

Are *Lotus creticus* and *Lotus cytisoides* (Leguminosae) closely related species? Evidence from nuclear ribosomal ITS sequence data

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Abstract *Lotus creticus* is a potentially important perennial legume for soil management in the Mediterranean climate. This plant is in focus of experimental research in different countries. The so-called *Lotus creticus* group is taxonomically problematic. Although some authors consider all members of the group as a single variable species, others segregate several species distinct from *L. creticus*, especially *L. cytisoides*.

Recent morphological studies suggested that *L. creticus* and *L. cytisoides* are taxonomically distinct at specific level and not even closely related to each other. Two molecular phylogenetic studies published so far and based on one accession of *L. creticus* each gave conflicting results on relationships of this species. In this paper, sequences of nuclear ribosomal ITS (nrITS) are produced from multiple accessions of both *L. creticus* and *L. cytisoides*. Taxonomic identity of each accession has been tested using morphology. The new nrITS sequences are inserted in a data matrix containing a representative set of *Lotus* species covering all the geographical range and all major taxonomic groups of *Lotus*. Phylogenetic analysis of nrITS sequence data showed that *L. creticus* is related to *L. assakensis* and other species of the section *Pedrosia* while *L. cytisoides* is related to *L. longisiliquosus* and other species of the section *Lotea*. These results are important for establishing future germplasm collection, breeding and selection programs of perennial *Lotus* species.

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Introduction

The genus *Lotus* L. (Leguminosae Juss.: Loteae DC.) contains about 120–130 species of perennials and

annuals native to Eurasia, Africa, Australia and adjacent islands, with the main centre of diversity in the Mediterranean region (reviewed in Degtjareva et al. 2006). Several of these species are of significant agricultural importance including the perennials *L. corniculatus* L., *L. pedunculatus* Cav. s.l. and *L. glaber* Mill. as well as the annual (to biennial or perennial—Kramina 2006) *L. subbiflorus* Lag. The genus also includes the model legume *L. japonicus* and ornamentals such as *L. berthelotii* Masf. and *L. maculatus* Breif. Another potentially important perennial *Lotus* is *L. creticus* L. because of its capacity to stabilise sand dunes (Tsurieil 1974), provide forage for livestock (Le Houerou 1979) and withstand low humidity and increased salinity (Sanchez-Blanco et al. 1998; Morales et al. 2000; Savé et al. 2000; Franco et al. 2002; Vignolio et al. 2002, 2005; Bañon et al. 2004; Zakhia et al. 2004; Rejili et al. 2007).

The so-called *Lotus creticus* group (Heyn and Herrnstadt 1968) is taxonomically problematic because of technical difficulties that sometimes occur in the identification of its species. Some authors accepted a single variable species *Lotus creticus* s.l. within this group (e.g. Quezel and Santa 1962), while others recognized more than one species. A detailed taxonomic revision of the *Lotus creticus* group, based on morphology, was published by Heyn and Herrnstadt (1968). These authors recognized four different mainly Mediterranean species, namely *L. polyphyllus* Clarke, *L. creticus* L. s.str. (= *Lotus commutatus* Guss.), *L. cytisoides* L. and *L. collinus* (Boiss.) Heldr. Currently, individuality of these four species is accepted in most taxonomic publications with a change that the name *L. collinus* is corrected to *L. longisiliquosus* de Roem. (Greuter et al. 1989; Kramina and Sokoloff 1999; Valdés 2000). The wide taxonomic concept is still accepted in some experimental studies, where *L. cytisoides* is treated as a subspecies of *L. creticus* (e.g., Savé et al. 2000; Franco et al. 2002). Rejili et al. (2007) compared effect of NaCl on the growth and the ionic balance K^+/Na^+ of two populations of *Lotus creticus* in Tunisia. However, *L. creticus* s.str. is very rare in this part of Africa and Sandral et al. (2006) were unable to trace any herbarium specimen from Tunisia. Since *L. cytisoides* is common in Tunisia and many herbarium collections of this species are misidentified as “*L. creticus*” we could hypothesize that Rejili et al. (2007) studied *L. cytisoides* rather than *L. creticus* s.str. Also, the

report on nodulating bacteria in wild *L. creticus* from Tunisia (Zakhia et al. 2004) might belong to *L. cytisoides*.

The question whether *L. cytisoides* is distinct from *L. creticus* at a specific level or should it be accepted as a subspecies was mainly of pure taxonomic interest when the *Lotus creticus* group was considered as a natural unit of perennial species within a section *Lotea* (Medik.) DC. of the genus *Lotus* (e.g., Ball and Chrtková-Žertová 1968). Things became more complicated when Kramina and Sokoloff (1999) suggested that *L. creticus* and *L. cytisoides* may not be even closely related to each other. They argued that *L. cytisoides*, together with *L. polyphyllus*, *L. longisiliquosus* and some other species belong to the section *Lotea*, while *L. creticus* belongs to the section (or subgenus) *Pedrosia* (Lowe) R.P. Murray. The section *Pedrosia* includes about 30 species occurring mostly in Canary Islands, Madeira, Cape Verde, the Azores, and Morocco (Sandral et al. 2006). Morphologically, the section (or subgenus) *Pedrosia* differs from the section *Lotea* in the presence of a distinct tooth on ventral side of a style (e.g., Monod 1980; Mader and Podlech 1989; Kramina and Sokoloff 1999; Sandral et al. 2006). Kramina and Sokoloff (1999) found a very short and often scarcely recognizable tooth on style in *L. creticus*. Since they considered presence of a tooth quite an important feature, Kramina and Sokoloff (1999) concluded that *L. creticus* should be included in the section *Pedrosia* rather than in the section *Lotea*. This taxonomic rearrangement extends considerably the geographical range of the section *Pedrosia*, because *L. creticus*, though occurring in the Azores and Morocco is distributed in various Mediterranean countries up to Israel in the eastern Mediterranean, i.e., in the regions where other members of the section *Pedrosia* are totally absent. Valdés (2000), like Kramina and Sokoloff (1999), illustrated presence of a tooth on style of *L. creticus*. Nevertheless, he placed the species near *L. cytisoides* within the section *Lotea*.

