

## New data on nrITS phylogeny of *Lotus* (Leguminosae, Loteae)

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*Summary:* Some new nrITS sequences of *Lotus* are produced and added to the data set analysed in DEGTJAREVA et al. (2006). *Lotus burttii* and *L. filicaulis* are revealed as members of the /*Lotus corniculatus* clade. *Lotus conimbricensis* is found to be sister to the entire /*Lotus corniculatus* clade; the /*Lotus pedunculatus* clade is more distantly related. The New Caledonian *Lotus anfractuosus* is closest among species sampled to two Australian endemics, though its molecular divergence is considerable. The NE African *Lotus torulosus* is close to some other red-flowered species from the same region; it does not group with any other *Lotus* species with dimorphic leaflets. In general, dimorphic vs. monomorphic leaflets is a quite homoplastic character in *Lotus*. Molecular divergence is weak within the /*Pedrosia* clade, where the morphological divergence is especially high. In contrast, molecular divergence is considerable but morphological differentiation is weak in the /*Lotus corniculatus* clade.

*Keywords:* Leguminosae, Loteae, *Lotus*, biogeography, nuclear ribosomal ITS sequences, morphology, phylogeny

The genus *Lotus* includes about 120–130 species native to Europe, Asia, Africa, Australia and some islands of Atlantic Ocean (many endemics in Macaronesia), Pacific Ocean (two endemic species in Ryukyu, Taiwan, New Caledonia, Vanuatu) and Socotra archipelago (two endemic species) in the Indian Ocean. All native New World species formerly placed in *Lotus* are now segregated in four (e.g. ARAMBARRI et al. 2005; SOKOLOFF & LOCK 2005; SOKOLOFF et al. 2007) or two (BROUILLET 2008) distinct genera. In the Old World, three monotypic segregate genera are accepted (*Kebirita*, *Podolotus* and *Pseudolotus*), while two commonly recognized genera (*Dorycnium* and *Tetragonolobus*) are placed in synonymy of *Lotus* (reviewed in DEGTJAREVA et al. 2006).

Molecular phylogenetic studies of *Lotus* were mainly concentrated on analyses of nrITS sequences (ALLAN & PORTER 2000; ALLAN et al. 2003, 2004; DEGTJAREVA et al. 2006). To-date, nrITS sequences of more than 80 *Lotus* species are published; they represent most traditionally recognized sections and species groups of the genus. This makes *Lotus* one of relatively well-explored legume genera with respect of nrITS phylogeny. Nevertheless, several critical species are not sampled so far, and phylogenetic placement of some other species is intriguing. Therefore, we produced new nrITS sequences for some *Lotus* species.

### Material and Methods

Most nrITS sequences used for phylogenetic analysis and all methods, respectively are the same as described in DEGTJAREVA et al. (2006). Information on new sequences is shown in Table 1. In addition, a GenBank sequence AY294302 of *Lotus mascaënsis* (referred here as 2) is used (first published by ALLAN et al. 2004).

Table 1: Voucher information and GenBank accession numbers of new nrITS sequences.

| Species   | Voucher  | GenBank number |
|---|--|----------------|
| <i>Lotus anfractuusus</i> (Bak. f.)<br>Kramina & D. D. Sokoloff | New Caledonia, Loyalty Is., Mare,<br>18.07.1951, <i>Baumann-Bodenheim</i> (Z)  | FJ411111       |
| <i>Lotus arinagensis</i> Bramwell                               | Canary Is., Gran Canaria, Punta Arinaga,<br>24.03.1996, <i>Royle 778</i> (B)   | FJ411112       |
| <i>Lotus burttii</i> Borsos                                     | B-303 (inbred line, plants grown from seeds<br>collected on 03.07.2006 by N. Sandal;<br>the origin of the seeds for establishment<br>of the inbred line: Kabul River, Peshawar,<br>Pakistan) | FJ411113       |
| <i>Lotus conimbricensis</i> Brot. (1)                           | Spain, 27.04.1966, <i>Segura Zubizarreta</i><br><i>960</i> (Z)   | FJ411114       |
| <i>Lotus conimbricensis</i> Brot. (2)                           | Israel, 19.04.1957, <i>Lorch &amp; Grizi 644</i> (Z)   | FJ411115       |
| <i>Lotus filicaulis</i> Durieu                                  | B37 (inbred line, plants grown from seeds<br>collected on 18.09.2002 by N. Sandal;<br>the origin of the seeds for establishment of the<br>inbred line: Algeria, W. F. Grant's collection)    | FJ411116       |
| <i>Lotus loweanus</i> Webb & Berthel.                           | Madeira, Porto Santo, 08.03.1985, <i>Luck</i><br><i>MD 33</i> (B)  | FJ411117       |
| <i>Lotus mascaënsis</i> Burchard (1)                            | Canary Is., Tenerife, Masca, 19.03.1985,<br><i>J.J. &amp; W. Greuter 286</i> (B)   | FJ411118       |
| <i>Lotus tetraphyllus</i> Murr.                                 | Baleares, Mallorca, Torrent de Sa Coma de<br>S'Arrom, 10.05.1986, <i>Orell 52</i> (B)  | FJ411119       |
| <i>Lotus torulosus</i> (Chiov.) Fiori                           | cultivated at the Botanical Garden of<br>Moscow University, 2006   | FJ411120       |

## Results and Discussion

The length of the ITS region (ITS1, 5.8S and ITS2) ranges from 587 to 617 bp for the 108 accessions of the ingroup (i.e. members of the tribe Loteae) and two outgroup (i.e., *Robinia* and *Sesbania*) taxa studied. In the 10 new generated sequences of *Lotus*, the length of the ITS ranges from 588 to 617 bp. The alignment of 110 ITS sequences resulted in a matrix of 599 nucleotide positions after excluding 256 ambiguous positions. Of the remaining sites, 262 characters were parsimony-informative, 82 characters were parsimony-uninformative and 255 were constant.

