods

Plant material. The main part of the material for morphological and molecular analyses were specimens of *L. glareosus*, *L. glacialis*, *L. alpinus*, *L. delortii* and *L. conimbricensis* from the Iberian Peninsula and adjacent regions stored in several large herbaria [B, BM, H, LE, MA, MHA and MW]. Additionally, samples from natural populations, collected from Spain in 2018 between Ahedo and La Revilla and near Barriosuso (Burgos province), and near Montenegro de Cameros and Laguna Negra de Urbión (Soria province), were included in the study.

Authentical specimens of *L. carpetanus* collected by Lacaita from Spain in 1923 and 1925 and kept in BM herbarium [BM 25710 and BM 28783] were included in morphological and molecular analyses.

The following type material of *L. glareosus* Boiss. et Reuter from the Herbarium of Conservatoire et Jardin botaniques de la Ville de Genève [G] was studied: Typus de *Lotus glareosus* Boiss. et Reut. Espagne, In glareosis humidis fluvii Darro prope Granatam, G.F. Reuter s.n., Jun.1849 [G00020259]; Typus de *Lotus glareosus* Boiss. et Reut. Espagne, In glareosis humidis fluvii Dar. prope Granatem, G.F. Reuter s.n., Jul.1849 [G00020258]; Lectotypus de *Lotus glareosus* Boiss. et Reut. var. *villosus* Boiss. et Reut., Espagne, Sa Nevada reg. media, ad fluv. Monachil, in glareosis, G.F. Reuter s.n., Jul.1849 [G00020261]. – Valdés Castrillón, B. – 1997; Typus de *Lotus glareosus* Boiss. et Reut. var. *villosus* Boiss. et Reut., Espagne, Sa Nevada reg. media, ad fluv. Monachil, in glareosis, G.F. Reuter s.n., Jul.1849 [G00020260]; Lectotype specimen of *L. glareosus* var. *glacialis* Boiss. et Reut., collected by P.E. Boissier in 1837 [G00020255]. All these specimens were used for morphological analysis only.

Phylogenetic reconstructions were conducted using studied material and a representative sampling of *Lotus* species, which covers thirteen sections of the genus. Molecular studies involved the ingroup: 52 species of *Lotus* represented by 93 and 92 specimens in ITS and *trn*L-F analyses,

respectively, or 55 species of *Lotus* represented by 78 specimens in ETS analyses. All analyses included three outgroups: *Cytisopsis pseudocytisus* (Boiss.) Fertig (Boissier 1843; Fertig 1970), *Hammatolobium kremerianum* (Coss.) Müll. Berol. (Cosson 1857; Müller 1870) and *Tripodion tetraphyllum* (L.) Fourr. (Linnaeus 1753; Fourreau 1868). Voucher information and GenBank accession numbers are presented in Appendix 1. Distribution map of studied specimens, constructed using SimpleMappr (Shorthouse 2010), is presented in Fig. 1.

Morphometric analyses. Measurements of 42 morphological characters were made using 22 herbarium specimens (Table 1). Statistical procedures were conducted using STATISTICA 7.1 software for Windows (StatSoft Inc. 2006). All characters were tested for normality. Then, two-sample tests were conducted to compare means in two groups corresponding to '*L. carpetanus*' and '*L. glareosus*', according to molecular data. T-test was applied for characters with distribution corresponding to normal and Mann-Whitney U test for those which distribution deviated from normal.

DNA extraction, amplification and sequencing. DNA was extracted from dry leaves taken from herbarium (20 mg leaf tissue) with NucleoSpin Plant Kit (Macherey-Nagel, Germany) according to the manufacturer's instructions or using the CTAB method (Doyle & Doyle 1987).

The sequences of the entire ITS1-5.8S-ITS2 region were amplified with primers NNC-18S10 and C26A (Wen & Zimmer 1996) and universal primers ITS2 and ITS3 (White et al. 1990). The sequences of *trn*L-*trn*F intergenic spacer (IGS) and *trn*L intron were amplified using standard primers 'c', 'd', 'e' and 'f' (Taberlet et al. 1991). PCRs were performed in a 0.02 ml mixture containing 10–20 ng DNA, 3.2 pmol of each primer and Mas^{DD}TaqMIX (Dialat LTD, Russia) containing 0.2 mM of each dNTP, 1.5 mM $MgCl_2$ and 1.5 units of SmarTaqDNA polymerase.

Amplification of nrDNA ITS1-2 and 5'ETS and cpDNA *trn*L intron and *trn*L-*trn*F IGS regions was performed under the following conditions: hold 94°C, 3 min; 94°C, 30 s; 57°C, 40 s; 72°C, 60 s; repeat 30 cycles; extend 72°C, 3 min.

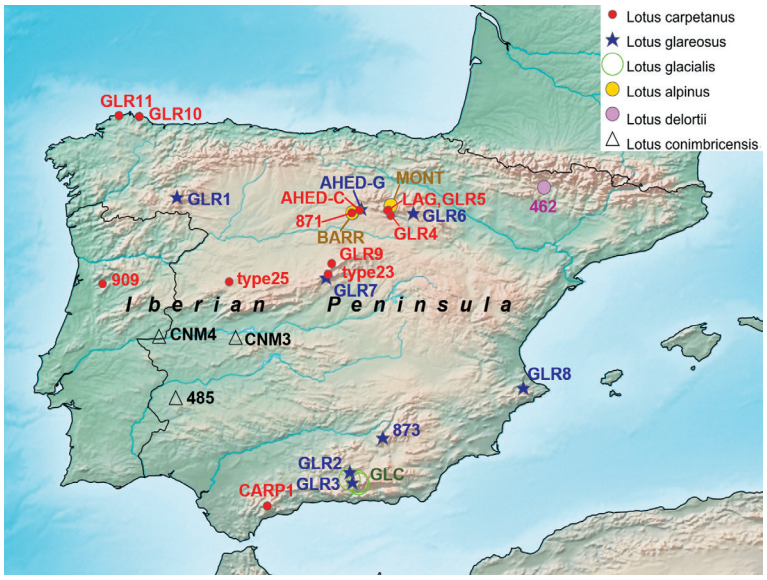

Figure 1. Distribution map of studied specimens of *L. carpetanus*, *L. glareosus* and related species in the Iberian Peninsula. Map: T. Kramina.

Table 1. Morphological characters measured in 22 specimens.

