

Phylogeny of *Ononis* in Iran using nuclear ribosomal DNA and chloroplast sequence data

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The genus *Ononis*, embraces more than 85 species worldwide. In the present study, materials of two subspecies of *O. spinosa* from different localities of Iran alongside some other native species of the genus were included in phylogenetic analyses. In addition, over 50 accessions were obtained from GenBank. In order to clarify the exact number of subspecies of *O. spinosa* in Iran, datasets were obtained from sequencing of nuclear ribosomal ITS (nr DNA ITS) and trnL-F plastid (cp DNA) regions and analysed. Three taxa belonging to *O. spinosa* L. complex (*O. spinosa* subsp. *leiosperma*, *O. spinosa* subsp. *antiquorum*, and *O. arvensis*=*O. spinosa* subsp. *arvensis*) were previously reported from Iran. Based on Maximum Parsimony and Bayesian analyses of the molecular datasets, it is shown that, unlike the previous reports, Iranian *O. spinosa* complex belongs to only two subspecies (i.e. *leiosperma* and *arvensis* subsp.). In addition, we also found that, *O. spinosa* subsp. *antiquorum* is not present in Iran. It was also demonstrated that, seed sculpturing does not provide valuable characters in diagnosing different subspecies of *O. spinosa*.

Keywords: nrDNA ITS, molecular phylogeny, species diversity, systematics, trnL-F**تبارزایی جنس *Ononis* براساس توالی‌های DNA ریبوزومی هسته‌ای و کلروپلاستی در ایران**

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جنس *Ononis* (باقلابیان)، دارای بیش از ۸۵ گونه در سرتاسر دنیا می‌باشد. در مطالعه حاضر، مواد دو زیرگونه از نقاط متفاوت ایران به همراه تعدادی از گونه‌های بومی دیگر جنس مورد تحلیل فیلوژنتیکی قرار گرفتند. به علاوه، بیش از ۵۰ توالی نیز از بانک ژن به دست آمد. به منظور تعیین تعداد دقیق زیرگونه‌های *O. spinosa* در ایران، مجموعه داده‌های حاصل از توالی‌یابی نواحی آی.تی.اس. از دی.ان.ای. ریبوزومی هسته‌ای و ناحیه پلاستییدی trnL-F (دی.ان.ای. کلروپلاستی) تحلیل شد. سه آرایه متعلق به کمپلکس گونه‌ای *O. spinosa* (*O. spinosa* subsp. *leiosperma*, *O. spinosa* subsp. *antiquorum* و *O. spinosa* subsp. *arvensis*=*O. spinosa* subsp. *arvensis*) قبلاً از ایران گزارش شده بودند. براساس تحلیل‌های بیشینه صرفه‌جویی و استنباط پیزین مجموعه داده‌های مولکولی، نشان داده شد که برخلاف گزارش‌های پیشین، کمپلکس گونه‌ای *O. spinosa* تنها متعلق به دو زیرگونه است (زیرگونه‌های *leiosperma* و *arvensis*). علاوه بر این، مشخص شد که زیرگونه *O. spinosa* subsp. *antiquorum* در ایران نمی‌روید. همچنین، نشان داده شد که تزیینات سطح دانه نمی‌تواند صفات مناسبی را در تشخیص زیرگونه‌های مختلف *O. spinosa* تأمین نماید.

واژه‌های کلیدی: تبارزایی مولکولی، تنوع گونه‌ای، آی.تی.اس. ریبوزومی هسته‌ای، سیستماتیک، trnL-F

Introduction

Ononis L. (*Fabaceae*) is a genus with more than 85 annual and perennial herbaceous species distributed in North Africa, West Asia, the Mediterranean region and Europe.

Linnaeus first described 12 species of *Ononis* in the Species Plantarum (Linnaeus 1753). Širjaev (1932) divided the genus into two major sections (i.e. *Ononis* and *Natrix*) and 22 subsections, while Turini *et al.* (2010) recognized five major lineages within the genus based on molecular phylogenetic approaches. In his later study, most of the subsections were well reflected by the molecular data (Turini *et al.* 2010). Furthermore, Turini *et al.* (l.c.) confirmed the results of Steele & Wojciechowski (2003) who had distinguished *Ononis* as a monophyletic group.

Rechinger (1984) reported 11 species of *Ononis* from the area of "Flora Iranica". According to him (l.c.), seven species i.e. *O. reclinata* L., *O. viscosa* L., *O. sicula* Guss., *O. pusilla* L., *O. arvensis* L., *O. spinosa* L., and *O. serrata* Forssk., occur in north, west and south of Iran. *Ononis spinosa* is the only perennial spinose species within the genus reported from Iran. This widespread species, which is known also from Europe, has lateral uniflorous flowers with sessile or sub-sessile glands on its calyx-lobes (Huber-Morath 1970). Overall, eight subspecies are reported for *O. spinosa* in the world (Kalvij 2012).

Greuter *et al.* (1989) considered four taxa under *Ononis spinosa*, i.e. *O. spinosa* subsp. *arvensis* (L.) Greuter & Burdet, *O. spinosa* subsp. *australis* (Širj.) Greuter & Burdet, *O. spinosa* subsp. *masquillierii* (Bertol.) Greuter & Burdet and finally *O. spinosa* subsp. *spinosiformis* (Simk.) Greuter & Burdet, but later, two of them i.e. subsp. *arvensis* and subsp. *spinosiformis* were considered as synonyms (Kalvij 2012).