Maximum parsimony analyses yielded 73000 shortest trees (1496 steps), with a consistency index of 0.4 and a retention index of 0.75. The strict consensus tree based on all shortest trees does not differ significantly in topology from the bootstrap consensus tree (Figs. 1, 2). The topology of the Bayesian tree (Fig. 3) is generally similar to those of the trees inferred from the maximum

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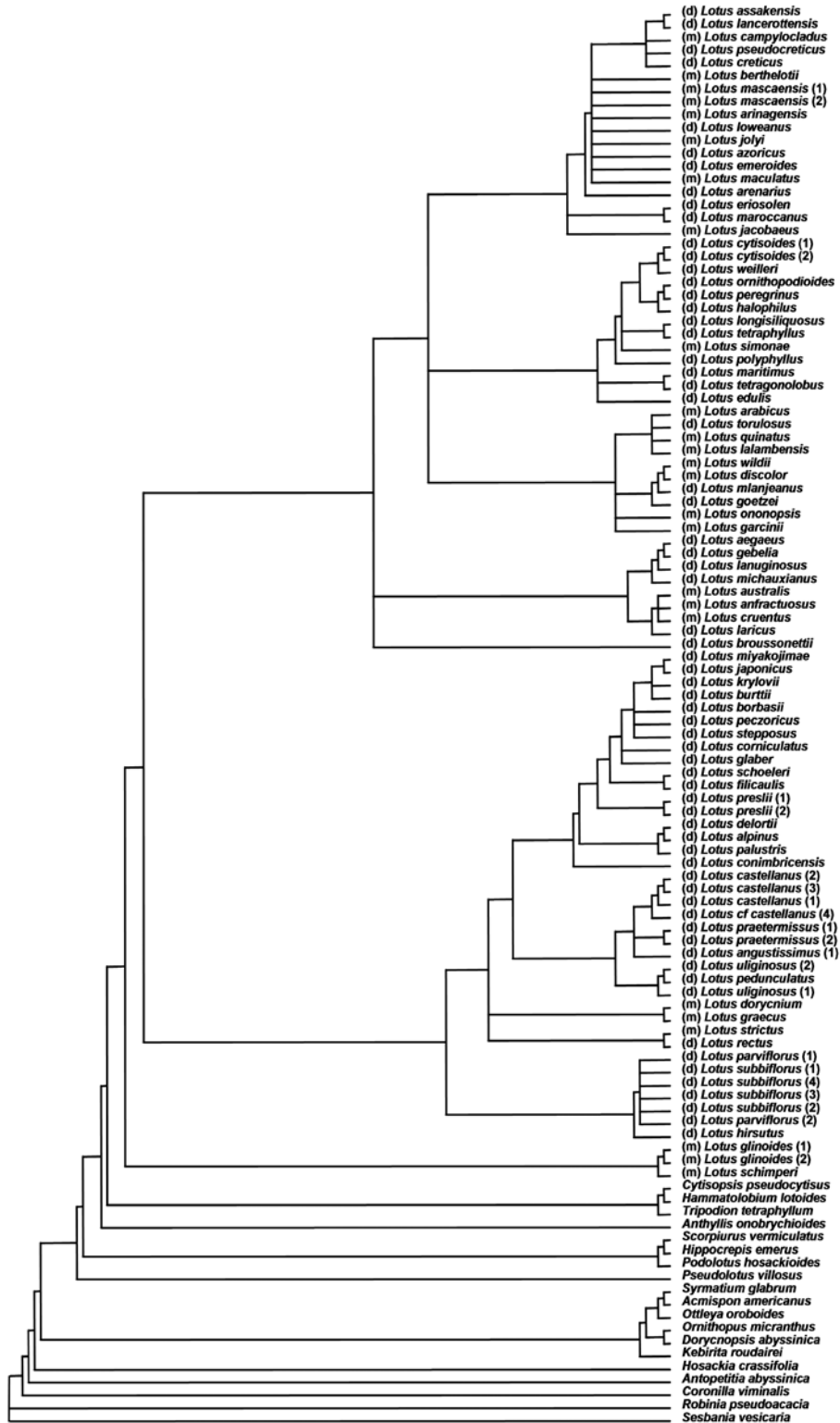


Figure 1: Strict consensus of most parsimonious trees with leaf morphology indicated for *Lotus* species: (m) = leaflets of a leaf are (almost) monomorphic, (d) = leaflets of a leaf are markedly dimorphic.