Character code	Character description
LML	Length of the terminal leaflet of a middle stem leaf (mm)
WML	Width of the terminal leaflet of a middle stem leaf (mm)
LMLMAX	Length from the base up to the position of maximal width of the terminal leaflet of a middle stem leaf (mm)
LUL	Length of the terminal leaflet of an upper stem leaf (mm)
WUL	Width of the terminal leaflet of an upper stem leaf (mm)
LLAT	Length of the lateral leaflet of a middle stem leaf (mm)
WLAT	Width of the lateral leaflet of a middle stem leaf (mm)
LLOW	Length of the basal leaflet of a middle stem leaf (mm)
WLOW	Width of the basal leaflet of a middle stem leaf (mm)
RACH	Length of rachis of a middle stem leaf (mm)
RTOP	Rachis tip length (mm)
COAV	Flower length (mm)
UMAV	Number of flowers per umbel
PEDUNC	Peduncle length (cm)
LST	Stem length (cm)
PBL	Pubescence density on leaves (grades 1 to 9)
PBCA	Pubescence density on calyces (grades 1 to 9)
HAIRL_L	Trichome length on leaves (mm)
HAIRL_C	Trichome length on calyces (mm)
ANG	Angle of trichome reflection on calyces (grades 1–4)
LCA	Calyx length (mm)
LTUB	Calyx tube length (mm)
UPLOBL	Length of the upper calyx lobe
LATLOBL	Length of the lateral calyx lobe
LOLOBL	Length of the lower calyx lobe
UPLOBW	Width of the upper calyx lobe
LATLOBW	Width of the lateral calyx lobe
LOLOBW	Width of the lower calyx lobe
KEELL	Keel length (mm)
KTOP	Keel beak length (mm)
KEELANG	Keel angle (grades 1–3)
STYLE	Style length (mm)
ULOBN	Portion of narrow part of calyx upper lobe (%)
LALOBN	Portion of narrow part of calyx lateral lobe (%)
LOLOBN	Portion of narrow part of calyx lower lobe (%)
CONUTE	Length of connate part of two upper calyx teeth (mm)
INDEX-L=LML/LLOW	Length of the terminal leaflet to length of the basal leaflet ratio of a middle stem leaf
FML=LML/WML	Length to width ratio of the terminal leaflet of a middle stem leaf (mm)
FUL=LUL/WUL	Length to width ratio of the terminal leaflet of an upper stem leaf (mm)
FLAT=LLAT/WLAT	Length to width ratio of the lateral leaflet of a middle stem leaf (mm)
FLOW=LLOW/WLOW	Length to width ratio of the basal leaflet of a middle stem leaf (mm)
IL1=LMLMAX/LML	Terminal leaflet index (i.e. length from the base up to the position of maximal width to the total leaflet length ratio) of a middle stem leaf

PCR products were purified using Cleanup Mini Kit (Evrogen, Moscow, Russia) following the manufacturer's instructions. Direct sequencing was performed on the ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA), using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 for cycle sequencing reactions following the manufacturer's instructions. Forward and reverse strands of all samples were sequenced. The polymorphism of ITS and ETS within one specimen was detected by direct sequencing (without cloning), by the presence of double peaks on chromatogram.

ITS and ETS sequences were aligned using MAFFT version 7.215 (Katoh et al. 2002; Katoh & Standley 2013) and then adjusted manually in BioEdit version 7.2.5 (Hall 1999), *trn*L-*trn*F sequences were aligned manually in BioEdit. Gap-rich and ambiguous positions were excluded from analyses. The aligned data matrices are available from the corresponding author on request.

Phylogenetic analyses. The Maximum Likelihood (ML) analyses were performed with MEGA X (Kumar et al. 2018) with TN93+G model of nucleotide substitutions for *trn*L-*trn*F sequences and GTR+G+I model for ITS and ETS sequences. The models were determined as the best choice for corresponding datasets following the Model Selection option implemented in MEGA X based on AICc information criteria. Bootstrap method with 500 bootstrap replications was used for phylogeny test.

Phylogenetic relationships were also inferred with Bayesian approach using MrBayes version 3.2.6 (Ronquist et al. 2012) with the GTR+G model of nucleotide substitutions for *trn*L-*trn*F and ETS, and SYM+G model for ITS sequences, the models were selected by AICc in PAUP version 4.0a (Swofford 2003). Bayesian inference was performed with two parallel runs with four Markov chains for each run. 20 000 000 steps were generated, trees were sampled every 1000 steps. The first 50 (0.25%) trees were discarded as burn-in, then a majority-rule consensus tree was constructed from the remaining trees. Trees were visualized using TreeView (Page 1996).

Generation of microsatellite data. 48 specimens were genotyped for seven microsatellite loci specified for *Lotus japonicus* (Kai et al. 2010). These loci are known as located on chromosomes #1 (locus TM0113), #3 (loci TM0035 and TM0127), #4 (locus TM0030), #5 (locus TM0186) and #6 (loci TM0014 and TM0055) of *L. japonicus* (Kai et al. 2010) and have been used for a study of *Lotus corniculatus* complex (Kramina et al. 2018). PCRs were conducted with fluorescent labeled primers, synthetized in Syntol company (Moscow, Russia) under the conditions described in Kai et al. (2010). Fragment analysis of PCR-products was conducted on 3500 Genetic Analyzer with size standard GeneScan-500 LIZ (Applied Biosystems). Spectrograms were then analyzed with GeneMapper program. Values of fragment length were then manually separated into discrete classes according to the expected size of tandem repeat.

Microsatellite data analyses. 48 specimens were organized in nine groups according to taxonomic identification and geographic location: *Lotus carpetanus*: 1. AHED-C (AHED61-66, 71-78, BUR1-2); 2. LAG (LAG1-6, GLR4-5); 3. GLR9; *Lotus glareosus*: 4. GLR-SOUTH (GLR2, 3, 8); 5. GLR-NORTH (GLR1, 6, 7); 6. AHED-G (AHED67-69); *Lotus alpinus*: 7. MONT8 (MONT81-85); 8. MONT9 (MONT91-94, 428); *Lotus glacialis*: 9. GLC (GLC1-3, 889) (Fig. 1).

To estimate the optimal number of genetic clusters, Bayesian approach based on the Markov chain Monte Carlo (MCMC) algorithm in STRUCTURE v.2.3.4 (Pritchard et al. 2000) was

applied. To access equal ploidy level for all individuals, diploid individuals were transformed into tetraploids by doubling, which did not affect allele frequencies. The aim of the analysis was to ascertain the degree of genetic similarity between four species and nine geographic groups and degree of population isolation within one geographic region. In the analysis, for each K value from 1 to 10, five independent Markov chain Monte Carlo (MCMC) runs with a burn-in length of 50 000 iterations followed by an additional 500 000 iterations were conducted using the admixture model with correlated allele frequencies and no prior population information. The optimal K was determined by the standardized second-order rate change of ln P(K), ΔK (Evanno et al. 2005) implemented in the STRUCTURE HARVESTER program (Earl & von Holdt 2012). Bar charts representing Structure results averaged for runs with the same K were generated using CLUMPAK (Kopelman et al. 2015).

Results

Sequences characteristics. The matrices obtained for nrITS, nrETS and cpDNA *trn*L-F included 96, 86 and 95 sequences, 93, 83 and 92 in the ingroup, respectively, and three in the outgroup. For four specimens two different ETS sequences were obtained. Characteristics of ITS/ETS/*trn*L-F alignments: alignment length 677/577/1018 bp, variable sites 264/289/110, parsimony informative sites 202/225/48.

Phylogenetic analyses by nrITS 1-2 and cpDNA *trn*L-*trn*F markers. The overall topology of ITS and *trn*L-F trees is different to some extent, but the positions of specimens which are in the focus of the present research are very similar (Figs 2, 3). In all phylogenetic analyses conducted by ITS and *trn*L-F, 15 or 16 specimens, originally determined as '*L. glareosus*', formed a well supported separate clade (ITS: Posterior Probability in Bayesian Analysis 1.00 / Bootstrap Support in ML Analysis 100; *trn*L-F: 1.00/95). This clade contains authentical specimens of *L. carpetanus* and samples, collected in Black Lagoon Urbión and, partially, between Ahedo and La Revilla, as well as individuals from Spanish Central System, Northern Iberian System, NW and S Spain. This clade, which will be later named *L. carpetanus*, joins with the clade (in ITS phylogenies) or grade (in *trn*L-F phylogenies) of the species *L. conimbricensis* (ITS: 1.00/100; *trn*L-F: 0.99/74). The other part of specimens of '*L. glareosus*', which will be later named *L. glareosus*, fell into the *Lotus corniculatus* complex clade (1.00 both markers/ITS: 100, *trn*L-F: 95). In the ITS phylogeny (Fig. 2), they form a separate western subclade within the *L. corniculatus* complex clade (0.98/89) together with specimens of *L. alpinus* (from Spain), *L. delortii* and *L. glacialis*. Individuals, collected from natural populations in La Revilla y Ahedo (partially), Barriosuso, Montenegro de Cameros and specimens of *L. glareosus*, collected across Spain, became a part of this western subclade.