Sandral et al. (2006), in the context of a detailed comparative morphological study of all species of the section *Pedrosia*, agreed with placement of *L. creticus* in this section. Kramina and Sokoloff (1999) and Sandral et al. (2006) noted close morphological similarity between *L. creticus* and a local endemic of SW Morocco, *L. pseudoreticus* Maire, Weiller et Wilczek. The main distinction between the

two species is the presence of a well developed style tooth in *L. pseudocreticus*, while in most other characters the two species are alike. Sandral et al. (2006) found that, morphologically, *L. pseudocreticus* is intermediate between *L. creticus* and another Moroccan member of the section *Pedrosia*, *L. assakensis* Brand. Since all three species occupy similar habitats in SW Morocco, Sandral et al. (2006) suggested hybrid origin of *L. pseudocreticus* (*L. assakensis* × *L. creticus*).

Molecular phylogenetic data based on nuclear ribosomal ITS sequences supported the idea that the section *Lotea* is distinct from the section *Pedrosia*. Indeed, according to nrITS data, the section *Lotea* is closer to the section (formerly genus) *Tetragonolobus* (Scop.) Benth. et Hook. f. than to *Pedrosia* (Allan et al. 2003, 2004; Degtjareva et al. 2006). However, placement of *L. creticus* was conflicting in phylogenies of Allan et al. (2003, 2004) and Degtjareva et al. (2006). According to Allan et al. (2003, 2004), *L. creticus* falls into the *Lotea* clade while according to Degtjareva et al. (2006) it is closely related to *Pedrosia* species. Only two accessions of *L. creticus* have been analyzed by molecular methods so far (one accession PI287858-64i, GenBank number AF450192 in Allan et al. 2003, 2004 and another accession *Severova* s.n., Portugal, June 2001 (MW), GenBank number DQ160279 in Degtjareva et al. 2006). Preliminary data on psbA-trnH phylogeny of *Loteae* (Degtjareva 2007) supported placement of *L. creticus* (*Severova* s.n.) together with members of the section *Pedrosia*.

Thus the available molecular data on phylogenetic placement of *Lotus creticus* are equivocal. The data in Allan et al. (2003, 2004) support the traditional view that *L. creticus* and *L. cytisoides* are closely related species. The data in Degtjareva et al. (2006) and in Degtjareva (2007) support an alternative view by Kramina and Sokoloff (1999). This study is aimed in clarification of this question by producing and analyzing nrITS sequences of *L. creticus* and *L. cytisoides* from multiple accessions.

Materials and methods

Both *Lotus creticus* and *Lotus cytisoides* were grown from seed samples of different geographical origin at South Australian Research and Development Institute, Adelaide, Australia. Taxonomic identity of each

specimen has been established using morphological characters listed in Heyn and Herrstadt (1968), Ball and Chrtková-Žertová (1968), Kramina and Sokoloff (1999) and Valdés (2000). Out of 30 accessions cultivated, 14 were identified as *L. creticus* and 15 as *L. cytisoides*. One accession (PI287858), which is listed in the seed bank collection under *L. creticus*, has been morphologically identified in this study as *L. arenarius* Brot. This accession has the same voucher details as the accession of *L. creticus* used by Allan et al. (2003). DNA was extracted from each accession. Voucher herbarium specimens are pressed from the same plant individuals from which DNA was extracted; they are deposited at Herbaria of Royal Botanic Gardens, Kew (K) and Moscow State University (MW). In addition, DNA was extracted from one herbarium specimen of *L. creticus*, one herbarium specimen of *L. cytisoides*, three herbarium specimens of *L. pseudocreticus* and two herbarium specimens of *L. assakensis*. Voucher information and GenBank accession numbers of all sequences used of *L. creticus*, *L. cytisoides*, *L. pseudocreticus*, *L. assakensis* and *L. arenarius* is given in Table 1. For all other sequences used, the accession numbers are the same as in Degtjareva et al. (2006).

For all new accessions, complete sequences of ITS1, ITS2 and the 5.8S region were generated. Total DNA was isolated from leaf tissue using NucleoSpin Plant isolation kit (Macherey–Nagel, Düren, Germany) following the manufacturer's instructions. Details of PCR amplifications of ITS1-2 region and sequencing strategies used (including primer locations and characteristics) are provided in Valiejo-Roman et al. (2002). PCR products were purified using the DNA cleaning kit from Cytokine (St.-Petersburg, 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 for cycle sequencing reactions following the manufacturer's instructions. Forward and reverse strands of all ITS samples were sequenced.

Resulting sequences were further checked by eye with the software CHROMAS 1.45 (<http://www.technelysium.com.au/chromas.html>). ITS sequences were aligned automatically using the program MUSCLE (Edgar 2004) and then manually adjusted using program BioEdit (Hall 1999). The aligned data matrices

Table 1 Accessions and GenBank accession numbers of *Lotus arenarius* Brot., *L. assakensis* Brand, *L. creticus* L., *L. cytisoides* L. and *L. pseudocreticus* Maire, Weiller & Wilczek used in this paper