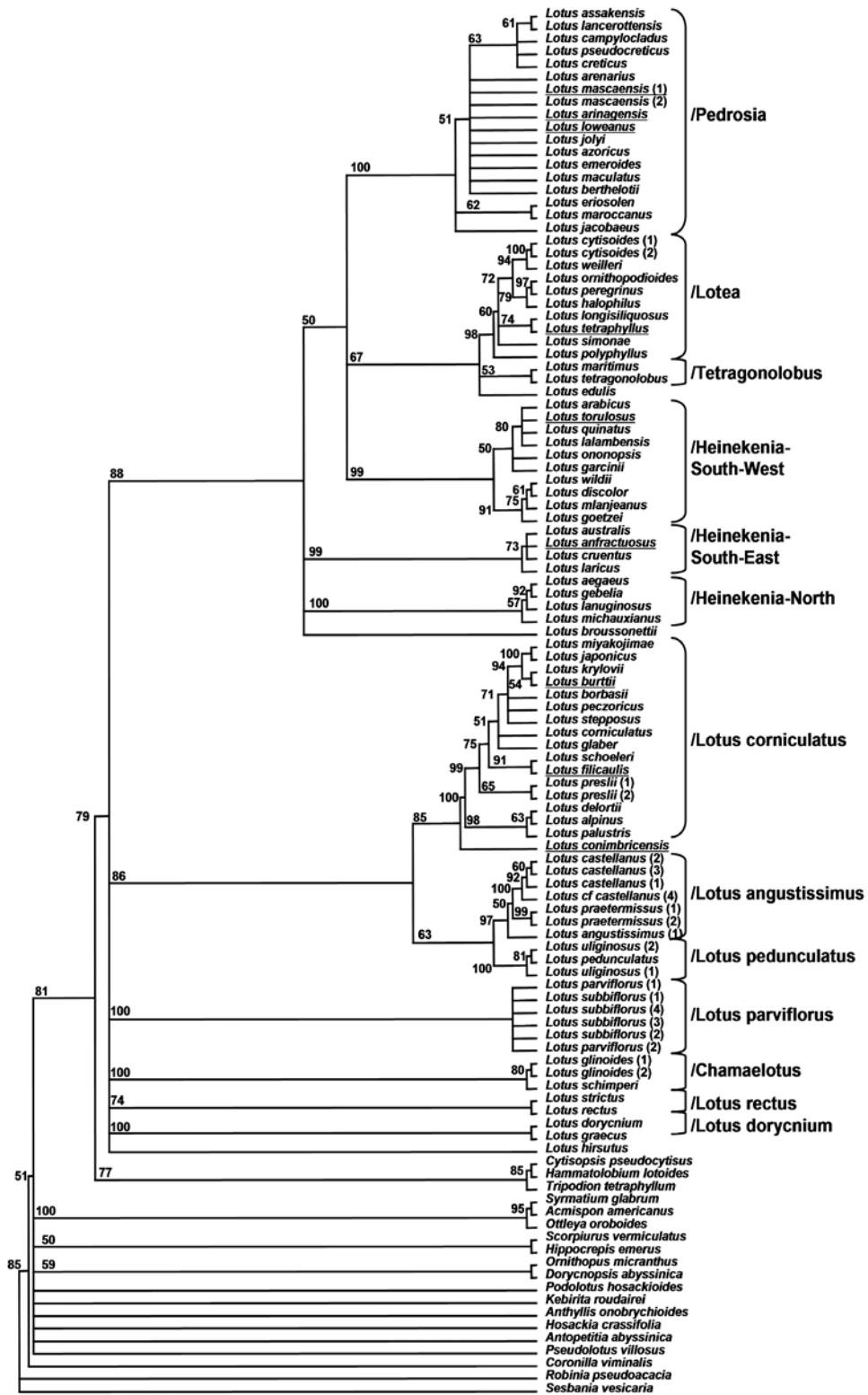


Figure 2: Majority-rule bootstrap consensus tree inferred from the maximum parsimony analysis. Numbers indicate bootstrap support of clades. New nrITS accessions are underlined. Clade names used in the text are outlined.

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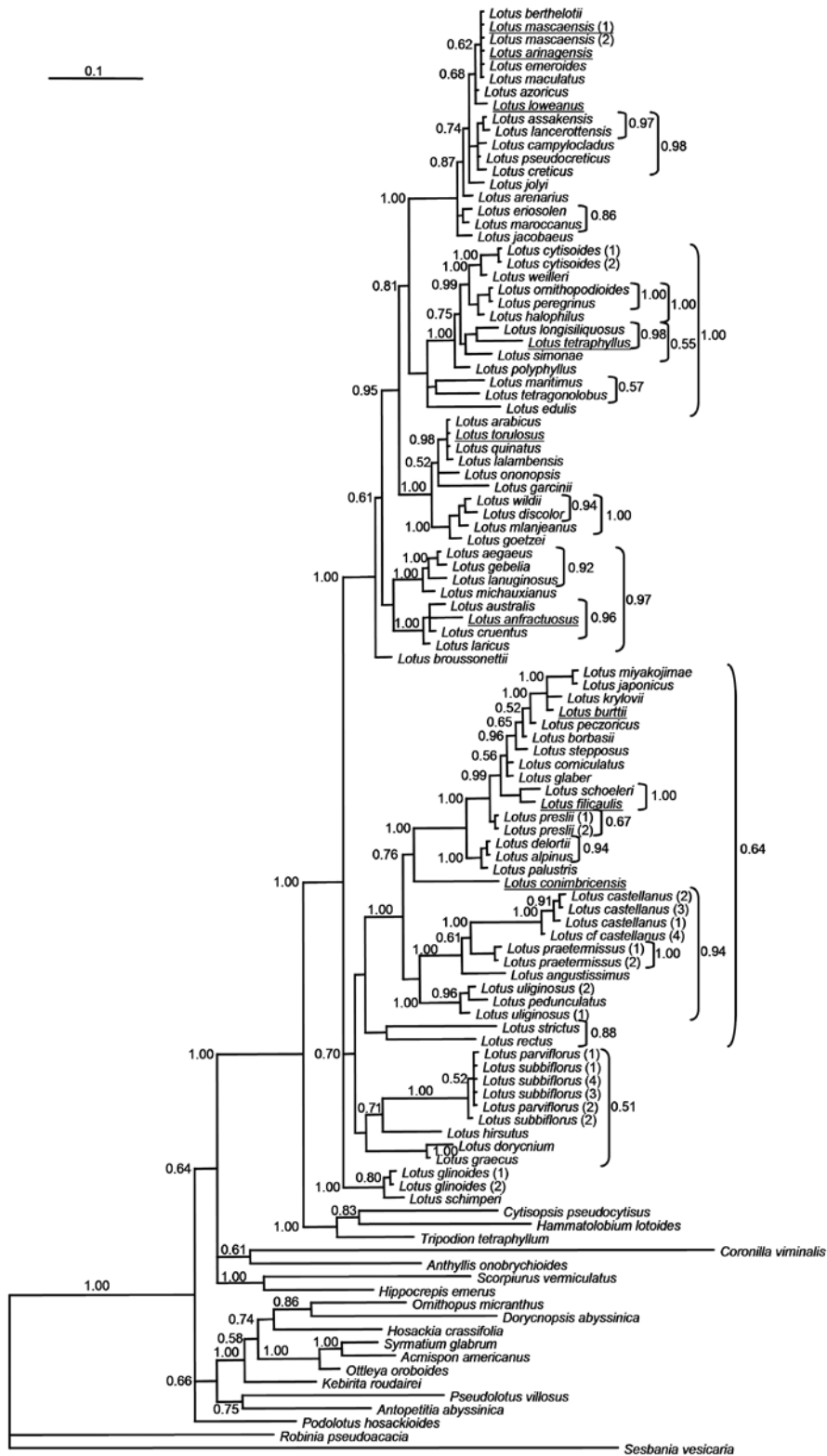