Two clades (i.e. the clade [*L. carpetanus* plus *L. conimbricensis*] and *L. corniculatus* complex clade) are included in the larger clade, exactly the section *Lotus* p. max p. clade in the ITS analyses (Fig. 2) and the *Lotus* North clade in the analyses of *trnL-trnF* (Fig. 3), however, in the latter case with very low support in ML analysis (1.00/62).

Number of substitutions between *L. carpetanus* and *L. glareosus* was 43 to 47 bp in ITS matrix and 12 to 14 bp in *trn*L-F matrix (p-distance 0.07-0.08 in ITS and 0.010-0.013 in *trn*L-F). Substitutions in ITS sequences are uniformly distributed in ITS1 (18–20 substitutions) and ITS2 (24–26 substitutions), one stable change was in 5.8S. In *trn*L-F sequences, more substitutions

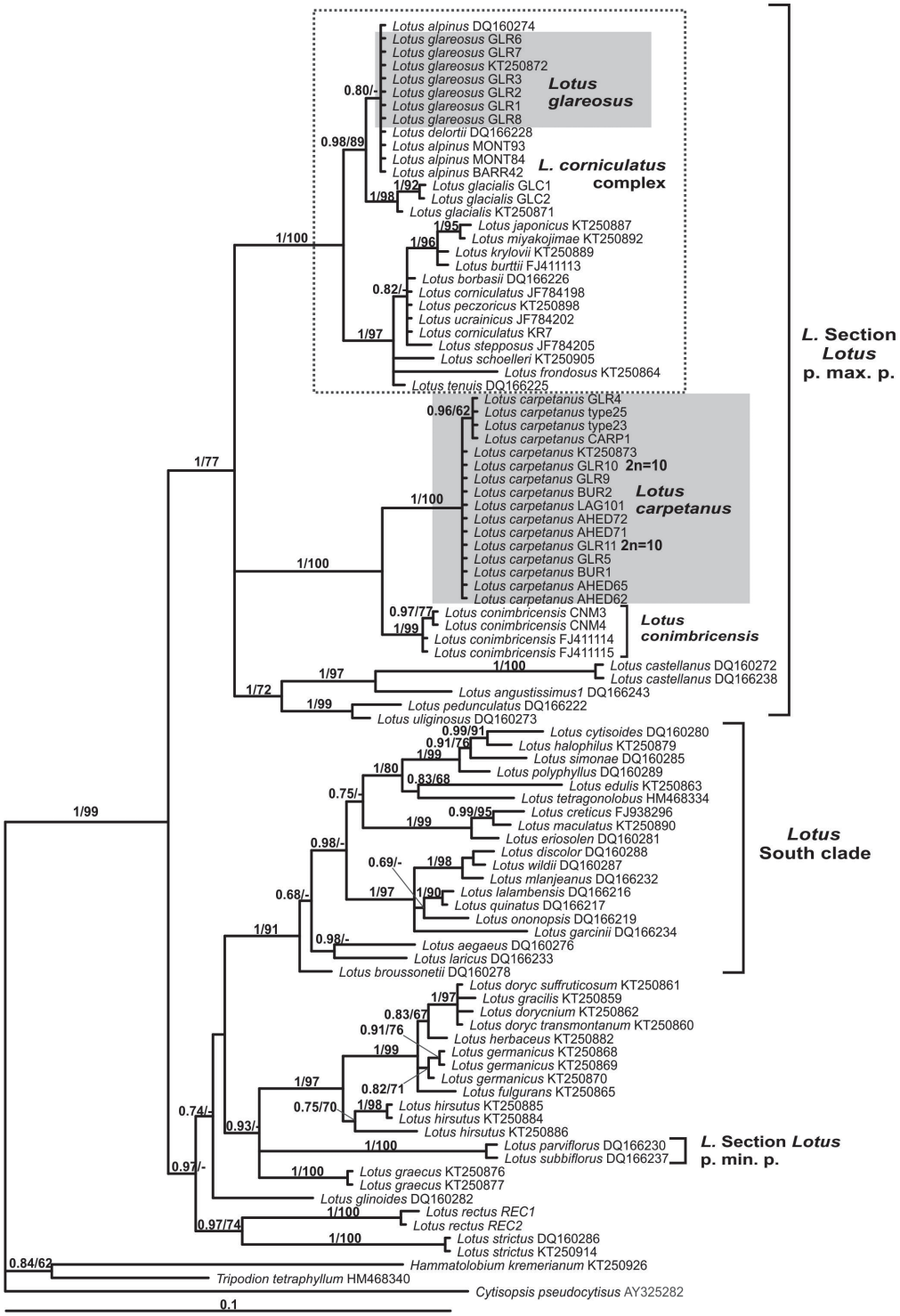

Figure 2. Phylogenetic relationships in *Lotus* inferred from Bayesian analysis of the nrITS dataset. Branch length is proportional to the number of expected nucleotide substitutions, scale bar corresponds to 0.1 substitutions per site. Numbers above branches are posterior probabilities. Numbers below branches or after slashes are bootstrap values found in Maximum Likelihood analysis of the same dataset (values equal or more than 0.6/60% shown). GenBank accession numbers for previously published sequences and sample codes for newly obtained sequences are given after species names. See Appendix 1 for voucher information.