Boissier (1872) introduced two further distinct perennial species, i.e. *O. Antiquorum*, and *O. leiosperma* for Iran which were later reduced to subspecies under *O. spinosa*, i.e. *O. spinosa* subsp. *antiquorum* (L.) Briq., and *O. spinosa* subsp. *leiosperma* (Boiss.) Širj., respectively. The two subspecies are distinguished from

each other by the presence of tuberculate seeds in subsp. *antiquorum* and the smooth ones in subsp. *leiosperma* as the only morphological key character. Rechinger (1984) reported *O. spinosa* subsp. *leiosperma*, originally described from south Turkey, as the only subspecies that grows wild in Iran.

Akhani (1996) reported *O. spinosa* subsp. *antiquorum* from Golestan National Park (northeast of Iran) and Yusef Naanaie (2009) confirmed the presence of this subspecies in central and western part of Iran. Akhani (l.c.) also stated that, *O. spinosa* group in Asia, Afghanistan and NE Iran presents a complicated taxonomic problem. *Ononis spinosa* subsp. *antiquorum*, has a Mediterranean distribution and it is retained as a subspecies of *O. spinosa* in many European publications (Širjaev 1932, Greuter *et al.* 1989), while most of the Russian botanists such as Muravjeva (1945) and Nikitin & Gel'dihanov (1988) preferred using the specific level for this taxon.

In this study, we have performed the phylogenetic analyses of datasets containing nr DNA ITS (Internal Transcribed Spacer) and *trnL-F* spacer sequences of *Ononis* taxa including all its Iranian members to clarify their taxonomic position. Subsequently, we have found the exact number of *Ononis* species and subspecies in Iran. Due to some uncertainty and ambiguity about the taxonomic status of *O. spinosa* in Iran, we especially focused on the material representing different subspecies of this species in our analyses, in order to find out whether those reports of different subspecies of *O. spinosa* are correct or not and also how many subspecies are distributed in Iran.

Materials and Methods

Leaf samples were taken from herbarium specimens deposited at TARI and IAUH (abbreviations according to Thiers, 2015+). Ten samples from six *Ononis* spp. were sequenced especially for this study: *Ononis serrata*, and *O. spinosa* from different localities of Iran (with either smooth or tuberculate seeds), *O. arvensis* (syn.: *O. spinosa* subsp. *arvensis*), *O. pusilla*, *O. reclinata* and finally *O. sicula*. Table 1 represents a

list of taxa already reported from Iran. No herbarium specimen matching the description of *O. viscosa* was found in two mentioned above herbaria and also those which we had access to.

Moreover, 51 and 53 accessions from GenBank were included in the analyses of *trnL-F* and ITS regions, respectively (Appendix 1). *Trigonella caerulea* (L.) Ser. was elected as outgroup (see Steele & Wojciechowski 2003, Wojciechowski *et al.* 2004, Turini *et al.* 2010). The sources of plant material for DNA isolation and GenBank accession numbers for ITS and *trnL-F* sequences used in the analyses are given in Appendix 1.

Following manufacturer's protocol, total genomic DNA was extracted from dried leaf specimens (approximately 0.5 g material per sample) using NucleoSpin Plant-Kit (Macherey-Nagel, Düren, Germany). The complete Internal Transcribed Spacer (ITS) region was amplified using forward primer AB101 (5'-ACGAATTCAAGGTCCGGTGAAGTGTTTCG-3') and reverse primer AB102 (5'-TAGAATTCCTCCCGGTTCTCGCTCGCCGTTAC-3') (Douzery *et al.* 1999). The *trnL-F* spacer region was amplified using forward (5'-CGAAATCGGTAGACGCTACG-3') and reverse (5'-ATTTGAACTGGTGACACGAG-3') primers (Taberlet *et al.* 1991). Following program used for amplification of nuclear regions (ITS1 and ITS2) in a PCR reaction: a pretreatment at 94° C for 2 min 30s; 40 cycles of denaturation at 94° C for 30s, annealing at 54.5° C for 30s and extension at 72° C for 1 min 15s; and a final extension at 72° C for 10 min. To amplify the plastid region *trnL-F*, following PCR parameters used: 40 cycles, each comprised of denaturation at 94° C for 30s, annealing at 55.3° C for 30s and extension at 72° C for 1 min 30s, the other steps stayed unchanged. Amplification products were purified using NucleoSpin® Extract II-Kit (Macherey-Nagel, Düren, Germany) following manufacturer's protocol. Purified PCR products were sequenced on an ABI 3730 automatic sequencer machine (Applied Biosystems/Hitachi) and achieved chromatograms were edited using Sequencher v. 4.8 software (Gene Codes Corporation). Manual sequence alignment was carried out using Macclade