Figure 3: Bayesian tree. Numbers indicate posterior probabilities of particular clades. Nodes with posterior probabilities less than 0.5 are shown as unresolved. Scale = 0.1 substitution/site. New nrITS accessions are underlined.

parsimony analyses. Adding new sequences to the data set analyzed by DEGTJAREVA et al. (2006) does not bring about significant changes of the overall tree topology. The general pattern of phylogenetic relationships within *Lotus* is still the same as revealed by DEGTJAREVA et al. (2006). Therefore, we discuss below details of relationships within some clades.

***Lotus corniculatus* clade.** The clade comprising the tetraploid *Lotus corniculatus* and its closest predominantly diploid relatives (*L. palustris*, *L. alpinus*, *L. delortii*, *L. preslii*, *L. schoelleri*, *L. glaber*, *L. stepposus*, *L. peczoricus*, *L. borbasii*, *L. krylovii*, *L. japonicus*, *L. miyakojimae*) was found to be highly supported in the molecular phylogenetic analysis by DEGTJAREVA et al. (2006). This study added to this clade two more species, namely *L. burttii* and *L. filicaulis*. The chromosome number of both species is the same,  $2n = 12$  (SZ.-BORSOS et al. 1972; CHENG & GRANT 1973).

*Lotus filicaulis* Dur. was described in 1846 from coastal sandy places in Oran and limestone slopes in Mascara (Mediterranean coast in northwestern Algeria). It is characterized by filiform stems and long peduncles, about ten times longer than subtending leaves (BRAND 1898). BRAND considered it as a variety of *Lotus corniculatus* (*L. corniculatus* L. var. *filicaulis* (Durieu) Brand).

*Lotus burttii* Borsos was described in 1972 on the basis of plants grown from seeds collected by Dr. B. L. Burtt (Royal Botanic Garden, Edinburgh) on the 3<sup>rd</sup> May 1958 from plants on the bank of Kabul River, Peshawar, West Pakistan (SZ.-BORSOS et al. 1972). Dr. Burtt identified those plants as *L. corniculatus* L. var. *minor* Bak. The plants of *L. burttii* are small, almost glabrous annual herbs with prostrate or ascending shoots, oblanceolate upper leaflets of a leaf and one- or, rarely, two-flowered umbels. The flowers are small, 7–9 mm long, with primrose to pale pink petals. They are also characterized by non-shattering behavior of pods (KAWAGUCHI et al. 2005).

Our observations on the plants of inbred lines of *L. burttii* B-303 (KAWAGUCHI et al. 2005) and *L. filicaulis* Dur. B-37 (GRANT et al. 1962) did not reveal deep morphological differences between them. Plants of *L. filicaulis* possessed slightly longer peduncles and leaflets than *L. burttii* plants, but the number of flowers in umbel was similar in two species. The petal colour seems to be a variable character in the *Lotus corniculatus* group. We observed pink petal colour more often in *L. burttii* than in *L. filicaulis*, but this character needs a more detailed investigation.

Very complicated relationships among diploid species of *Lotus corniculatus* complex were demonstrated in a series of experimental works carried out by W.F. Grant and collaborators. SOMAROO & GRANT (1971) reported that diploid and self-fertile species such as *L. burttii*, *L. alpinus*, *L. krylovii*, *L. filicaulis*, *L. japonicus*, and *L. schoelleri* could cross more or less with each other in artificial conditions and produce F1 hybrids. The success of crossing was counted as a percent of developed pods from the total number of pollinated flowers. Pollen fertility in hybrids was also analysed. It was shown that species with close geographical distribution crossed worse than species distributed distantly from each other. For example, crosses *L. schoelleri* × *L. burttii* (and the reciprocal combination) were unsuccessful, crosses *L. schoelleri* × *L. filicaulis* had 14% success and pollen fertility was 17% or less. *L. filicaulis* could cross as a male parent with *L. burttii* with 50% success, pollen fertility in this hybrid was 32%. *L. krylovii* was crossed as a female parent with *L. filicaulis*, *L. schoelleri*, and *L. burttii* with success of 41%, 37%, and 10%, respectively. The most geographically distant species *L. japonicus* (as a female parent) produced hybrids with other diploid species more successfully, thus F1 hybrids between *L. japonicus* and

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*L. filicaulis*, *L. schoelleri*, *L. burttii* and *L. krylovii* had 57%, 40%, 10–30% and 13% success, respectively (SOMAROO & GRANT 1971), which is negatively correlated with geographical distance between type localities of these species.