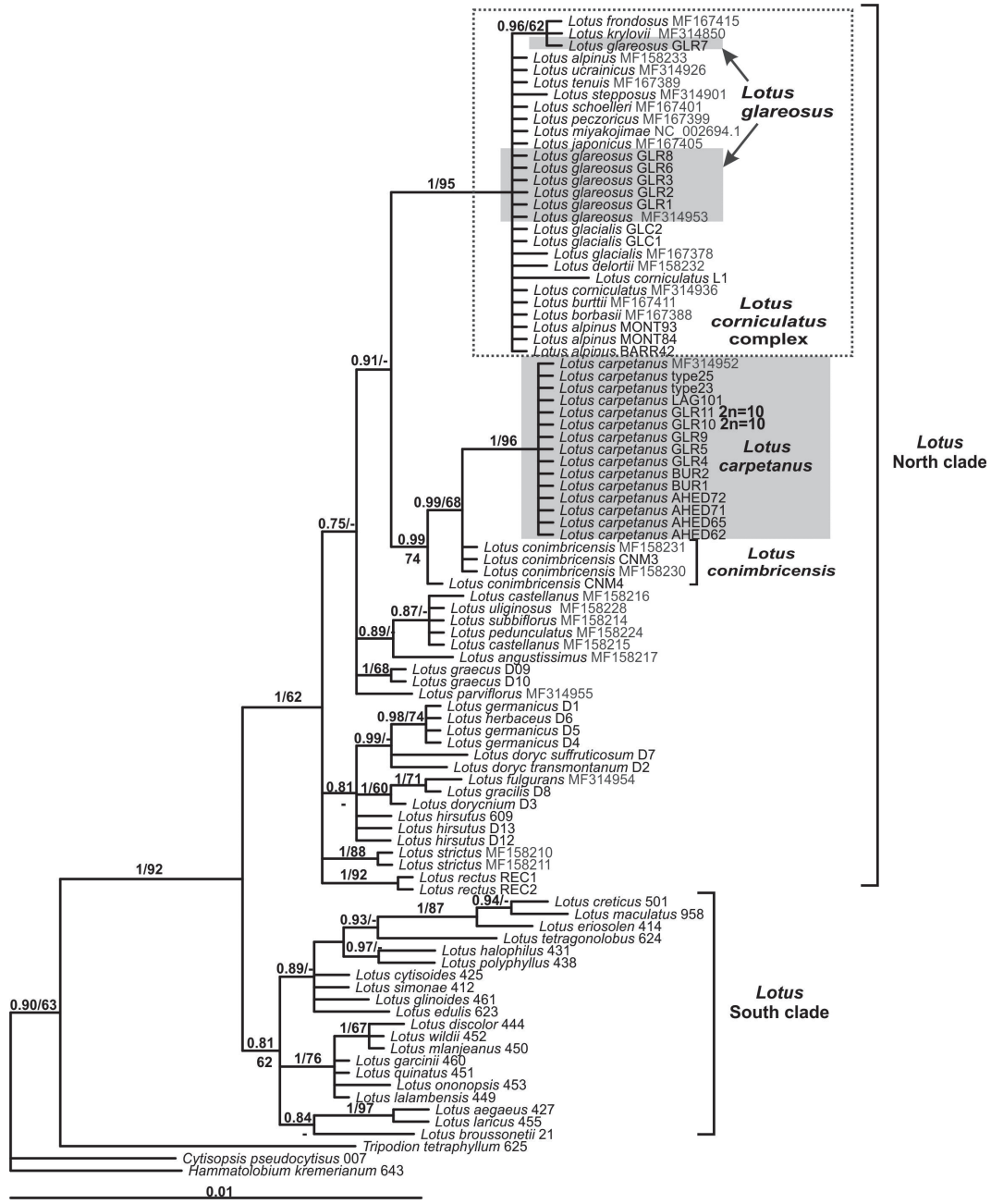

Figure 3. Phylogenetic relationships in *Lotus* inferred from Bayesian analysis of the cpDNA *trn*L-*trn*F dataset. Branch length is proportional to the number of expected nucleotide substitutions, scale bar corresponds to 0.01 substitutions per site. Numbers above branches are posterior probabilities. Numbers below branches or after slashes are bootstrap values found in Maximum Likelihood analysis of the same dataset (values equal or more than 0.6/60% shown). GenBank accession numbers for previously published sequences and sample codes for newly obtained sequences are given after species names. See Appendix 1 for voucher information.

were observed in *trn*L intron (eight stable substitutions) than in *trn*L-F IGS (only two stable ones). Differences between *L. carpetanus* and *L. conimbricensis* were smaller, i.e. 16–18 bp and 4 bp in ITS and *trn*L-F matrices, respectively. The difference between ITS sequences of *L. carpetanus* and *L. glareosus* (43–47 substitutions) is comparable with that between sequences of *L. corniculatus*

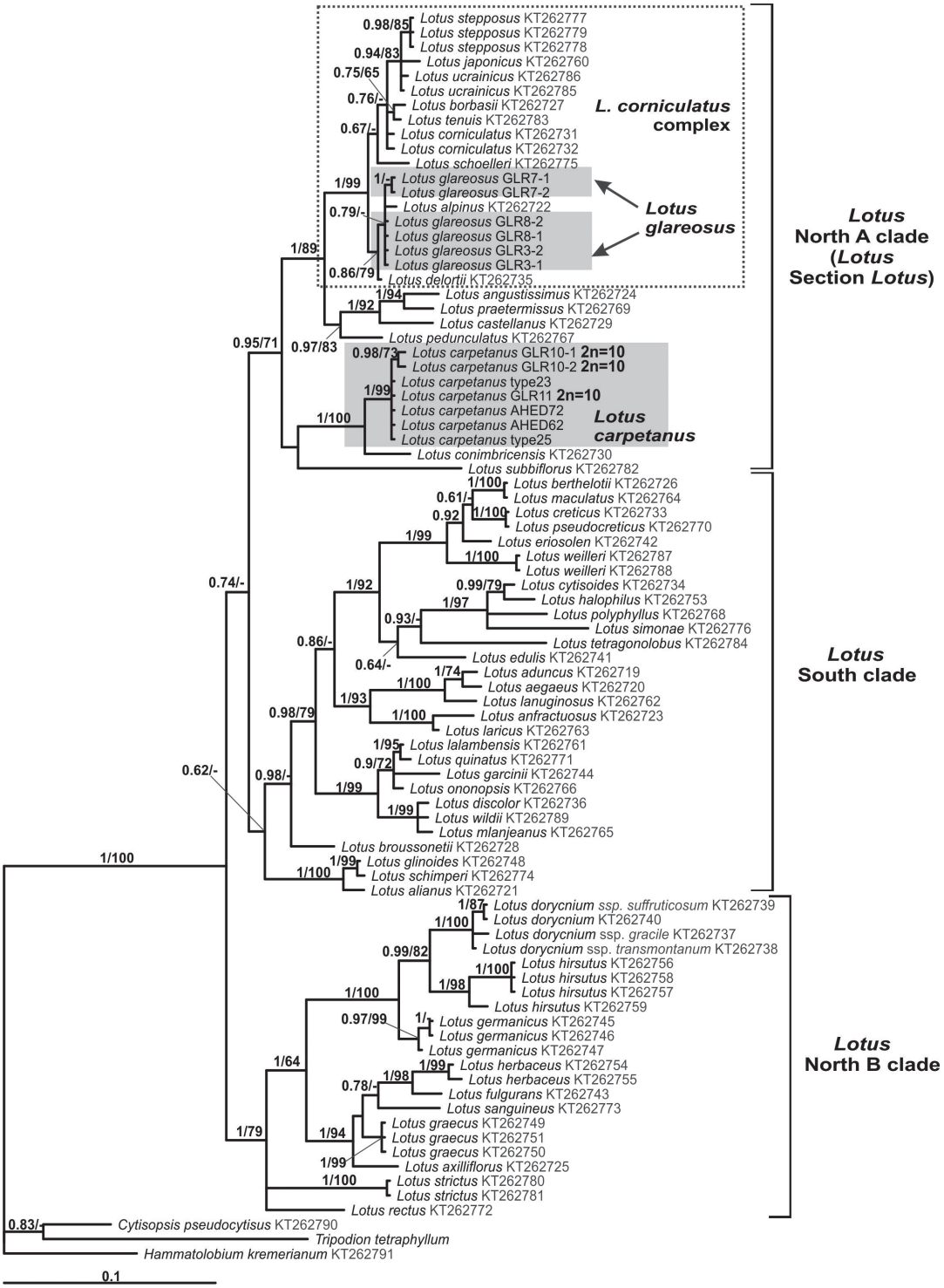

Figure 4. Phylogenetic relationships in *Lotus* inferred from Bayesian analysis of the nrETS dataset. Branch length is proportional to the number of expected nucleotide substitutions, scale bar corresponds to 0.1 substitutions per site. Numbers above branches are posterior probabilities. Numbers below branches or after slashes are bootstrap values found in Maximum Likelihood analysis of the same dataset (values equal or more than 0.6/60% shown). GenBank accession numbers for previously published sequences and sample codes for newly obtained sequences are given after species names. See Appendix 1 for voucher information.

KR7 and *L. graecus* D09&D10 (43 substitutions), which belong to different *Lotus* sections and are often treated as members of different genera *Lotus* and *Dorycnium*, respectively.

Phylogenetic analyses by nrETS. P-distances between 5'ETS sequences of *L. carpetanus* and those of *L. glareosus* were comparatively high, 0.060–0.067. Number of substitutions varied within 22–24 bp.