Sample (species name according to the present study)	GenBank accession number	Voucher information ^a	Seed donator	Initial identification according to records of seed donator (when available)
<i>L. arenarius</i> (1)	FJ938295	Seed collection: Spain, SA37652, cultivated in Adelaide, 2007	United States Department of Agriculture (PI287858), originally donated by Instituto Forestal de Investigaciones y Experimentias Madrid, Spain	<i>L. creticus</i>
<i>L. arenarius</i> (2)	AF218528 (Allan and Porter 2000)	Morocco, Podlech 46130 (RSA)	n/a	
<i>L. assakensis</i> (1)	DQ160277 (Degjareva et al. 2006)	Morocco, Podlech 40448 (M)	n/a	
<i>L. assakensis</i> (2)	FJ938331	Morocco, Agadir, VIII 2008 Nuraliev (MW)	n/a	
<i>L. creticus</i> (1)	FJ938296	Seed collection: Azores Is., Terceira, 38°43'942" N, 27°03'221" W, alt 52 m, 3.VIII 2002, Sandral SA39213, cultivated in Perth 2005	Agricultural Research Institute, Wagga Wagga, NSW, Australia	<i>L. creticus</i>
<i>L. creticus</i> (2)	FJ938297	Seed collection: Morocco, Agadir, Reserve Biologique Permanente de Massa, 30°05' N, 9°40' W, alt. 40 m, 10.VI 1990, Prendergast, SA34273, cultivated in Perth, 2005	Royal Botanic Gardens, Kew, United Kingdom	<i>L. creticus</i>
<i>L. creticus</i> (3)	FJ938298	Seed collection: Azores Is., Terceira, 38°43'597" N, 27°03'697" W, alt 10 m, 3.VIII 2002, Sandral SA39207, cultivated in Perth 2005	Agricultural Research Institute, Wagga Wagga, NSW, Australia	<i>L. creticus</i>
<i>L. creticus</i> (4)	FJ938299	Morocco, 15.VI 1993, Lewalle 13914 (NSW)	n/a	
<i>L. creticus</i> (5)	FJ938300	Seed collection: Portugal, between Praia das Macas and Praia Grande, 38° N, 9° W, alt. 50 m, 20.VI 1967, Crawford, SA2317, cultivated in Adelaide, 2007	South Australian Research and Development Institute, Adelaide, SA, Australia	<i>L. commutatus</i>
<i>L. creticus</i> (6)	FJ938301	Seed collection: Israel, Sand Dune Nursery S.C.S. Kiryat Shmuel, 17.V 1967, Crawford, SA2318, cultivated in Perth, 2005	South Australian Research and Development Institute, Adelaide, SA, Australia	<i>L. commutatus</i>
<i>L. creticus</i> (7)	FJ938302	Seed collection: Morocco, Agadir, Reserve Biologique Permanente de Massa, 30°05' N, 9°40' W, alt. 40 m, 10.VI 1990, Prendergast, SA34273, cultivated in Adelaide, 2007	Royal Botanic Gardens, Kew, United Kingdom	<i>L. creticus</i>
<i>L. creticus</i> (8)	FJ938303	Seed collection: Portugal, 11 km N of Vila do Bispo, alt. 120 m, 23.V 1974, O'Brien, SA36538, cultivated in Adelaide, 2007	Commonwealth Scientific and Industrial Research Organisation, St Lucia, Queensland, Australia	<i>L. creticus</i>

Table 1 continued

Sample (species name according to the present study)	GenBank accession number	Voucher information ^a	Seed donor	Initial identification according to records of seed donator (when available)
<i>L. creticus</i> (9)	FJ938304	Seed collection: Spain, 4.5 km NE of San Diego, 36°02' N, 50°14' W, alt. 20 m, 31.XII.2000, Sandral & Loi, SA 37340, cultivated in Adelaide, 2007	Agricultural Research Institute, Wagga Wagga, NSW, Australia	<i>L. creticus</i>
<i>L. creticus</i> (10)	FJ938305	Seed collection: Portugal, Fortaleza SW of Sagres, alt. 30 m, 23.V.1974, O'Brien, SA37350, cultivated in Adelaide, 2007	Department of Agriculture Western Australia	
<i>L. creticus</i> (11)	FJ938306	Seed collection: Spain, SA37657, cultivated in Adelaide, 2007	United States Department of Agriculture, originally donated by Instituto Forestal de Investigaciones y Experiencias Madrid, Spain	<i>L. creticus</i>
<i>L. creticus</i> (12)	FJ938307	Seed collection: Israel, SA37659, cultivated in Perth, 2006	Soil Conservation Service Ministry of Agriculture Kiriyat Shmuel Haifa, Haifa, Israel.	<i>L. creticus</i>
<i>L. creticus</i> (13)	FJ938308	Seed collection: Portugal, SA37757, cultivated in Adelaide, 2007	Margot Forde Forage Germplasm Centre, New Zealand	<i>L. creticus</i>
<i>L. creticus</i> (14)	FJ938309	Seed collection: Azores Is., Terceira, 38°04'3" N, 27°00'3" W, alt. 10 m, 03.VIII.2002, Sandral, SA39199, cultivated in Adelaide, 2007	Agricultural Research Institute, Wagga Wagga, NSW, Australia	<i>L. creticus</i>
<i>L. creticus</i> (15)	FJ938310	Seed collection: Israel, SA39319, cultivated in Perth, 2006	The Israeli Gene Bank for Agricultural Crops, Bet-Dagan, Israel.	<i>L. creticus</i>
<i>L. creticus</i> (16)	DQ160279 (Degjareva et al. 2006)	<i>Severova</i> s.n., Portugal, June 2001 (MW)	n/a	
<i>L. cytisoides</i> (1A)	DQ166241 (Degjareva et al. 2006)	Cyprus, Akrotiri, III.2004, Seregin & Sokoloff 280 (MW)	n/a	
<i>L. cytisoides</i> (1B)	DQ160280 (Degjareva et al. 2006)	As above		
<i>L. cytisoides</i> (2)	FJ938312	Seed collection: Crete, Vihadia, alt. 50 m, SA37747, cultivated in Adelaide, 2007	UNITHES, Department Botany, Aristoteleion University Thessalonica, Thessalonica, Greece	<i>L. creticus</i>
<i>L. cytisoides</i> (3)	FJ938313	Seed collection: Crete, Moore, SA38065, cultivated in Adelaide, 2007	Department of Agriculture Western Australia	<i>L. cytisoides</i>