v. 4.08a software (Maddison & Maddison 2005). After editing and aligning the sequences, resulting data matrix was analyzed using PAUP* v.4.0b10 (Swofford 2003) and MrBayes v.3.1.2 program (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). Maximum Parsimony (MP) analysis was carried out in PAUP* using heuristic search strategies that following these settings: simple sequence addition, TBR branch swapping. All characters were unordered and equally weighted and the relative level of support was assessed by bootstrap analysis for individual clades in strict consensus tree (Felsenstein 1985). The ModelTest software v. 3.7 was used in order to select the best substitution model (Posada & Crandall 1998, Posada & Buckley 2004). Bayesian analysis of the ITS1+ITS2, *trnL-F* and combined ITS1+ITS2+*trnL-F* datasets were performed using following parameters: for ITS datasets: [Model selected: TVM+I+G, frequency of base A=0.2293, C=0.2174, G=0.2425 and T=0.3107; Substitution model: (A-C=1.3972, A-G=2.9763, A-T=1.2096, C-G=0.6831, C-T=2.9763, G-T=1.0000), Invariable sites proportion (I): 0.2736, Gamma distribution shape parameter: 0.5878]; For *trnL-F* datasets: [Model selected: TVM+I+G, The base frequencies (A=0.3616, C=0.1739, G=0.1649 and T=0.2995); Substitution model: (A-C=0.7082, A-G=0.6724, A-T=0.4147, C-G=0.8420, C-T=0.6724, G-T=1.0000), Invariable sites proportion (I): 0.4553; Gamma distribution shape parameter: 0.9248]; For combined datasets: [Model selected: TIM+I+G; Base frequencies: A=0.2955, C=0.1929, G=0.2091 and T=0.3025; Substitution model: (A-C=1.0000, A-G=1.6847, A-T=0.7499, C-G=0.7499, C-T=2.3530, G-T=1.0000), Invariable sites proportion (I): 0.4227, Gamma distribution shape parameter: 0.6795]. The Markov Chain Monte Carlo process was set and four chains were run for the total of 3,000,000 generations simultaneously. Overall, 30,000 trees were saved in the primary choices (one tree out of 100 generations). After discarding 25% of primary trees, posterior probability of the phylogeny and its branches was determined from the remaining 22500 trees. Internodes with 0.95 posterior probabilities were considered as well supported. Bayesian analysis led to a 50% majority rule consensus tree.

Table 1. List of *Ononis* species accompanied by their morphology and distribution in Iran

Taxon	Morphology	Distribution in Iran	Reference
<i>O. pusilla</i> L.	Perennial, stems erect to ascending erect, glandular-hairy. Flowers axillary, overtopped by floral leaves, forming dense terminal racemes. Peduncle inconspicuous. Calyx lobes ovate-lanceolate to lanceolate. Corolla yellow. Legume ovate-rhomboid. Seeds orbicular, slightly tuberculate.	N, W	Rechinger 1984, Huber-Morath 1970
<i>O. reclinata</i> L.	Annual, without spines, mainly branched from the base, glandular-villous. Flowers scattered or forming a short leafy raceme. Calyx lobes sub equal, linear-lanceolate, peduncle conspicuous (5-10mm), awnless. Corolla pink to purple. Legume linear-oblong, glandular-villous. Seeds orbicular, rough-tubercled.	N, W, S	"
<i>O. serrata</i> Forssk.	Annual, viscid pubescent, branched from the base, with decumbent or prostrate stems, without spines. Flowers sessile or sub sessile, forming dense oblong terminal racemes. Peduncle conspicuous. Calyx lobes lanceolate-subulate, corolla pink, glandular-hairy. Legume ovate, glandular-pubescent. Seeds elliptic to sub-orbicular, tuberculate.	W, S	"
<i>O. sicula</i> Guss.	Annual, commonly erect, branched from the base, with dense glandular hairs. Flowers solitary, arranged in a leafy raceme. Peduncle conspicuous (10-30 mm). Corolla yellow, 6-9 mm long, legume linear-oblong. Seeds reniform-orbicular.	W, S	"
<i>O. arvensis</i> L. [= <i>O. spinosa</i> subsp. <i>arvensis</i> (L.) Greuter & Burdet]	Perennial, spinose, with erect ascending stems. Flowers shortly pedicellate, mainly congested into ovate terminal raceme. Calyx lobes linear-lanceolate, with numerous long eglandular and shorter glandular hairs. Corolla pink with purple stripes. Seeds orbicular, tuberculate.	C	"
<i>O. spinosa</i> L. subsp. <i>leiosperma</i> (Boiss.) Širj.	Perennial, spinose, with erect ascending stems. Flowers shortly pedicellate, solitary or raceme. Calyx lobes with sessile or sub-sessile glands, sometimes glabrescent. Seeds smooth.	W, S	"
<i>O. viscosa</i> L.	Annual, often branched from the base. Flowers forming long leafy racemes. Peduncle conspicuous (10-25 mm), long awned, one flowered. Calyx lobes linear acuminate. Corolla pale yellow, often striped with red. Legume linear to linear oblong, glandular- hairy. Seeds kidney-shaped to orbicular, tuberculate.	W, S	"

Results

- ITS datasets

In Maximum Parsimony (MP) analysis of ITS datasets, 10000 shortest trees with the length of 577 evolutionary steps, CI=0.633 and RI=0.867 were combined to form a strict consensus tree with 588 steps, CI=0.621 and RI=0.860. For *trnL-F* datasets, 10000 shortest trees with the length of 164 evolutionary steps, CI=0.793 and RI=0.911 were combined to form a strict consensus tree of 178 steps, CI=0.730 and RI=0.874. In analysis of combined (ITS1+ITS2+trnL-F) datasets 10000 shortest trees with 670 steps length, CI=0.676 and RI= 0.874 were obtained and combined into a strict consensus tree with 680 evolutionary steps length, CI=0.666 and RI=0.869.