The 5'ETS tree topology (Fig. 4) is in good agreement with the one obtained earlier (Kramina et al. 2016). Three main clades are revealed on the tree: *Lotus* South clade and two *Lotus* North clades, A and B. As on ITS tree, highly supported *L. carpetanus* clade (1.00/99) is sister to *L. conimbricensis*. All studied accessions of *L. glareosus* belong to the *L. corniculatus* complex clade (1.00/99). Both the clade [*L. carpetanus* plus *L. conimbricensis*] and the *L. corniculatus* complex clade belong to the large *Lotus* North A clade, i.e. the clade of *Lotus* section *Lotus* (0.95/71). A sister group to the *L. corniculatus* complex is a clade that includes members of *L. angustissimus* and *L. pedunculatus* groups.

In all phylogenetic analyses, two specimens with known chromosome numbers 2n = 10, determined earlier (Lago Canzobre & Castroviejo 1992), as well as both authentical specimens of *L. carpetanus*, belong to the *L. carpetanus* clade (Figs 2–4).

Morphological comparison of two groups of specimens. Common characters of *L. glareosus* and *L. carpetanus*: plants 8–35 cm high, corolla yellow, sometimes partially orange or with red stripes, flower 9–11.5 mm long; leaves, calyces and stems are covered with ± appressed or patent trichomes 0.5–1.5 mm long, of varying density; style 5–7 mm long.

Slight morphological differences were revealed between two species (Table 2, Fig. 5). *L. carpetanus* and *L. glareosus* can be better discriminated by the length and index of lower pair leaflets, length of leaflets of upper stem leaves, calyx tube length, average number of flowers per umbel and calyx lobe shape (i.e. the portion of the subulate upper part of the lobe). Less significant were indumentum density, calyx length, trichome length, pubescence density on leaves and angle of trichome reflection as well as some other leaf characters (LML, INDEX-L, FUL). One of the discriminating characters, which can be hardly formalized, is the total plant colour: darker, grayish green in *L. carpetanus* and light green in *L. glareosus*.

Relying on these characters, we carefully tested the type material of *L. glareosus* from herbarium G. Morphological investigation revealed that all individuals of *L. glareosus* and *Lotus glareosus* var. *villosus* from studied type specimens correspond well to the characters of *L. glareosus*, but not of *L. carpetanus*. Lectotype specimen of *L. glareosus* var. *glacialis* appeared to be more distant morphologically from the two other varieties of *L. glareosus* as well as from *L. carpetanus*. On the contrary, both authentical specimens of *L. carpetanus* are in a good agreement with diagnostic characters of this species.

Analysis of microsatellite data. According to the number of alleles in each SSR locus, the supposed ploidy level of studied species is the following: all studied specimens of *L. carpetanus* are presumably diploids, as they have two alleles in maximum in each SSR locus; specimens of other species (i.e. *L. glareosus*, *L. glacialis*, *L. alpinus*) are putative tetraploids, but some specimens of *L. glareosus* (GLR8) and *L. glacialis* (GLC2, 889) can be diploids.

Evanno method applied to the results obtained in Structure revealed K = 2 as optimal number of genetic clusters and K = 3 as less optimal number, but Mean Estimated Ln Prob of Data for

Table 2. Morphological characters distinguishing between *L. carpetanus* and *L. glareosus*.

Character	*L. carpetanus* (n = 12)	*L. glareosus* (n = 10)	T-test (p-value)	U-test (p-value)
MOST SIGNIFICANT				
Length of the basal leaflet of a middle stem leaf (mm)	3.79±0.22 (3.3–4.3)	6.05±0.41 (5.1–7.0)	<0.0001	
Calyx tube length (mm)	3.64±0.07 (3.5–3.8)	2.92±0.11 (2.7–3.2)	<0.0001	
Average number of flowers per umbel	2.2±0.13 (1.9–2.5)	3.8±0.3 (3.1–4.5)	<0.0001	
Length of the terminal leaflet of an upper stem leaf (mm)	5.5±0.22 (5–6)	7.7±0.53 (6.5–8.9)	<0.001	
Length to width ratio of the basal leaflet of a middle stem leaf (mm)	1.35±0.05 (1.2–1.5)	2.24±0.24 (1.7–2.8)	<0.001	
Portion of narrow part of calyx upper lobe (%)	24±1.7 (19–30)	52±1.3 (40–55)		<0.0001
Portion of narrow part of calyx lateral lobe (%)	37±2 (32–41)	63±2.2 (60–70)		<0.0001
Portion of narrow part of calyx lower lobe (%)	54±2 (50–60)	75±1.2 (71–78)		<0.0001
LESS SIGNIFICANT				
Length of terminal leaflet of a middle stem leaf (mm)	5.16±0.30 (4.5–5.8)	6.78±0.52 (5.6–7.9)	<0.01	
Calyx length (mm)	7.0±0.19 (6.6–7.4)	5.9±0.29 (5.3–6.6)	<0.01	
Length of the terminal leaflet to length of the basal leaflet ratio of a middle stem leaf	1.4±0.06 (1.3–1.5)	1.1±0.03 (1.1–1.2)	<0.01	
Length to width ratio of the terminal leaflet of an upper stem leaf (mm)	2.0±0.14 (1.7–2.3)	3.2±0.29 (2.5–3.8)	<0.01	
Trichome length on leaves (mm)	0.8±0.04 (0.7–0.9)	0.6±0.05 (0.5–0.7)	<0.01	
Trichome length on calyces (mm)	0.9±0.06 (0.8–1.0)	0.6±0.06 (0.5–0.8)	<0.01	
Pubescence density on leaves (grades 1 to 9)	8.2±0.11 (8) middle to high density	5.8±0.61 (5–7) low to middle density		<0.01
Angle of trichome reflection on calyces (grades 1 to 4)	3.2±0.14 (2.5–3.5)	2.4±0.17 (2.0–3.0)		<0.01
Plant colour	greyish	green		

Mean ± standard error is presented for each quantitative character, after which a 95% confidence interval for the mean (for characters with a normal distribution) or interquartile range (for characters whose distribution deviates from normal) is given in parentheses. The results of mean comparison (T-test) or mean rank comparison (U-test) between two species are presented in two last columns.

K = 3 was a little higher (-1771.10) than for K = 2 (-1852.04). Analyses with K = 2 revealed two pure groups, the first corresponding to *L. carpetanus* and the second to all remaining species (i.e. *L. glareosus*, *L. glacialis* and *L. alpinus*), respectively (Fig. 6a). This result was stable, Mean (similarity score) = 1.000. No specimens of mixed (or intermediate) genetic structure were revealed.