Table 1 continued

Sample (species name according to the present study)	GenBank accession number	Voucher information ^a	Seed donor	Initial identification according to records of seed donator (when available)
<i>L. cytisoides</i> (4)	FJ938314	Seed collection: Mainland Italy, 1999, Moore, SA38068, cultivated in Adelaide, 2007	Department of Agriculture Western Australia	<i>L. cytisoides</i>
<i>L. cytisoides</i> (5)	FJ938315	Seed collection: Yugoslavia, Seget, 2 km west Trogir, SA37951, cultivated in Adelaide, 2007	Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany	<i>L. cytisoides</i>
<i>L. cytisoides</i> (6)	FJ938311	Seed collection: Tunisia, Akrimi SA27513, cultivated in Perth, 2006	Ministry of Agriculture, Tunisia	<i>L. creticus</i>
<i>L. cytisoides</i> (7)	FJ938317	Seed collection: Tunisia, Akrimi SA27513, cultivated in Adelaide, 2007	Ministry of Agriculture, Tunisia	<i>L. creticus</i>
<i>L. cytisoides</i> (8)	FJ938318	Seed collection: Sicily, 37°03'6" N, 14°03'3" E, alt 548 m, 27.X 2000, Sandral & Ewing, SA37214, cultivated in Adelaide, 2007	Agricultural Research Institute, Wagga Wagga, NSW, Australia	<i>L. creticus</i>
<i>L. cytisoides</i> (9)	FJ938319	Seed collection: Sicily, just out of Scoglitti, 37°02'8" N, 14°04'6" E, alt 43 m, 28.X 2000, Sandral & Ewing, SA37218, cultivated in Adelaide, 2007	Agricultural Research Institute, Wagga Wagga, NSW, Australia	<i>L. creticus</i>
<i>L. cytisoides</i> (10)	FJ938325	Seed collection: Tunisia, Belvedere Park-Tunis, Tunis Prov., 36°05'0" N, 10°01'1" E, alt. 50 m, 10.VI 1981, Graves, SA45039, cultivated in Adelaide, 2007	United States Department of Agriculture (PI535593)	<i>L. creticus</i>
<i>L. cytisoides</i> (11)	FJ938324	Seed collection: Sicily, ¾ way from Palermo towards airport, alt 40 m, 31.XII 2000, Sandral & Loi, SA37337, cultivated in Adelaide, 2007	Agricultural Research Institute, Wagga Wagga, NSW, Australia	<i>L. creticus</i>
<i>L. cytisoides</i> (12A)	FJ938320	Seed collection: Turkey SA34062, cultivated in Adelaide, 2007	M. Forti, Division of Live Sciences, Negev Institute for Arid Research, Beer Sheva, Southern Israel.	<i>L. collinus</i>
<i>L. cytisoides</i> (12B)	FJ938321	As above		
<i>L. cytisoides</i> (13A)	FJ938326	Seed collection: Tunisia, Kef Prov., 36°01'4" N, 08°04'5" E, alt. 750 m, 16.VI 1981, Graves, SA 45040, cultivated in Adelaide, 2007	United States Department of Agriculture (PI 535591)	<i>L. creticus</i>
<i>L. cytisoides</i> (13B)	FJ938327	As above		
<i>L. cytisoides</i> (14A)	FJ938328	Seed collection: Tunisia, Kairouan to Ousseltia road on C46, 4 km N of C99, Kairouan Prov., 35°05'3" N, 09°05'3" E, alt. 450 m, 15.V 1981, Graves, SA45038, cultivated in Adelaide, 2007	United States Department of Agriculture (PI535592)	<i>L. creticus</i>
<i>L. cytisoides</i> (14B)	FJ938329	As above		

Table 1 continued

Sample (species name according to the present study)	GenBank accession number	Voucher information ^a	Seed donator	Initial identification according to records of seed donator (when available)
<i>L. cytisoides</i> (15)	FJ938322	Seed collection: Sicily, 4 km N of Piazza Armerina, towards Valguanera, 37°02'5" N, 14°02'3" E, alt. 740 m, 18.VI 1986, Auricht & Guarino, SA26222, cultivated in Adelaide, 2007	South Australian Research and Development Institute, Adelaide, SA, Australia	
<i>L. cytisoides</i> (16)	FJ938323	Seed collection: Sicily, 20 km W of Scoglitti, 37°00'1" N, 14°01'2" E, alt. 18 m, 31.XII 2000, Sandral & Loi, SA 37335, cultivated in Adelaide, 2007	Agricultural Research Institute, Wagga Wagga, NSW, Australia	
<i>L. cytisoides</i> (17)	FJ938316	Seed collection: Spain, SA37654, cultivated in Adelaide, 2007	United States Department of Agriculture, originally donated by Instituto Forestal de Investigaciones y Experiencias Madrid, Spain	<i>L. creticus</i>
<i>L. pseudocreticus</i> (1)	DQ160284 (Degjareva et al. 2006)	Morocco, prov. Essaouira, 01.IV 1995, Podlech 52358 (M)	n/a	
<i>L. pseudocreticus</i> (2)	FJ938330	Morocco, Cap Rhir, 03.IV 1994, Lewalle 13958 (AD)	n/a	
<i>L. pseudocreticus</i> (3)	FJ938332	Morocco, Agadir, VIII 2008 Nuraliev (MW)	n/a	

^a Voucher numbers starting with the letters "SA" are referring to the accession numbers of germplasm, which are preserved as seeds for long term conservation at the South Australian Genetic Resource Center in Adelaide. Voucher herbarium specimens for plants cultivated in Perth and Adelaide are deposited in MW; duplicates of most specimens are in K

are available from the corresponding author on request. The ITS data matrix was analyzed using maximum parsimony (MP) and the Bayesian inference of phylogeny (BI). Maximum parsimony analysis involved a heuristic search conducted with PAUP* 4.08b (Swofford 2000) using TBR branch swapping with character states specified as equally weighted. Hundred replicates with random addition of sequences were performed and all shortest trees were saved. All gaps were treated as missing data. Bootstrap (Felsenstein 1985) values were calculated from 100 replicate analyses with TBR branch swapping and random addition sequence of taxa. One thousand most parsimonious trees from each replicate were saved.