The strict consensus tree obtained from Maximum Parsimony (MP) analysis of the nrDNA ITS, is shown in Fig. 1. Both bootstrap support (BS) (derived from MP analysis) and posterior probability (PP) (resulted from Bayesian analysis) are also shown in Fig. 1. In the strict consensus tree, *Ononis* taxa including the Iranian ones and those obtained from GenBank are grouped into two

major clades: 1) Clade A: This well-supported clade (BS: 100%, PP: 1.00) comprise three species i.e. *O. rotundifolia* L., *O. fruticosa*, and *O. tridentata* subsp. *tridentata*. All members of this clade belong to the same section, i.e. section *Natrix*. 2) Clade B: This clade is divided into four subclades (i.e. B1, B2, B3 and B4). Two sub-clades, B1 and B3, are strongly supported (BS: 100%, PP: 1.00). Clade B1 consists of *O. reuteri* Boiss. & Reuter and *O. speciosa* Lagasca, both belonging to subsection *Chrysanthae* Willkomm. Clade B3 contains only one Iranian specimen of *O. pusilla* along with *O. pusilla* L. subsp. *saxicola* (Boiss.) Malag., *O. cephalotes* Boiss., *O. minutissima* L. and finally, *O. striata* Gouan, all obtained from GenBank. All these taxa belong to the same subsection i.e. *Bugranoides* (see Table 1). Clade B2 is weakly supported and includes only one species i.e. *O. ornithopodioides* L. Clade B4 consists of two major lineages among which only B4a is well supported (BS: 100%, PP: 1.00) and contains only one Iranian specimen of *O. sicula* but also *O. aurasica* Förther & Podlech, *O. ramosissima* Desf., *O. tazaensis* Förther & Podlech, *O. hesperia* (Maire) Förther &

Podlech, *O. mogadorensis* Förther & Podlech, *O. paralias* Förther & Podlech, *O. angustissima* Lam. subsp. *longifolia* (Willd.) Förther & Podlech, *O. pubescens* L., *O. antennata* Pomel subsp. *antennata*, *O. pseudoserotina* Batt. & Pit., *O. serotina* Pomel, *O. zygantha* Maire & Wilczek, *O. polyphylla* Ball and finally *O. polysperma* Barratte & Murb. In subclade B4b, *O. adenotricha* Boiss., is weakly supported as sister to other species nested in this clade. The secondary lineage (B4b) is also divided into two clades, one of which is strongly supported (BS: 90%). *Ononis christi* Bolle, and *O. laxiflora* Desf. are supported as sisters (Fig. 1; BS: 100%, PP: 1.00). In this clade, the two species *O. leucotricha* Coss., and *O. pinnata* Brot., belonging to the subsection *Pinnatae*, are sisters (MP BS= 100%, PP: 1.00). Group I, placed in clade B4b (BS: 100%, PP: 1.00), is divided into two subgroups, i.e. Ia and Ib. *Ononis alba* Poir. subsp. *monophylla* (Desf.) Murb. is the only species placed in subgroup Ia, while other *Ononis* species containing *O. nuristanica* Podlech (group A), *O. spinosa* subsp. *spinosa* (group B), *O. spinosa* subsp. *masquillierii*, *O. spinosa* subsp. *maritima*, and *O. spinosa* subsp. *antiquorum* (group C), four specimens of *O. spinosa* species (group D), with either smooth or tuberculate seeds, collected from different localities of Iran and finally three specimens of *O. spinosa* subsp. *arvensis* (two of which are Iranian samples shown in bold in Fig. 1; groups F, G) are placed in clade Ib, with high bootstrap support (BS: 100%). Four specimens of *O. spinosa* strongly nested in a separate group (group D; BS: 80%, PP: 0.95) and three *O. spinosa* subsp. i.e. *masquillierii*, *antiquorum*, and *maritima* (group C) (obtained from GenBank) are clustered in a separate clade with moderate bootstrap support (BS: 80%) and full maximum posterior probability (PP: 1.00), among which two first subspecies were sisters (BS: 70%, PP: 0.95). Moreover, the clade encompassing all

O. spinosa subspecies accompanied by *O. alba* subsp. *monophylla*, and *O. nuristanica* are well supported by full Bayesian posterior probabilities (PP: 1.00) (Fig. 1).

- trnL-F datasets

The *trnL-F* matrix included 51 *Ononis* taxa and only one species as outgroup. The species were grouped into six major lineages (A–F) (Fig. 2). Most of the results of trnL-F datasets were in line with those obtained from ITS.

Clade C, a well-supported clade, (BS: 100%, PP: 1.00) shows polytomy. Three taxa i.e. *O. biflora* Desf., *O. polysperma* and *O. zygantha* with strong bootstrap support and posterior probability are placed in this clade (BS: 70%, PP: 0.56) one of which i.e. *O. biflora* was absent in ITS topology. All *Ononis spinosa* subspecies containing Iranian ones (subsp. *leiosperma*, *arvensis*, *maritima*, and *spinosa*), together with *O. nuristanica*, have constructed a polytomy (BS: 80%, PP: 0.82).

- Combined (ITS+trnL-F) datasets

The results of combined datasets are shown in Fig. 3. Species are divided into two major clades A and B. Clade B is divided into four subclades B1, B2, B3 and B4. The Iranian *O. spinosa* subsp., i.e. *O. spinosa* subsp. *arvensis*, *O. spinosa* and *O. spinosa* subsp. *leiosperma*, together with *O. spinosa* subsp. *maritima*, *O. spinosa* subsp. *masquillierii*, *O. spinosa* subsp. *spinosa*, *O. nuristanica*, and *O. alba* subsp. *monophylla* gathered from GenBank, are placed in the same clade and are well supported (B4bII) (BS: 100%, PP: 1.00). In Figure 3, it is quite obvious that, four specimens of *Ononis spinosa* collected from different localities of Iran (two of which were previously distinguished as *O. spinosa* subsp. *antiquorum* by morphological characters) are grouped in a well-supported monophyletic clade (BS: 100%, PP: 1.00). *Ononis spinosa* subsp. *masquillierii*, and *O. spinosa* subsp. *maritima* are also placed in the same subgroup (BS: 90%, PP: 1.00).