In the variants of analysis with K = 3, the resulting clusterization was also stable, Mean (similarity score) = 0.994 (Fig. 6b). In these variants, *L. carpetanus* genetic cluster (cluster 1) remained unchanged, and the second group splits into two clusters (clusters 2 and 3), which were not genetically isolated from each other. A part of groups (i.e. groups 5–7) of *L. glareosus* and *L. alpinus* represent genetic cluster 2. *L. glacialis* (group 9) almost completely consists of individuals from

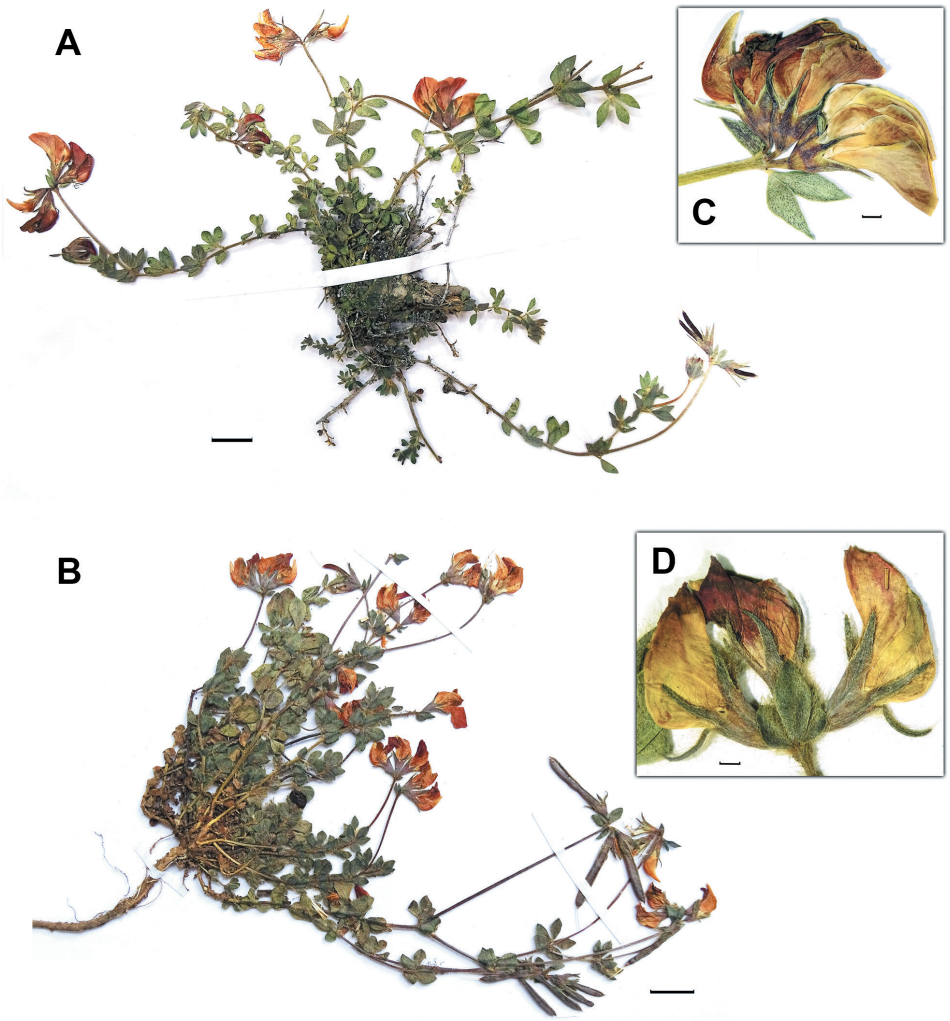

Figure 5. Whole plants and partial inflorescences of *L. glareosus* and *L. carpetanus*. A – Whole plant of *L. glareosus* (specimen GLR3, SPAIN: Granada, Sierra Nevada, Trevenque, 02.VII.1980, Pérez Raya & Martínez Parras s.n. [MA 336589]); B – *L. carpetanus* (specimen GLR9, SPAIN: Segovia, Santiuste de Pedraza, 22.VI.1985, R. García 1086 [MA 634557]). C – Partial inflorescence of *L. glareosus* (specimen GLR8, SPAIN, Alicante: Vall d'Alcala, 08.VI.1996, J.X. Soler & M. Signes s.n. [MA 587153]); D – *L. carpetanus* (specimen BUR2, SPAIN, Burgos: Santibañes del Val, Barriosuso, 11.VII.1979, Pons-Sorolla & Susanna 270 [MA 413052]). Scale bars = 1 cm (A, B); 1 mm (C, D). Images: T. Kramina.

cluster 3. Group 4 (*L. glareosus*) and, to some extent, group 8 (*L. alpinus*) demonstrate mixed genetic nature.

The most significant difference between *L. carpetanus* and other species was observed in the length of alleles in loci TM0030 and TM0113. In the locus TM0030, all studied specimens of *L. carpetanus* possessed unique allele of 104 bp length, whereas specimens of other species contain alleles of varying length from 110–205 bp. In the locus TM0113, alleles of *L. carpetanus* were 139–142 bp and those of other species 103–130 bp.

Geographical distribution and habitats of *L. carpetanus* and *L. glareosus*. Distribution of two species in the Iberian Peninsula is rather wide and sympatric. Specimens of both species

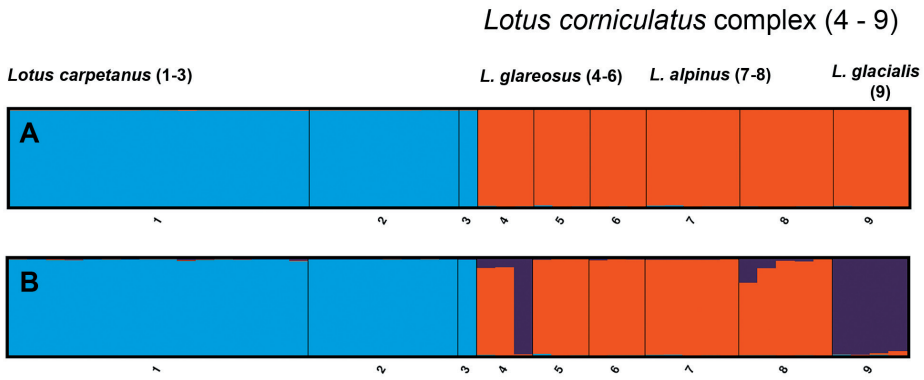

Figure 6. Genetic assignment of *Lotus carpetanus* (groups 1–3), *L. glareosus* (groups 4–6), *L. alpinus* (groups 7–8) and *L. glacialis* (group 9) based on SSR data. Results from STRUCTURE analysis, Admixture model. A – Expected number of genetic clusters K = 2; B – Expected number of genetic clusters K = 3.

were collected in the peninsula mainly in mountains, sometimes at lower altitudes (Fig. 1). Plants of *L. carpetanus* were collected in the Spanish Central System (GLR9, type23, type25), Northern Iberian System (871, BUR1–2, AHED62, 65, 71–72, LAG1, GLR4–5), Penibaetic System (CARP1), Central Portugal (909) and on the capes of NW Spain (GLR10–11). Studied specimens of *L. glareosus* were also distributed widely across the Iberian Peninsula and occurred in Cantabrian Mountains (GLR1), Northern Iberian System (GLR6), Spanish Central System (GLR7), Penibaetic System (873, GLR2, GLR3) and in Alicante on lower altitudes (GLR8).

Habitats of two species are similar with slight differences. Habitats of *L. carpetanus*: altitudes 100–1200 m a.s.l.; substrates: rocky, mainly siliceous or sandy, acid substrates; vegetation: grasslands (often mountain meadows), pastures, dry fields, coastal cliffs, forests (especially pine and oak), bushes, thyme communities. A photograph of *L. carpetanus* plants in their natural habitat in pine forest is presented in Fig. 7. Habitats of *L. glareosus*: altitudes 650–1800 m a.s.l.; substrates: schists, screes, rocky places, calcareous or siliceous substrates; vegetation: grasslands (usually wet) and pastures.

Discussion

Analyses of studied *Lotus* material from the Iberian Peninsula based on various types of data (i.e. sequences of nrITS, nrETS and cpDNA *trn*L-F, nuclear microsatellites) confirmed that it is subdivided into two genetically very distant groups: *L. carpetanus* and members of *L. corniculatus* complex (i.e. *L. glareosus*, *L. glacialis*, *L. alpinus*, *L. delortii*). Morphologic analysis more or less supports this conclusion, however determination of specimens based on morphology only is rather difficult and should be confirmed by sequencing of marker DNA regions. These groups can be interpreted as cryptic species sensu Bickford et al. (2007), i. e. lines that are distinct by molecular and other data, but were erroneously classified within one species due to morphological similarity.