Bayesian inference of phylogeny was explored using the MrBayes program (version 3.1; Ronquist and Huelsenbeck 2003). The GTR+I+G model was selected by the Akaike Information Criterion in the program Modeltest (Posada and Crandall 1998). A total of 25,000,000 generations were performed and trees from first 17,000,000 generations were discarded. The number of generations to be discarded was determined using a convergence diagnostic.

To recognize taxa and molecular clade nomenclature we followed Alverson et al. (1999) marking clades by foregoing slash as, for example, /Pedrosia clade.

Results

All sequences of *L. creticus* analysed in this paper were identical to each other and had length of 601 base pair (ITS1 + 5.8S + ITS2). One sequence of *L. pseudocreticus* differs considerably from two other sequences of the same species. Sequence *L. pseudocreticus* (1) was identical to those of *L. creticus* while two other sequences (*L. pseudocreticus* 2 and 3) were identical to both sequences of *L. assakensis* analysed here (Table 2). Sequences of *L. assakensis*, *L. lancerottensis* Webb et Berthel. and *L. pseudocreticus* (only accessions 2 and 3) share two substitutions that are not recorded in any other sequence used in this paper. Morphologically, vouchers of all specimens of *L. pseudocreticus* are alike. Our data on differences in nrITS sequences between two specimens of *L. pseudocreticus* are congruent with the hypothesis (Sandral et al. 2006) that *L. pseudocreticus* is a hybrid between *L. assakensis* and *L. creticus*. All three species co-occur in the same habitats (sandy sea shores) in

Table 2 Summary of differences between nrITS sequences of examined accessions of *L. assakensis*, *L. pseudocreticus* and *L. creticus*

Taxon	Site position in the alignment	
	ITS2	
	4	4
	0	2
	7	2
<i>L. assakensis</i>	C	T
<i>L. pseudocreticus</i> (2, 3)	C	T
<i>L. pseudocreticus</i> (1)	–	A
<i>L. creticus</i>	–	A

SW Morocco, and *L. pseudocreticus* is morphologically clearly intermediate between *L. assakensis* and *L. creticus*. Of course, much more robust molecular data (such as those from SSR analysis) are required for precise testing of this hypothesis. We should note that *L. lancerottensis* is an unlikely parent species for *L. pseudocreticus* because of non-overlapping geographical ranges of the two species.

Sequences of *L. cytisoides* were not identical to each other but all distinct from those of *L. creticus* (Table 3). Sequence length (ITS1 + 5.8S + ITS2) varied between 601 and 607 base pairs in *L. cytisoides*. Most sequences had a length of 603 base pairs. Out of 17 accessions of *L. cytisoides*, four had two matrices of nrITS (labelled as A and B in Table 1). In one of these four accessions the second matrix had a duplication of four nucleotides in the ITS1, while in other three accessions the second matrix had a deletion of two nucleotides in the same position in the ITS2.

According to the alignment of nrITS sequences, all accessions of *L. cytisoides* could be divided into two main groups. Group 1 includes accessions *L. cytisoides* 1, 2, 3, 4. These accessions are from Cyprus, Crete and Italy.

Accessions *L. cytisoides* 6–17 form Group 2, in which several subgroups can be segregated. These accessions are from Sicily, Tunisia, and Spain. Subgroup 2.1 includes accessions *L. cytisoides* 6, 7, 8, 9, 10, 11, which are from Sicily and Tunisia. Sequences of these accessions are identical with each other. The only available accession of *L. weilleri* Maire (which is from GenBank) was identical to sequences of *L. cytisoides* from the subgroup 2.1. Morphologically, *L. weilleri* is well-differentiated from *L. cytisoides*,

Table 3 Summary of differences between nrITS sequences of examined accessions of *L. creticus*, *L. cytisoides* and *L. assakensis*