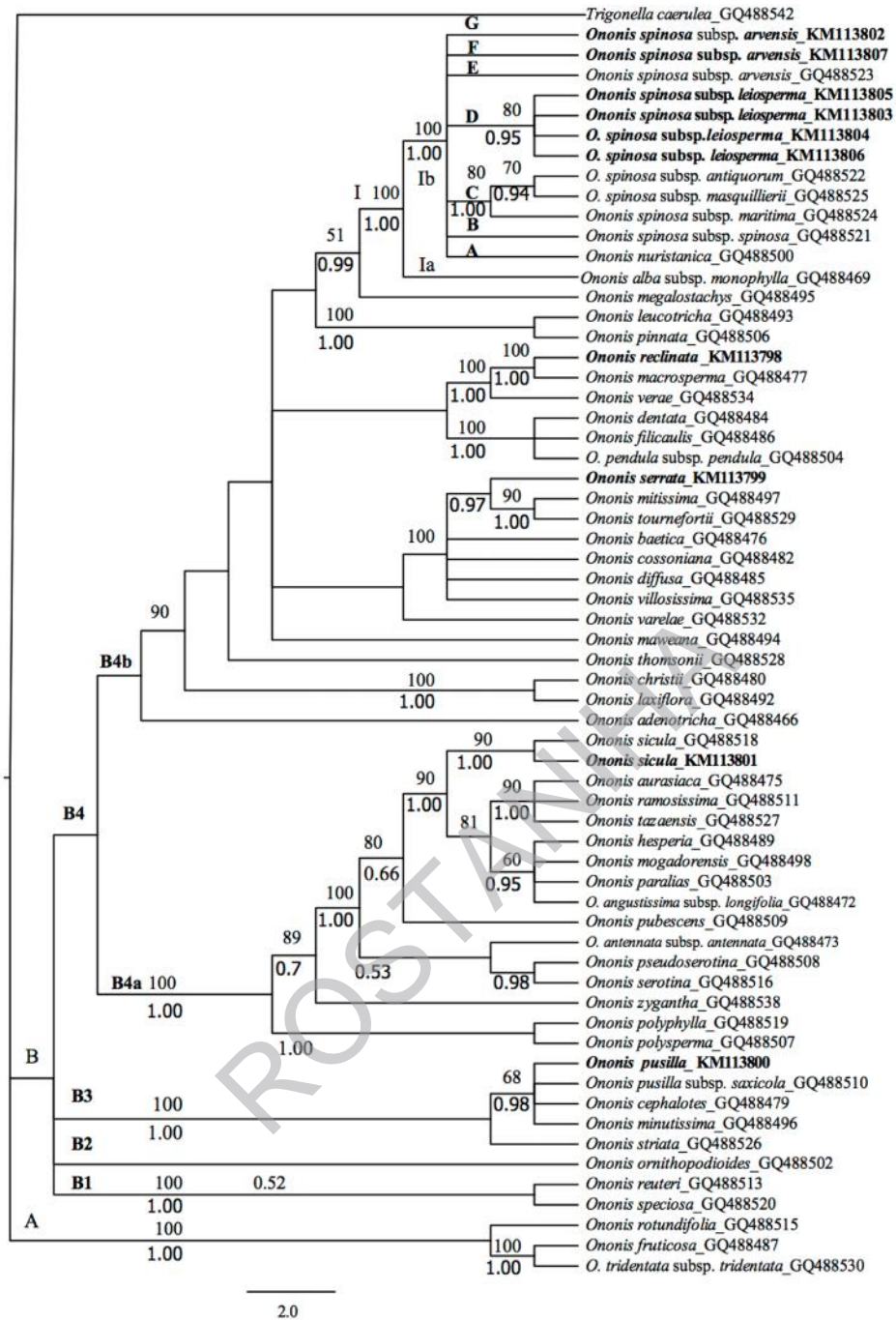


Fig. 1. Strict consensus tree resulted from the Maximum Parsimony analysis of the nrDNA ITS dataset (The newly sequenced accessions are in Bold-Italic). All other sequences are taken from the GenBank. Numbers above branches represent Bootstrap values (BS) and numbers below branches show posterior probabilities (PP) (only values greater than 50 % for BS and greater than 0.50 for PP are shown). *Trigonella caerulea* is outgroup.