Microsatellite data obtained in the present study let to assume that *L. carpetanus* is a diploid taxon. The combination of molecular phylogenetic data obtained in this study and chromosome numbers directly counted in the GLR10 and GLR11 samples of *Lotus carpetanus* (Lago Canzobre & Castroviejo 1992) allowed us to conclude that this species has chromosome number 2n = 10.

Figure 7. Plants of *Lotus carpetanus* in their natural habitat, pine forest (specimen LAG1, SPAIN, Soria: ca. 4 km S from Laguna Negra de Urbión, 08.VI.2018, T. Kramina & L. Koppel s.n. [MW]). Image: L. Koppel.

We did not conduct molecular analyses of any type specimens of *L. glareosus* Boiss. & Reuter, because of rather ancient collection dates (1837 and 1849). Morphological features of studied type specimens allowed to suggest that they all belong to *L. glareosus*. The calyx teeth shape in the type specimens of *L. glareosus* var. *villosus* [G00020260, G00020261] raised some doubts because of a very wide lower part of the upper tooth. However, these specimens should be assigned formally to *L. glareosus* due to the majority of characters. Specimens collected not far from locus classicus of *L. glareosus* var. *glabrescens* and *L. glareosus* var. *villosus*, i.e. specimens GLR2 and GLR3, belong to *L. glareosus* and not to *L. carpetanus* in morphological and molecular features.

Lotus carpetanus and *L. glareosus* occupy similar habitats with a slight tendency for *L. carpetanus* to prefer drier conditions. The contemporary sympatric distribution of *L. carpetanus* and *L. glareosus* in a combination with no evidence of genetic exchange between them suggests that they represent two deeply diverged lineages that should be treated as two separate species. Even if we accept *L. glareosus* at subspecific rank within *L. corniculatus* (e.g. Valdés 2000), *L. carpetanus* is recommended to be accepted as separate species.

Despite the fact that the composition of the *Lotus corniculatus* complex is interpreted differently, there are at least two more examples of the inclusion of obviously more distant representatives in this complex. The first case concerns *L. uliginosus* – *L. pedunculatus* group, which was assigned to the *L. corniculatus* complex by some authors (e.g. Ball & Chrtková-Žertová 1968; Greuter et al. 1989), but excluded from it by others (e.g. Heyn 1970a; Valdés 2000). Molecular evidences based on both nuclear and plastid DNA markers supported the second opinion (Kramina

et al. 2016). However, hybridization supposed between *L. corniculatus* and *L. pedunculatus* / *L. uliginosus* (Valdés 2000) cannot be ruled out and sometimes was confirmed by molecular data (Kramina et al. 2018). The second example concerns the relationships of the species *Lotus palustris* Willd., which was included either in *L. corniculatus* group (Ball & Chrtková-Žertová 1968) or in *L. angustissimus* group (Heyn 1970b; Kramina 2006; Pina & Valdés 2009). As in the previous case, molecular phylogenetic data clearly confirmed more distant relations between *L. palustris* and *L. corniculatus* complex (Kramina et al. 2016).

Similarly, *L. carpetanus* is apparently not a member of the *Lotus corniculatus* complex. To estimate the time of divergence event between [*L. carpetanus* plus *L. conimbricensis*] clade and *L. corniculatus* clade, a dated phylogenetic analysis is needed and that is the task for future studies.

Taxonomic conclusion

The plant material from the Iberian Peninsula earlier determined as '*L. glareosus*' belong to two separate species:

***Lotus carpetanus* Lacaita**

1928, in Cavanillesia 1: 10. – *L. corniculatus* ssp. *carpetanus* (Lacaita) Rivas Mart., 1964, in Anales Inst. Bot. Cavanilles 21: 240. – *L. corniculatus* var. *carpetanus* (Lacaita) Castrov., 1982 [1981 publ. 1982], Anales Jard. Bot. Madrid 38(2): 509.

Lectotypus: Cercedilla at the foot of Sierra de Guadarrama in rocky grass (Castile), 10.VI.1923, Lacaita [BM25710]. – designated here by T.E. Kramina.

***Lotus glareosus* Boiss. et Reuter**

1852, Pugillus Pl. Afr. Bor. Hispan.: 36; Ball et Chrtková-Žertová, 1968, Fl. Europ. 2: 174; Fernandes, 1981, Bol. Soc. Broteriana 55: 29–86; Greuter et al. 1989, Med-Checklist, 4: 130.

Lectotypus: Espagne, Sa Nevada reg. media, ad fluv. Monachil, in glareosis, G.F. Reuter s.n., 0.7.1849 [G00020261]. – designated by H.M. Burdet, A. Charpin & F. Jacquemoud (1988) (Burdet et al. 1988).

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Conflict of interest. The authors declare that they have no conflict of interest.

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Appendix 1. Taxa, collection sites and GenBank accession numbers used in this study. Herbarium codes according to Index Herbariorum. This list includes all studied specimens of *Lotus alpinus, L. carpetanus*, *L. conimbricensis*, *L. delortii*, *L. glareosus* and *L. glacialis*. For other species, voucher information is presented only for those samples, for which at least one new sequence has been obtained, for the others Genbank accession numbers are indicated on the trees (Figs 2–4).

***Taxon*, sample code**, locality, voucher information [herbarium code], GenBank accession numbers for ITS, ETS, *trn*L-F; new sequences indicated by an asterisk; m-dash (—) denotes a missing marker.