Both *L. filicaulis* and *L. burttii* were used as crossing partners to *L. japonicus* in experimental studies aimed to a construction of chromosomal and genetic linkage maps of the model legume *L. japonicus* (PEDROSA et al. 2002; SANDAL et al. 2002; KAWAGUCHI et al. 2005). The occurrence of inversions on chromosomes 1 and 3 between the closely related species *L. filicaulis* and *L. japonicus* was demonstrated, suggesting that these chromosomal rearrangements are early events in speciation of this group (PEDROSA et al. 2002). Besides, *L. filicaulis* has a very high level of DNA polymorphism by AFLP (amplified fragment length polymorphism) analysis (up to 49%) (SANDAL et al. 2002). The general chromosome morphology and genome size of *L. burttii* are similar to those of *L. japonicus*. The translocation event, involving chromosomes 1 and 2, which was observed in *L. miyakojimae*, is not present in *L. burttii*. However, the long arm of chromosome 1 of *L. burttii* was altered in comparison to that of *L. japonicus*. The close proximity between two BAC clones on the long arm of *L. burttii* chromosome 1 is similar to the position of these clones in *L. filicaulis*. This observation may suggest that those clones had been in close proximity in the ancestor of the two species and an inversion in the long arm of chromosome 1 might have occurred during the speciation of *L. japonicus* (KAWAGUCHI et al. 2005).

Thus, *L. burttii* and *L. filicaulis* are morphologically rather similar species showing small morphological differences, however, some chromosomal rearrangements exist between them. In phylogenetic trees presented in this paper, *L. filicaulis* is grouped with *L. schoelleri*, and *L. burttii* is placed in a subclade with *L. krylovii*, *L. japonicus* and *L. miyakojimae*. This grouping is well corresponding with geographical distribution of the species, i.e. closely distributed species are clustered together.

Generally, recognition of the highly supported (100% bootstrap support in the parsimony analysis) *Lotus corniculatus* clade does not contradict traditional views and morphological data. All species that belong to this clade have been traditionally classified within the section *Lotus*. The section, in its traditional circumscription (e.g. BALL & CHRTKOVÁ-ŽERTOVÁ 1968; KRAMINA 1999a; see also DEGTJAREVA et al. 2006) comprises both annuals and perennials. Most members of the *Lotus corniculatus* clade are perennials or plants that can be either annuals or perennials. In the classification of KRAMINA (1999b) other perennial members of the section *Lotus* are *L. pedunculatus*, *L. uliginosus*, and *L. granadensis*. Morphologically, these three species are closely related to each other and known as a *Lotus pedunculatus* group. Members of this group have subterranean shoots (stolons) with reduced scaly leaves. In contrast, members of the *Lotus corniculatus* clade, with very rare exceptions, do not have subterranean shoots with reduced leaves. Species of *L. uliginosus* group occur in wet and swampy places, which is not typical to species of *L. corniculatus* group. *L. uliginosus* and related species are characterized by a series of morphological, cytological and biochemical distinctions from *L. corniculatus* group. They have hollow stems, long ciliate trichomes on calyx teeth, small usually one-coloured seeds. It was shown that two pairs of chromosomes of *L. pedunculatus* possessed satellites (ZANDSTRA & GRANT 1968). This species differs from *L. corniculatus*, *L. krylovii* and their relatives by phenolic compounds (ZANDSTRA & GRANT 1968) and isoenzymes of leaves and pollen grains (RAELSON & GRANT 1988, 1989). Molecular phylogenetic data show that the *Lotus pedunculatus* group (two of three species are

studied so far – the /*Lotus pedunculatus* clade) is not sister to the /*Lotus corniculatus* clade; instead, the *Lotus pedunculatus* group appears to be sister to a clade of three annual members of the section *Lotus*, namely the /*Lotus angustissimus* clade (DEGTJAREVA et al. 2006; this study). This suggests a complex picture of evolution of life form in *Lotus*.

**The *Lotus angustissimus* complex and *Lotus conimbricensis*.** The *Lotus angustissimus* complex, as traditionally circumscribed, comprises a group of morphologically similar species of the section *Lotus*. The plants are mostly annual, sometimes biennial or rarely perennial. Morphological diagnostic characters of different species are overlapping, and their identification is sometimes problematic (KRAMINA 2006). Nevertheless, (mostly) annuals of the section *Lotus* do not form a clade on molecular trees. They form two clades that are not closely related: the /*Lotus angustissimus* clade and the /*Lotus parviflorus* clade. In the previous analysis (DEGTJAREVA et al. 2006), we used a GenBank accession of *L. conimbricensis*, and this species was found to be a member of the /*Lotus parviflorus* clade (together with *L. parviflorus* and *L. subbiflorus*). In the current analysis, we used two new sequences of *L. conimbricensis* based on different vouchers. Both new sequences were identical to each other but quite different from the GenBank sequence. In the current phylogeny, *L. conimbricensis* does not group with *L. parviflorus* and *L. subbiflorus*. Instead, it is sister to the /*Lotus corniculatus* clade.

Traditionally, *L. conimbricensis* was classified within the section *Erythrolotus*. As defined by BRAND (1898), the section *Erythrolotus* is characterized by red (to white) flowers. Members of the section *Lotus* have predominantly yellow flowers. KRAMINA & SOKOLOFF (2003) re-defined the section *Erythrolotus* as comprising only *L. conimbricensis* and placed other red-flowered species to the section *Heinekenia*. The presence of the basic chromosome number  $x=6$  (instead of  $x=7$ ) is an obvious synapomorphy for members of the section *Lotus* plus *L. conimbricensis*; besides, data on biogeography and hair morphology agree with a placement of *L. conimbricensis* among members of the section *Lotus* (see DEGTJAREVA et al. 2006).