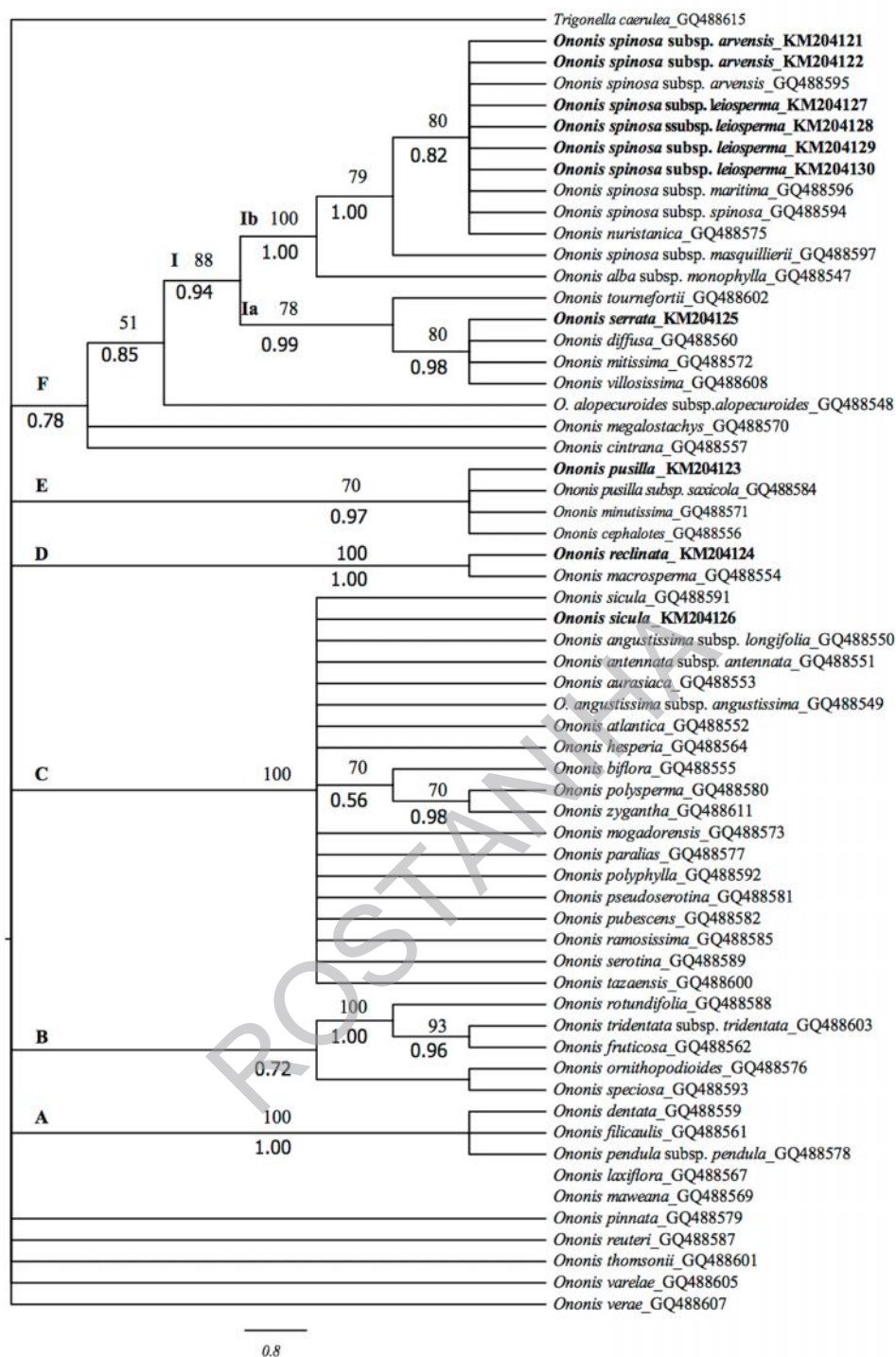


Fig. 2. Strict consensus tree resulted from the Maximum Parsimony analysis of the plastid *trnL*_F spacer dataset (The newly sequenced accessions are in Bold-Italic). All other sequences are taken from the GenBank. Numbers above branches represent Bootstrap values (BS) and numbers below branches show posterior probabilities (PP) (only values greater than 50 % for BS and greater than 0.50 for PP are shown). *Trigonella caerulea* is outgroup.