Taxon	Site position in the alignment																	
	ITS1											5,8S rDNA						
	0	0	0 0	0	0	0	0	1	1	1	1	1	2	3				
	1	1	2-2	3	5	5	7	5	6	7	8	8	1	6				
	0	9	4 7	0	0	5	7	8	9	0	2	4	9	1				
<i>L. assakensis</i>	T	T	–	A	C	–	C	A	A	G	C	G	A	A				
<i>L. creticus</i>	T	T	–	A	C	–	C	A	A	G	C	G	A	A				
<i>L. cytisoides</i> (1)	C	A	CCAA/–	T	T	A	T	T	T	A	T	A	A	T				
<i>L. cytisoides</i> (2)	T	A	–	T	T	A	T	T	T	A	T	A	G	T				
<i>L. cytisoides</i> (3)	T	A	–	T	T	A	T	T	T	A	T	A	A	T				
<i>L. cytisoides</i> (4)	T	A	–	T	T	A	T	T	T	A	T	A	A	T				
<i>L. cytisoides</i> (6, 7, 8, 9, 10, 11)	T	A	–	T	T	A	T	T	T	A	T	A	A	T				
<i>L. cytisoides</i> (12, 13, 14)	T	A	–	T	T	A	T	T	T	A	T	A	A	T				
<i>L. cytisoides</i> (15, 16)	T	A	–	T	T	A	T	T	T	A	T	A	A	T				
<i>L. cytisoides</i> (17)	T	A	–	T	T	A	T	T	T	A	T	A	A	T				
<i>L. cytisoides</i> (5)	T	A	–	T	T	A	T	T	T	A	T	A	A	T				
Taxon	Site position in the alignment																	
	ITS2																	
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4		
	0	1	1	1	1	2	2	2	3	3	3	3	4	6	6	7	8	8
	7	1	2	6	7	0	2	8	1	4	6	7	3	0	6	3	3	7
<i>L. assakensis</i>	C	A	T	A	T	C	T	A	T	T	G	T	C	C	T	A	G	T
<i>L. creticus</i>	–	A	T	A	T	C	A	A	T	T	G	T	C	C	T	A	G	T
<i>L. cytisoides</i> (1)	–	G	C	T	C	T	A	T	T	C	T	G	T	T	C	G	T	A
<i>L. cytisoides</i> (2)	–	G	C	A	C	T	A	T	T	C	T	G	T	T	C	G	T	A
<i>L. cytisoides</i> (3)	–	G	C	A	C	T	A	T	T	C	T	G	T	T	C	G	T	A
<i>L. cytisoides</i> (4)	–	G	C	A	C	T	A	T	C	C	T	G	T	T	C	G	T	A
<i>L. cytisoides</i> (6, 7, 8, 9, 10, 11)	–	G	C	A	C	T	A	T	T	C	T	G	T	T	C	G	T	A
<i>L. cytisoides</i> (12, 13, 14)	–	G	C	A	C	T	A	T	T	C	T	G	T	T	C	G	T	A
<i>L. cytisoides</i> (15, 16)	–	G	C	A	C	T	A	T	T	C	T	G	T	T	C	G	T	A
<i>L. cytisoides</i> (17)	–	G	C	A	C	T	A	T	T	C	T	G	T	T	C	G	T	A
<i>L. cytisoides</i> (5)	–	G	C	A	C	T	A	T	C/T	C	T	G	T	T	C	G	T	A
Taxon	Site position in the alignment																	
	ITS2																	
	5	5	5	5 5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	0	0	0	0-0	2	5	5	6	6	6	6	7	7	9	9	9	9	9
	1	2	3	7 8	5	1	3	3	4	5	6	1	7	3	7	8	9	9
<i>L. assakensis</i>	A	T	G	TG	T	G	A	C	T	A	C	G	A	–	C	C	A	A
<i>L. creticus</i>	A	T	G	TG	T	G	A	C	T	A	C	G	A	–	C	C	A	A
<i>L. cytisoides</i> (1)	G	A	A	TG	C	C	T	T	C	G	T	A	T	T	A	A	A	G
<i>L. cytisoides</i> (2)	G	A	A	TG	C	C	T	T	C	G	C	A	T	T	A	A	A	G
<i>L. cytisoides</i> (3)	G	A	A	TG	C	C	T	T	C	G	C	A	T	T	A	A	A	G
<i>L. cytisoides</i> (4)	G	A	A	TG	C	C	T	T	C	G	C	A	T	T	A	A	A	G
<i>L. cytisoides</i> (6, 7, 8, 9, 10, 11)	G	A	A	TG	C	G	T	T	C	G	C	G	T	T	A	T	A	G

Table 3 continued

Taxon	Site position in the alignment																
	ITS2																
	5	5	5	5 5	5	5	5	5	5	5	5	5	5	5	5	5	5
	0	0	0	0-0	2	5	5	6	6	6	6	7	7	9	9	9	
	1	2	3	7 8	5	1	3	3	4	5	6	1	7	3	7	8	
<i>L. cytisoides</i> (12, 13, 14)	G	A	A	TG/-	C	G	T	T	C	G	C	G	T	T	A	T	G
<i>L. cytisoides</i> (15, 16)	G	A	A	T G	C	G	T	T	C	G	C	G	T	T	A	T	G
<i>L. cytisoides</i> (17)	G	A	A	T G	C	G	T	T	C	G	C	G	T	T	A	A	G
<i>L. cytisoides</i> (5)	G	A	A	T G	C	C/G	T	T	C	G	C	G/A	T	T	A	A/T	G

although morphology also suggests close relationship between these two species.

Subgroup 2.2 includes accessions *L. cytisoides* 12, 13, 14, which are from Tunisia and Turkey. Each accession of Subgroup 2.2 contains two matrices, one identical to those of Subgroup 2.1 and another differing in a two nucleotide deletion in ITS2.

Subgroup 2.3 contains accessions *L. cytisoides* 15 and 16, both from Sicily. Sequences of accessions 15 and 16 are identical with each other. They differ from sequences of the Subgroup 2.1 only in the presence of one polymorphic A/C site. The polymorphism is of potential interest, because only limited variation is observed at this site of alignment across all studied species of the genus *Lotus*. Presence of C in this site is characteristic to all studied *Lotus* species of sections *Lotus*, *Krokeria* (Moench) Ser., *Erythrolotus* Brand, *Dorycnium* (Mill.) D.D. Sokoloff, *Bonjeanea* (Rchb.) D.D. Sokoloff, and *Chamaelotus* Kramina et D.D. Sokoloff (see Degtjareva et al. 2006, for review of sectional boundaries). Presence of C in this site was not recorded so far (data from Degtjareva et al. 2006, 2008) in any member of sections *Lotea* or *Pedrosia*. All accessions of *L. cytisoides* except 15 and 16 have A in this site.

Subgroup 2.4 contains accession *L. cytisoides* 17, which is the only accession from Spain studied here. The sequence is similar to those of the Subgroup 2.1 but contain single nucleotide substitution, which is shared with sequences of the Group 1.

The accession *L. cytisoides* 5, which is the only accession from former Yugoslavia, has a sequence that suggests its hybrid origin with parents belonging to Groups 1 and 2. The sequence possesses polymorphisms in all sites in which those two main groups differ from each other.

Uncorrected pairwise sequence divergence values ranged from 0 to 1.0% within *L. cytisoides* and from 6.2 to 7.2% between *L. creticus* and *L. cytisoides*. For comparison, p-distances between all studied *Lotus* species vary from 0 to 13.2%.

Maximum parsimony analysis yielded 13,010 shortest trees (615 steps). Strict consensus of shortest trees (not shown) and 50%-majority rule bootstrap consensus tree (Fig. 1) are very similar to each other and to the trees produced earlier for the genus *Lotus* by Degtjareva et al. (2006). The Bayesian tree (Fig. 2) is also similar to the tree in Degtjareva et al. (2006), with minor exceptions, such as different position of *L. schimperi* Boiss. These small differences with topology in Degtjareva et al. (2006) are probably due to reduced taxon sampling in the present analysis. The differences do not affect position of *L. creticus* and *L. cytisoides*.