Grouping of *L. conimbricensis* with *L. parviflorus* and *L. subbiflorus* agreed with the fact that all three species are annual plants (*L. subbiflorus* is annual to perennial). However, it was curious that *L. conimbricensis* has probably the greatest fruit length/width ratio in the genus *Lotus*, while the fruits of *L. parviflorus* and *L. subbiflorus* are, instead, short. *Lotus parviflorus* is one of few species with shortest fruits in the genus *Lotus*. The current tree topology is more congruent with fruit morphology. Interestingly, the /*Lotus parviflorus* clade shows weak association with *L. hirsutus*, a species formerly classified as a member of the genus *Dorycnium*. Other former members of the genus *Dorycnium* (*L. dorycnium*, *L. rectus*, *L. graecus*) are in an unresolved position close to the /*Lotus parviflorus* clade. One of the features used to characterize *Dorycnium* was fruit length: the fruits are generally short in *Dorycnium* and sometimes 1-seeded (*L. dorycnium*). SERINGE (1825) placed both *L. subbiflorus* and *L. parviflorus*, apparently on the basis of short fruits, in the genus *Dorycnium*, a view that was rejected by almost all subsequent authors. The current molecular data show that the SERINGE'S view was not completely misleading.

**/Heinekenia clades.** Members of the section *Heinekenia*, as defined by KRAMINA & SOKOLOFF (2003) belong to three highly supported clades. Relationships between these clades and some other clades of *Lotus* are unresolved. As found by DEGTJAREVA et al. (2006) phylogenetic placement of members of the section *Heinekenia* agrees with pattern of geographical distribution. For this



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study, we added two more species (*L. anfractuosus* and *L. torulosus*), and their placement supports the earlier view.

The /Heinekenia-North clade includes *L. aegaeus*, *L. gebelia*, *L. lanuginosus*, *L. michauxianus*. These species occur mainly in the south-western part of the Irano-Turanian floristic region (sensu TAKHTAJAN 1978) and partly in the Arabian peninsula and in the Mediterranean region.

The /Heinekenia-South-East clade comprises *L. laricus* (along around the Persian Gulf to Pakistan), two Australian species (*L. australis* and *L. cruentus*) and the newly sequenced *L. anfractuosus* from New Caledonia. The morphologically close *L. taitungensis* from Japan and Taiwan may also belong to this clade, but no molecular data are available.

The members of the /Heinekenia-South-East clade are characterized by an unique deletion (Fig. 4). All members of this clade have very short ITS sequences (588 bp). Some other species of *Lotus*, such as *L. dorycnium*, *L. garcinii*, *L. graecus* also possess short ITS sequences (587–588 bp), but these species have other deletions, non homologous to the deletion in the /Heinekenia-South-East clade.

The /Heinekenia-South-West clade is most diverse and comprises the rest of the members of the section *Heinekenia* sequenced so far plus two members of the traditional section *Ononidium* (*L. ononopsis*, *L. garcinii*). The members of this clade occur in Africa, except its Mediterranean part, and in the western and southern part of the Arabian peninsula. Placement of *L. torulosus* (Djibouti, Ethiopia, Eritrea, Sudan) within this clade further supports importance of the biogeographic pattern found by DEGTJAREVA et al. (2006). On the basis of morphology, *L. torulosus* was thought to be relatively close to *L. laricus*, in particular because in both species basal leaflets of a leaf differ in shape from the terminal leaflets and have their maximum length in the basal part. In our phylogenetic trees *L. torulosus* groups with *L. arabicus*, *L. quinatus* and *L. lalambensis*. These three species (especially *L. quinatus*) are close to *L. torulosus* in terms of biogeography, but differ in leaf morphology (all five leaflets of about the same shape). In general, species of *Lotus* with dimorphic leaflets do not form a clade in our phylogeny. Rather, they are scattered and ‘mixed’ with species

## ITS2

|                           |                                    |
|---------------------------|------------------------------------|
| <b>Lotus arabicus</b>     | CCAACGTCAAACGCTCTAGTGCAATGTGGCGTGT |
| <b>Lotus torulosus</b>    | CCAACGTCAAACGCTCTAGTGCAATGTGGCGTGT |
| <b>Lotus quinatus</b>     | CCAACGTCAAACGCTCTAGTGCAATGTGGCGTGT |
| <b>Lotus lalambensis</b>  | CCAACGTCAAACGCTCTAGTGCAATGTGGCGTGT |
| <b>Lotus ononopsis</b>    | CCAACGTCAAACGCTTTAGTGCAATGTGGCGTGT |
| <b>Lotus garcinii</b>     | CCAACGTCAAACGCTCTAGTGCAATGTGGCGTGT |
| <b>Lotus wildii</b>       | CCAACGTCAAACGCTCTAGTGCAATGTGGCGTGT |
| <b>Lotus discolor</b>     | CCAACGTCAAATGTCTAGTGCAATGTGGCGTGT  |
| <b>Lotus mlanjeanus</b>   | CCAACGTCAAACGCTCTAGTGCAATGTGGCGTGT |
| <b>Lotus goetzei</b>      | CCAACGTCAAACGCTCTAGTGCAATGTGGCGTGT |
| <b>Lotus australis</b>    | CCAATGCCAAT-----ATGGCGTGT          |
| <b>Lotus anfractuosus</b> | CCGATGCCAAT-----ATGGCGTGT          |
| <b>Lotus cruentus</b>     | CCAATGCCAAT-----ATGGCGTGT          |
| <b>Lotus laricus</b>      | CCAATGCCAAT-----ATGGCGTGT          |
| <b>Lotus aegaeus</b>      | CCAACGCCAAACGCCTAGTGCAATGTGGCGTGT  |
| <b>Lotus gebelia</b>      | CCAACGCCAAACGCCTAGTGCAATGTGGCGTGT  |
| <b>Lotus lanuginosus</b>  | CCAACGCCAAATGCCTAGTGTAATGTGGCGTGT  |
| <b>Lotus michauxianus</b> | CCAACGCCAAACGCCTAGTGCAATGTGGCGTGT  |

Figure 4: A portion of the alignment showing a deletion that characterized the /Heinekenia-South-East clade.

having monomorphic leaflets (Fig. 1). At the higher level, a tendency to have dimorphic leaflets is a very important feature of the genus *Lotus*. However, this character is quite homoplastic within *Lotus* and does not represent an unequivocal synapomorphy of the genus.