***Cytisopsis pseudocytisus* (Boiss.) Fertig, 7**, Turkey, C1, Mugla, Datca, Knidos, 29–31.V.1995, A.P. Khokhryakov & M.T. Mazurenko s.n. [MHA], AY325282, KT262790, MK751647*; ***Hammatolobium kremerianum* (Coss.) Müll. Berol., 643**, Morocco, Podlech 51378 [MHA], KT250926, KT262791, MK751648*; ***Lotus aegaeus* Boiss., 427**, Turkey, C3, Antalya Korkuteli, Termesos, Buyukkumluc, Cakilli gecidi, 04.VI.1995, A.P. Khokhryakov & M.T. Mazurenko 1135 [MHA], DQ160276, KT262720, MK751649*; ***Lotus alpinus* (Ser.) Schleich. ex Ramond, 428**, Spain, Soria, Montenegro de Cameros, 28-May-1995, Segura Zubizarreta 43694 [MHA], DQ160274, KT262722, MF158233; **BARR42**, Spain, Burgos, near Barriosuso, 07.VI.2018, T. Kramina & L. Koppel s.n. [MW], MK780138*, —, MK751650*; **MONT84**, **MONT93**, Spain, Soria, near Montenegro de Cameros, 08.VI.2018, T. Kramina & L. Koppel s.n. [MW], MK780139-MK780140*, —, MK751651-MK751652*; ***Lotus broussonetii* Choisy ex Ser., 21**, Cultivated at Royal Botanic Gardens, Kew: introduced from Canary Is., DQ160278, KT262728, MK751653*; ***Lotus carpetanus* Lacaita, 871**, Spain, Burgos, Santibáñez del Val, Barriosuso, 11.VII.1979, A. Pons-Sorolla & A. Susanna 270 (B), KT250873 as *L. glareosus* Boiss. et Reuter, MF314952 as *L. glareosus*; **AHED62**, **AHED65**, **AHED71**, **AHED72**, Spain, Burgos, between La Revilla and Ahedo, 07.VI.2018, T. Kramina & L. Koppel s.n. [MW], MK780141-MK780144*, MN807782-MN807783*, MK695867-MK695870*; **BUR1**, Spain, Burgos, Peñas de Cervera y aledaños, La Revilla, camino de Ahedo, alt. 1000 m, 17.VII.1979, Muños Garmendia, Pons-Sorolla, Sanches Garcia & Susanna 476 [MA 643064], MK780145*, —, MK695871*; **BUR2**, Spain, Burgos, Santibañes del Val, Barriosuso, alt. 1100 m, 11.VII.1979, Pons-Sorolla & Susanna 270 [MA 413052], MK780146*, —, MK695872*; **CARP1**, Spain, circa Igualeja, Sierra de la Nieve, 19.VI.1972, L. Bernardi 13821 [LE], MN795612*, —, —; **GLR4**, Spain, Soria, Santa Inés, Vivero, 1.200 m, 09.VIII.1981, A. Segura Zubizarreta 22.704 [MA 361071], MK780147*, —, MK695873*; **GLR5**, Spain, Soria, Vinuesa, el Bardo, 1.150 m, 04.IX.1974, A. Segura Zubizarreta 14.131 [MA 361140], MK780148*, —, MK695874*; **GLR9**, Spain, Segovia, Santiuste de Pedraza, 1180 m, 22.VI.1985, R. García 1086 [MA 634557], MK780149*, —, MK695875*; **GLR10**, Spain, Lugo, Punta del Roncadoiro, 26.VIII.1987, E. Lago, C. Ferreiro & A. Paz 1290EL [MA 459768], MN795613*, MN807784-MN807785*, MN807793*; **GLR11**, Spain, La Coruña, Punta dos Aguillons, E. Lago, C. Ferreiro & A. Paz 1129EL [MA 459769], MN795614*, MN807786*, MN807794*; **LAG1**, Spain, Soria, ca. 4 km S from Laguna Negra de Urbión, 08.VI.2018, T. Kramina & L. Koppel s.n. [MW], MK780150*, MK695876*; **type23**, Spain, Cercedilla at the foot of Sierra de Guadarrama in rocky grass (Castile), 10.VI.1923, Lacaita [BM 25710], MK780151*, MK695877*; **type25**, Spain, Peña Gudina prope Veguillas (Salamanca), 5.VI.1925, Lacaita [BM 28783], MK780152*, MK695878*; **909**, Portugal, 25 km W of Viseu, Cambarinho, Reserve do Cambarinho, Alt. 500 m, 30.V.1972, Jaakko Jalas 1768 [H 1046639], KT250874 as *L. glareosus*, —, —; ***Lotus conimbricensis* Brot., 485**, Spain, Badajoz,

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MN807790*, MK751675*; **GLR8**, Spain, Alicante: Vall d'Alcala, 650 m, 08.VI.1996, J.X. Soler & M. Signes [MA 587153, MK780163*, MN807791-MN807792*, MK751676*; ***Lotus glinoides* Del., 461**, Egypt, 7.V.1962, Bochantsev s.n. [LE], DQ160282, KT262748, MK751677*; ***Lotus graecus* L., D9**, Turkey, A3, Bolu, Düzce-Akçakoca, 24.V.1990, R. Lampinen 7871 [H], KT250876, KT262749, MK751678*; **D10**, Greece, East Macedonia, Thasos, Glifada, 18.V.1986, T. Raithalme s.n. [H], KT250877, KT262750, MK751679*; ***Lotus halophilus* Boiss. & Spruner, 431**, Greece, Karpathos, Pigadia, 19.IV.1984, Th. Raus 9307 [MHA], KT250879, KT262753, MK751680*; ***Lotus herbaceus* (Vill.) Jauzein, D6**, Austria, Steirisches Hügelland, Steiermark, Umgebung von Radkersburg, 7.VII.1976, H. Mayrhofer & H. Teppner s.n. [H], KT250882, KT262755, MK751681*; ***Lotus herbaceus* (Vill.) Jauzein ssp. *gracilis* (Jord.) Jauzein, D8**, France, dép. Pyrénées-Orientales, Canet, 02.VII.1981, J. Lambinon, R. Renard & L. Smeets 81/287 [H], KT250859, KT262737, MK751682*; ***Lotus hirsutus* L., 609**, Spain, VII.2006, Beer & Beer s.n. [MW], KT250886, KT262759, MK751683*; **D12**, Greece, East Macedonia, Thasos, Glifada, 19.V.1986, T. Raithalme s.n. [H], KT250885, KT262758, MK751684*; **D13**, Croatia, Korčula island, SW of Pupnat, 23.VI.1971, L. Hämet-Ahti 2225 [H], KT250884, KT262757, MK751685*; ***Lotus lalambensis* Schweinf., 449**, Saudi Arabia, East of Rabigh, 100 km SW of Akhal, 21.II.1992, I.S. Collenette 7908 [E], DQ166216, KT262761, MK751686*; ***Lotus laricus* Rech. f., Aellen & Esfand., 455**, Abu Dhabi, Abu Dhabi Island. Abandoned camel stables in Al Mushrif Palaca, 04.V.1982, R.A. Western 275 [E], DQ166233, KT262763, MK751687*; ***Lotus maculatus* Breitf., 958**, Canary Is. (cult.), Tenerif. Municipio de la Orotava, Puerto de la Cruz, 14.IV.2000, H. Väre 10894 & H. Kaipiainen [H 1702795], KT250890, KT262764, MK751688*; ***Lotus mlanjeanus* J.B. Gillett, 450**, Malawi, Southern Region, Mt. Mulanie, Muloza River between Madzeka and Nayawani Shelf, 20. VIII.1987, J.D. Chapman & E.G. Chapman 8807 [E], DQ166232, KT262765, MK751689*; ***Lotus ononopsis* Balf. f., 453**, Yemen, Muqadrihon Pass, c. 10 km SW of Hadiboh, 26.I.1990, A.G. Miller et al. 10097 [E], DQ166219, KT262766, MK751690*; ***Lotus polyphyllus* Clarke, 438**, Egypt, Ras El Hekma, 6.IV.1962, Bochantsev s.n. [LE], DQ160289, KT262768, MK751691*; ***Lotus quinatus* (Forssk.) J.B. Gillett, 451**, Yemen, 45 km from Taizz along road to Ibb, 15.XI.1995, M. Thulin, M. Ghebrehiwet & A.N. Girfi 9374 [E], DQ166217, KT262771, MK751692*; ***Lotus rectus* L., REC1**, Spain, Alicante, Rio Guadalest, 02.VII.1958, A. Rigual s.n. [MA 373077], MK780164*, —, MK751693*; **REC2**, Spain, Castelló, Burriana, PNM "El Clot de la Mare de Déu", 04.V.2006, R. Roselló Gimeno s.n. [MA 741964], MK780165*, —, MK751694*; ***Lotus simonae* Maire, Weiller & Wilczek, 412**, Morocco, 5°48' W, 30°20' N, 10.IV.1990, D. Podlech 49444 [M], DQ160285, KT262776, MK751695*; ***Lotus tetragonolobus* L., 624**, Cyprus, to E from Limassol, Amathus, 08.III.2004, A. Seregin et al. A-110 [MW], HM468334, KT262784, MK751696*; ***Lotus wildii* J.B. Gillett, 452**, Zimbabwe, Inyanga, Eastern Highlands, 11.X.1984, R.D. Bayliss 110166 [E], DQ160287, KT262789, MK751697*; ***Tripodion tetraphyllum* (L.) Fourr., 625**, Cyprus, 7.5 km to N from Limassol, 11.III.2004, A. Seregin & D. Sokoloff A-240 [MW], HM468340, MN832851*, MK751698*.