The sequences of *L. creticus* belong to a clade with bootstrap support 100% and posterior probability 1.00 that comprises all analysed members of the sections *Pedrosia* and *Rhyncholotus* (/Pedrosia clade, as defined by Degtjareva et al. 2008). These sections are characterised by the presence of a ventral tooth on a style. Thus, monophyly of the /Pedrosia clade strongly supports the idea on a single origin of the ventral style tooth in the genus *Lotus*. This feature is unique to this clade among members of the tribe Loteae. We do not know reports of a similar structure from other legume tribes (see also Mönch 1910). Placement of *L. creticus* outside of the /Pedrosia clade in phylogenetic trees of Allan et al. (2003, 2004) implied independent origin of style tooth in two different lineages of the genus *Lotus*.

Within the /Pedrosia clade, sequences of *L. creticus* fall in our analysis into a subclade with bootstrap support 83% and posterior probability 1.00

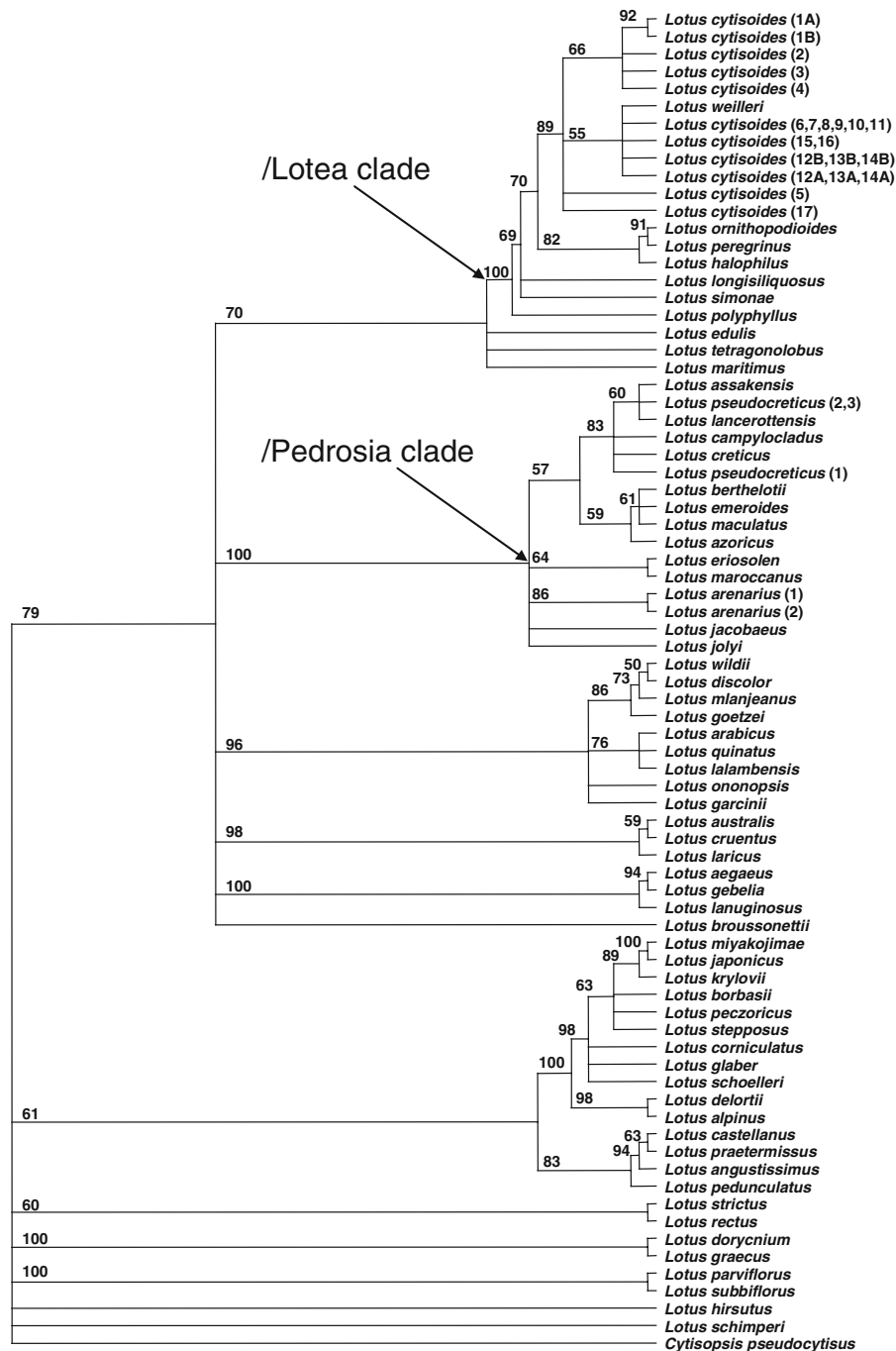


Fig. 1 Phylogenetic tree inferred from maximum parsimony analysis of nrITS data with bootstrap resampling showing relationships between *L. creticus*, *L. cytisoides* and other species of the genus *Lotus*. Numbers above branches show bootstrap support values

that includes also *L. assakensis*, *L. pseudocreticus*, *L. lancerottensis* and *L. campylocladus* Webb et Berthel. These species are morphologically close to *L. creticus* (Sandral et al. 2006).