**/Lotea clade.** This clade was found by DEGTJAREVA et al. (2006) as including all members of the section *Lotea* (except *L. creticus*) plus *L. simonae*. The present analysis added to this clade *L. tetraphyllus*. Traditionally, *L. tetraphyllus* has been classified within a monospecific section *Quadrifolium* (BRAND 1898; BALL & CHRŤKOVÁ-ŽERTOVÁ 1968; VALDÉS 2000). Our data suggest that *L. tetraphyllus* can be placed within the section *Lotea*. It seems that presence of four rather than five leaflets is the only significant morphological difference between sections *Lotea* and *Quadrifolium*. BRAND (1898) described the section *Quadrifolium* within the framework of his general ideas on classification of *Lotus*. Since the vast majority of *Lotus* species constantly possess five leaflets, BRAND (1898) considered this character as an important one and therefore segregated species of his subgenus *Edentolotus* with leaflet numbers other than five into two separate sections. All four species with three leaflets that were known at the time of BRAND (1898) formed the section *Ononidium*, while the only species with four leaflets (*L. tetraphyllus*) formed the section *Quadrifolium*. Molecular data showed that *Lotus* species with three leaflets do not form a natural monophyletic group. Two species constantly showing presence of three leaflets studied so far (*Lotus ononopsis* and *L. simonae*) do not form a monophyletic group (DEGTJAREVA et al. 2006). Besides, leaflet number varies in some *Lotus* species (3–5 in *L. brunneri*, *L. jacobaeus*, *L. garcinii* and *L. quinatus*, 4–5 in *L. hebranicus*, 6–9 in *L. benoistii*, 5–9 in members of the section *Rhyncholotus*); some *Lotus* species that normally possess five leaflets (*L. taitungensis* [= *L. pacificus*], *L. corniculatus*, *L. weilleri*, *L. australis*, *L. cruentus*) sporadically produce leaves with other leaflet numbers (LASSEN 1986; SOKOLOFF 2003; KRAMINA & SOKOLOFF 2004; SANDRAL et al. 2006). Finally, leaves with five leaflets can be rarely found in *L. tetraphyllus* (VALDÉS 2000; KRAMINA pers. obs.). In summary, leaflet number cannot be effectively used as a criterion for sectional delimitation in *Lotus*. In the re-circumscribed section *Lotea*, most species have five, one species (*L. tetraphyllus*) has four, and one species (*L. simonae*) has three leaflets.

**/Pedrosia clade.** This clade comprises all members of sections *Pedrosia* and *Rhyncholotus* studied so far. Monophyly of this clade and its detailed phylogeny were discussed by ALLAN et al. (2004). Although *Pedrosia* and *Rhyncholotus* have been traditionally accepted as two subgenera or sections (see MONOD 1980 and SANDRAL et al. 2006, for discussion), molecular data show that *Rhyncholotus* is derived from the paraphyletic *Pedrosia*. The section *Rhyncholotus* is endemic to Canary Islands, while the section *Pedrosia* has main diversity in Canary Islands, Madeira, Cape Verde and Morocco (SANDRAL et al. 2006).

An important gap in molecular sampling of *Pedrosia* was the absence of data on *Lotus loweanus*, which is endemic to the Island of Porto Santo (Madeira). This is probably the most remarkable species of the section. It differs from the rest of the section by a combination of such features as very short one-flowered peduncles (flowers appear to be solitary in axils of foliage leaves), blackish-purple to pink flowers, hairy outer surface of the standard, fruits densely pubescent along whole surface of valves and usually with one or several very deep constrictions between seeds (SANDRAL et al. 2006). Our present phylogenetic data clearly confirm placement of *L. loweanus* in the /*Pedrosia* clade.

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Two other new sequences of the section *Pedrosia* produced for this paper are those of *L. arinagensis* and *L. mascaënsis*. These two species have been analyzed by ALLAN et al. (2004). We sequenced them again because we were intrigued by the fact that they formed an unresolved position with species of the section *Rhyncholotus* (ALLAN et al. 2004). The section *Rhyncholotus* differs significantly from *Pedrosia* in floral morphology. The new sequence of *L. arinagensis* is identical to the sequence published by ALLAN et al. (2004). In contrast, the sequence of *L. mascaënsis* slightly differs from the previously published one. Figure 5 shows a portion of alignment of nrITS sequences of *L. mascaënsis* and its closest relatives of the /*Pedrosia* clade. For this figure, only those parts of the alignment are selected that possess parsimony informative substitutions. Sites with unique substitutions are not considered. In contrast to the previously published sequence (*Lotus mascaënsis* 2), our new sequence (*Lotus mascaënsis* 1) shows two polymorphic sites (Y = C/T). In the first site, where *Lotus mascaënsis* 1 is polymorphic, most examined members of the tribe Loteae have C, while T is much less common (though the presence of T is not unique for the /*Pedrosia* clade). In the second site, where *Lotus mascaënsis* 1 is polymorphic, most members of the tribe Loteae have G, while T is less common; the presence of C is recorded only from within the /*Pedrosia* clade and from a totally unrelated monotypic *Kebirita*.