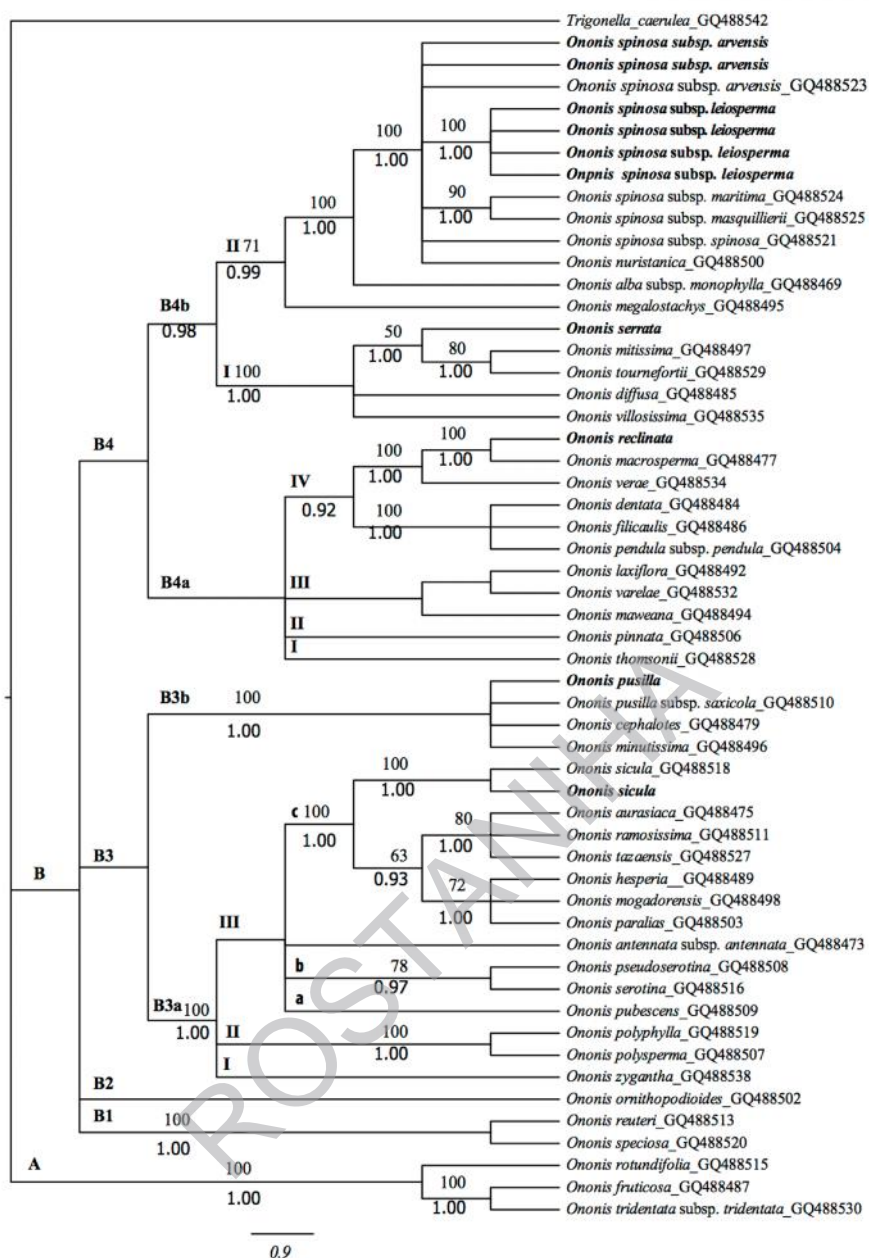


Fig. 3. Strict consensus tree resulted from the Maximum Parsimony analysis of the combined nrDNA ITS+plastid *trnL*_F spacer datasets (The newly sequenced accessions are in Bold-Italic). All other sequences are taken from the GenBank. Numbers above branches represent Bootstrap values (BS) and numbers below branches show posterior probabilities (PP) (only values greater than 50 % for BS and greater than 0.50 for PP are shown). *Trigonella caerulea* is outgroup.

Discussion

The results of this study corroborate those of Turini et al. (2010) in following points: placing the Iranian *Ononis serrata* (subject. *Diffusae*) close to *O. mitissima* (subject. *Mitissimae*) and *O. tournefortii* (subject. *Diffusae*); placing the Iranian *O. reclinata* close to *O. macrosperma* and *O. verae*, and finally, placing *O. pusilla* close to *O. pusilla* subsp. *saxicola*,

O. cephalotes, and *O. minutissima* in the combined datasets. Four specimens of *O. spinosa* from different parts of Iran were investigated. These specimens were previously distinguished as two separate subspecies (i.e. *O.* subsp. *leiosperma* with “smooth” seed coat and subsp. *antiquorum* with “tuberculated” seed coat) (Akhani 1996). The phylogenetic results showed that, all the mentioned specimens formed a monophyletic clade with

robust support (Figs 1 & 3). It is shown that, Iranian samples of *O. spinosa* with different seed coat ornamentation could not be separated as different taxa. However, *O. spinosa* subsp. *antiquorum*, from GenBank, was placed in a different clade in the ITS analysis (Fig. 1). This indicates that, despite a few morphological differences among the Iranian taxa, there is no considerable phylogenetic difference between them (Figs 1 & 3). Having granular or smooth seed surface is not regarded as a diagnostic character to differentiate these closely related Iranian taxa into two separate subspecies (subsp. *leiosperma* and *antiquorum*). Therefore, we concluded that, identification of these subspecies is not correct and both must be identified under a single subspecies. Considering the geographical distribution of *O. spinosa* subsp. *leiosperma*, which is described based on the type material collected from southwest Asia (Boissier 1872), it is more reliable to accept the presence of this subspecies instead of subsp. *antiquorum*. Type material of *O. spinosa* subsp. *antiquorum* is collected from south Europe (Mediterranean region; Huber-Morath 1970). Therefore, *Ononis spinosa* subsp. *leiosperma* could be considered as the only subspecies occurs in Iran. Further investigations based on the materials from south Europe, Iran, Afghanistan and central Asia are needed to clarify taxonomic position of subspecific taxa of *O. spinosa* complex. One of the *Ononis* species that is endemic to Afghanistan and Pakistan, i.e. *O. afghanica* (Syn.: *O. spinosa* subsp. *afghanica* (Širj. & Rech.f.)

Kitamura) reported in Flora Iranica is very complicated and could not be easily separated from those reported from the Mediterranean region under the name *O. spinosa* subsp. *antiquorum*. *Ononis spinosa* subsp. *arvensis* (Syn.: *O. arvensis*) that was collected from northwest Iran is placed in a group encompassing all *Ononis spinosa* subspp. This issue confirms the morphological results of Greuter *et al.* (1989) who have introduced a new combination, i.e. *O. spinosa* subsp. *arvensis* (L.) Greuter & Burdet. With respect to the results obtained in this study, two subspecies have been recognized for *O. spinosa* (i.e. subsp. *arvensis*, and subsp. *leiosperma*), and *O. spinosa* subsp. *antiquorum* is not present in Iran. In conclusion, our molecular phylogenetic data suggest the occurrence of three species of *Ononis* in Iran among which, *O. spinosa* itself is further represented by three subspecies.