All analysed sequences of *L. cytisoides* form a clade (bootstrap support 89%, posterior probability 1.00) together with the GenBank accession of *L. weilleri*. This clade is a member of a highly supported

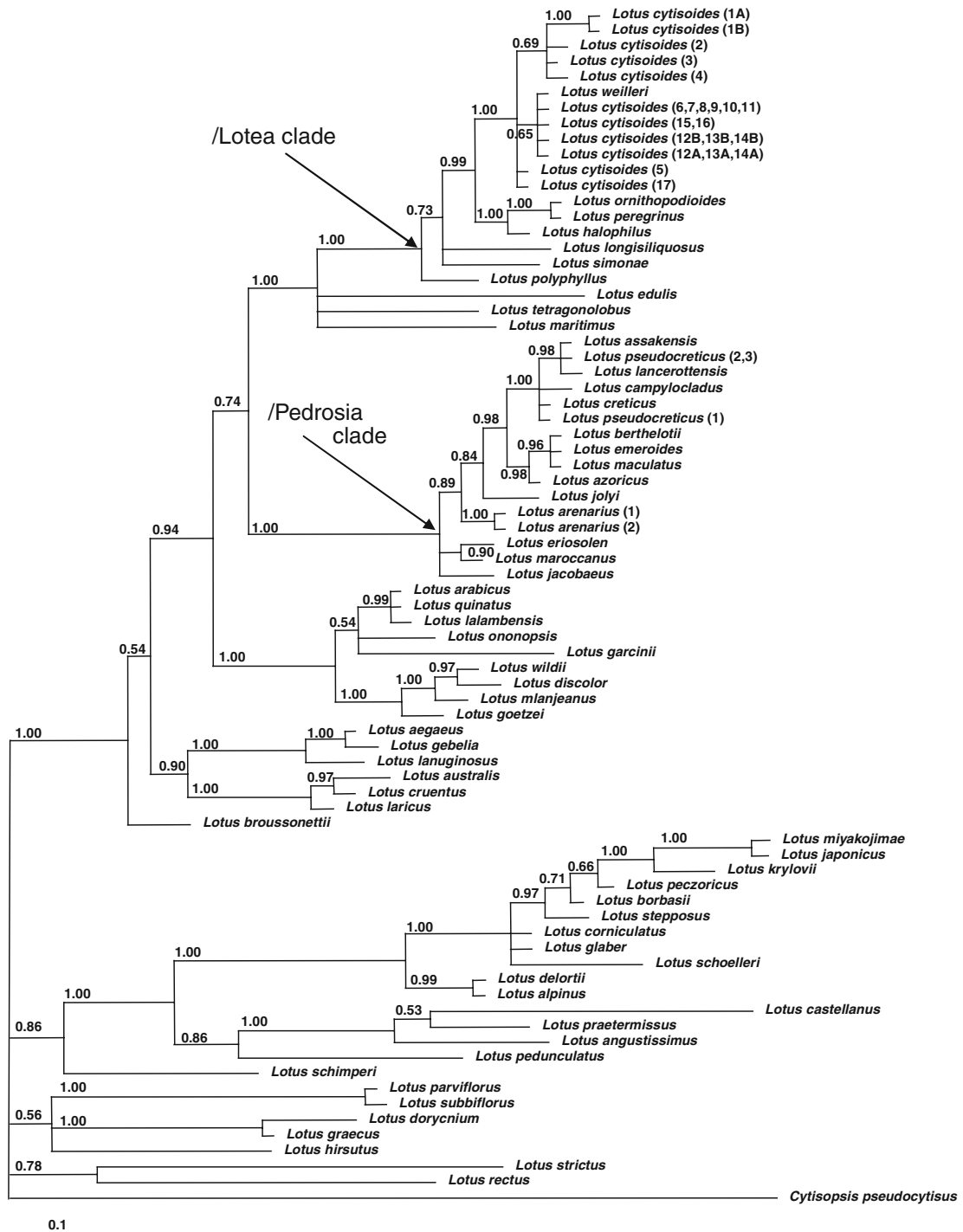


Fig. 2 Bayesian tree showing relationships between *L. creticus*, *L. cytisoides* and other species of the genus *Lotus*. Numbers above branches are posterior probabilities. Scale = 0.1 substitution/site

(bootstrap support 100%, posterior probability 1.00) / *Lotea* clade (clade name after Degtjareva et al. 2008). This clade includes all members of the traditionally

recognized section *Lotea* plus *L. simonae* Maire, Weiller et Wilczek and minus *L. creticus*. The *Lotea* clade is phylogenetically closer to former members of

the genus *Tetragonolobus* Scop. (*Lotus tetragonolobus* L. and *L. maritimus* L.) and *L. edulis* L. (section *Krockeria*) than to the *Pedrosia* clade. Therefore, our data clearly show that *L. creticus* is not closely related to *L. cytisoides*.

Discussion

Our data strongly support the earlier hypothesis (Kramina and Sokoloff 1999) that *Lotus creticus* is not related to *Lotus cytisoides*. As suggested by Kramina and Sokoloff (1999) and Sandral et al. (2006), *L. creticus* should be assigned to the section *Pedrosia*, while *L. cytisoides* belongs to another natural group within the genus *Lotus* and should be included in the section *Lotea*. Furthermore, the present study supports the idea of Sandral et al. (2006) that the NW African species *Lotus pseudocreticus* might represent hybrids between *L. creticus* and *L. assakensis*.

Our results are important for establishing future germplasm collection and conservation, as well as breeding and selection programs of perennial *Lotus* species.

We analyzed accessions of *Lotus creticus* from extreme eastern part of the species range (Israel) and from the extreme west of its range (Azores). All produced sequences of *L. creticus* were identical to each other. This suggests that wide distribution area of *L. creticus* might be very recently formed and that infraspecific genetic variation of *L. creticus* is low. Therefore it is probably not worthwhile searching for new germplasm variation of *L. creticus* in natural populations. On the other hand, hybridization with further breeding and selection between *L. creticus*, *L. assakensis* and other species of the section *Pedrosia* might be helpful for producing new and useful genotypes.

In contrast to *L. creticus*, *L. cytisoides* appears to be genetically quite a differentiated species. Our data suggests presence of a geographical genetic variation in *L. cytisoides*. Therefore, collecting new germplasm of *L. cytisoides* from previously not explored areas could be quite promising. In addition, the discovery of non-shattering pods on some whole plants of some accessions of *L. cytisoides* makes it attractive to plant breeding efforts (G. Sandral, pers. comm.). This trait of course is very important in the domestication of

plant species as it allows mechanized seed production to be undertaken.

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