The discovery of variation in nrITS sequences of *L. mascaënsis* is important because of a very limited distribution range of this species, which is restricted to just one valley (Valle de Masca) on Tenerife. Very few collections of this species have been made. Interestingly, within these few collections a morphological variation is also observed (leaf rachis present or absent). Presence/absence of a leaf rachis is an important taxonomic character in the section *Pedrosia* (SANDRAL et al. 2006). Morphological and molecular variation may suggest possible hybrid origin of *L. mascaënsis* or an increased evolution rate in a small population. *L. mascaënsis* is an endangered species (BRAMWELL & BRAMWELL 2001). SANDRAL et al. (2006) suggested that the species might be even extinct in the wild.

Apart from incongruence between the two sequences of *Lotus mascaënsis*, Figure 5 shows absence of any differences in this portion of the alignment between species of the section *Rhyncholotus* (e.g. *L. maculatus*, *L. berthelotii*) and their closest relatives of the section *Pedrosia* (*L. arinagensis*, *L. mascaënsis*, *L. emeroides*). This shows that nrITS sequences are not enough informative for detailed phylogeny reconstruction within the /*Pedrosia* clade.

|                             | ITS 1  | ITS 2 |
|-----------------------------|--|-------|
| <i>Lotus berthelotii</i>    | TTAGTTGTACTCTT . . . . TGCCCC-AACGCCAAATGCCTAGTGCTATGTGGTGGCTGGGGCGA |       |
| <i>Lotus mascaënsis1</i>    | TTAGTTGTACTCTT . . . . TGCCCC-AACGYCAAATGCCTAGTGCTATGTGGTGGYTGGGGCGA |       |
| <i>Lotus mascaënsis2</i>    | TTAGTTGTACTCTT . . . . TGCCCC-AACGCCAAATGCCTAGTGCTATGTGGTGGCTGGGGCGA |       |
| <i>Lotus arinagensis</i>    | TTAGTTGTACTCTT . . . . TGCCCC-AACGCCAAATGCCTAGTGCTATGTGGTGGCTGGGGCGA |       |
| <i>Lotus emeroides</i>      | TTAGTTGTACTCTT . . . . TGCCCC-AACGCCAAATGCCTAGTGCTATGTGGTGGCTGGGGCGA |       |
| <i>Lotus maculatus</i>      | TTAGTTGTACTCTT . . . . TGCCCC-AACGCCAAATGCCTAGTGCTATGTGGTGGCTGGGGCGA |       |
| <i>Lotus azoricus</i>       | TTAGTTGTACTCTT . . . . TGCCCC-AACGCCAAATGCCTAGTGCTATGTGGTGGTTGGGGCGA |       |
| <i>Lotus loweanus</i>       | TTAGTTGTACTCTT . . . . TGCCCC-AACGTCAAATGCCTAGTGCTATGTGGTGGTTGGGGCGA |       |
| <i>Lotus assakensis</i>     | TTAGTTGCACTCTT . . . . TGCCCCAACATCAAATGCCTTGTGCTATGTGGTGGTTGGGGCGA  |       |
| <i>Lotus lancerottensis</i> | TTAGTTGCACTCTT . . . . TGCCCCAACATCAAATGCCTTGTGCTATGTGGTGGTTGGGGCGA  |       |
| <i>Lotus campylocladus</i>  | TTAGTTGYACTCTT . . . . TGCCCC-AACATCAAATSCCTAGTGCTATGTGGTGGTTGGGGCGA |       |
| <i>Lotus pseudoreticus</i>  | TTAGTTGCACTCTT . . . . TGCCCC-AACATCAAATGCCTAGTGCTATGTGGTGGTTGGGGCGA |       |
| <i>Lotus creticus</i>       | TTAGTTGCACTCTT . . . . TGCCCC-AACATCAAATGCCTAGTGCTATGTGGTGGTTGGGGCGA |       |

Figure 5: A portion of the alignment showing differences between the two sequences of *Lotus mascaënsis* and sequences of its closest relatives of the /*Pedrosia* clade. See text for the details.

**Unequal levels of molecular differentiation within different clades.** Data on nrITS sequences show considerable differences between patterns of molecular variation within different clades. In particular, differences between /Pedrosia and /Lotus corniculatus clades are remarkable. Morphologically, the /Pedrosia clade is extremely diverse, with taxa differentiated in such substantive characters as corolla shape (as an adaptation to insect versus bird pollination), presence/absence of hairs on standard, presence/absence of leaf rachis, leaflet shape (all range of leaflet length/width ratio, also leaflets dimorphic vs. monomorphic), flower colour (all range from dark brown to pale yellow), growth form (annuals to shrubs), inflorescence architecture (including differences in plant axiality), umbel peduncle length, fruit shape, presence/absence of hairs on fruits (e.g. SANDRAL et al. 2006). Nevertheless, very little resolution is found in the nrITS trees within the /Pedrosia clade. No clade with bootstrap support more than 80% is found within the /Pedrosia clade. In contrast, species of the /Lotus corniculatus clade differ in such quantitative characters as leaflet shape (the range of variation here is much narrower than in /Pedrosia), flower size, calyx shape, pubescence of the calyx and vegetative parts of the plant, and some others (e.g. KRAMINA 1999a, b). In terms of nrITS sequences, differentiation within the /Lotus corniculatus clade is considerable, and several well-supported subclades can be identified. This is unexpected from a group with reportedly occurrence of limited or wide hybridization in wild populations (e.g. GAUTHIER et al. 1998; KRAMINA 2000).

Within the /Pedrosia clade, molecular differences between mainland and insular taxa (from the Azores, Madeira, Canary Is., and Cape Verde) are low (see also ALLAN et al. 2004). In contrast, *L. anfractuosus* from New Caledonia differs considerably in nrITS sequence from its closest relatives in Australia (*L. australis* and *L. cruentus*). This is clear because of a relatively long branch leading to *L. anfractuosus* in Figure 3.

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