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Appendix 1. List of the 67 *Ononis* species and one outgroup, accompanied by other information of each specimen [including GenBank accession number, locality (for Iranian species), collector name, collection number, section/subsection]. The two last column on the right show the presence of each species in either ITS or *trnL-F* sequences analyses (by the signs of + and -)

No.	Taxon	GenBank No. (ITS/ <i>trnL-F</i>)	Collector & collection No.	Locality	Section/subsection	ITS seq.	<i>trnL-F</i> seq.
1	<i>O. spinosa</i> L. subsp. <i>leiosperma</i> (Boiss.) Širj.	KM113804-KM204129	Nowroozi & Etemadi, 1280 (TARI)	Isfahan: above Bojn Cheshmeh Rashid, 2475 m	<i>Ononis / Ononis</i>	+	+
2	<i>O. spinosa</i> L. subsp. <i>leiosperma</i> (Boiss.) Širj.	KM113806-KM204130	Buttler & Buthmer, 22817 (TARI)	Tehran: Firuzkuh-Pol-e sefid, C. 4 km NE of Firuzkuh. 1950 m	<i>Ononis / Ononis</i>	+	+
3	<i>O. spinosa</i> L. subsp. <i>leiosperma</i> (Boiss.) Širj.	KM113805-KM204127	Sanandaji, 1700 (IAUH)	Kurdistan: Sanandaj, Saral-Chattan, 2041 m.	<i>Ononis / Ononis</i>	+	+
4	<i>O. spinosa</i> L. subsp. <i>leiosperma</i> (Boiss.) Širj.	KM113803-KM204128	Rahmani, 7680 (IAUH)	Qazvin: Bashgol, 1900 m	<i>Ononis / Ononis</i>	+	+
5	<i>O. spinosa</i> L. subsp. <i>arvensis</i> (L.) Greuter & Burdet	GQ488523-GQ488595	Heubl, <i>n/a</i>	Spain	<i>Ononis / Ononis</i>	+	+
6	<i>O. spinosa</i> L. subsp. <i>antiquorum</i> (L.) Arcang.	GQ488522	Berger, 4284	Spain	<i>Ononis / Ononis</i>	+	-
7	<i>O. spinosa</i> L. subsp. <i>maritima</i> (Dumort.) P. Fourn.	GQ488524-GQ488596	Schuhwerk, 05/272	Spain	<i>Ononis / Ononis</i>	+	+
8	<i>O. spinosa</i> L. subsp. <i>masquillieri</i> (Bertol.) Greuter & Burdet	GQ488525-GQ488597	Ferrari, <i>s.n.</i>	Spain	<i>Ononis / Ononis</i>	+	+
9	<i>O. spinosa</i> L. subsp. <i>spinosa</i>	GQ488521-GQ488594	Heubl, <i>n/a</i>	Spain	<i>Ononis / Ononis</i>	+	+
10	<i>O. spinosa</i> L. subsp. <i>arvensis</i> (L.) Greuter & Burdet	KM113807-KM204122	Bairami, 5937 (TARI)	Azarbayjan: Kaleybar, Khoda Afarin Arasbaran protected area, Kalaleh village, 1200 m	<i>Ononis / Ononis</i>	+	+
11	<i>O. spinosa</i> L. subsp. <i>arvensis</i> (L.) Greuter & Burdet	KM113802-KM204121	Mozaffarian, 43600 (TARI)	Azarbayjan: 90 km from Varzeghan to Jofa, 1000 m	<i>Ononis / Ononis</i>	+	+
12	<i>O. serrata</i> Forssk.	KM113799-KM204125	Mozaffarian, 62663 (TARI)	Khuzestan: Susangerd, Bostan, Allahoakbar, 60 m	<i>Ononis / Diffusae</i> Širj.	+	+
13	<i>O. pusilla</i> L.	KM113800-KM204123	Hamze & Asri, 81601 (TARI)	Azarbayjan: Arasbaran protected area, Vaighan to Kalaleh, Research forests, 1150 m	<i>Ononis / Bugranoides</i> Willk.	+	+
14	<i>O. pusilla</i> L. subsp. <i>saxicola</i> (Boiss. & Reut.) Malag.	GQ488510-GQ488584	Reverchon, 1333	Spain	<i>Ononis / Bugranoides</i> Willk.	+	+
15	<i>O. reclinata</i> L.	KM113798-KM204124	Mozaffarian, 63202 (TARI)	Khuzestan: Elize Parke Jangali, 850 m	<i>Natrix</i> (Moench) Griseb / <i>Reclinatae</i> Širj.	+	+
16	<i>O. sicula</i> Guss.	KM113801-KM204126	Mozaffarian, 59161 (TARI)	Bandar-Abbas: Genu Mts., 1200-2200 m	<i>Natrix / Reclinatae</i> Širj.	+	+
17	<i>O. sicula</i> Guss.	GQ488518-GQ488591	Lobin, 7	Morocco	<i>Natrix / Reclinatae</i> Širj.	+	+
18	<i>O. angustissima</i> Lam. subsp. <i>longifolia</i> (Willd.) Förther & Podlech	GQ488550	Ginové <i>et al.</i> , 16.222	Tenerife	<i>Natrix / Natrix</i>	-	+

Appendix 1 (contd.)

19	<i>O. angustissima</i> Lam. subsp. <i>angustissima</i>	GQ488549	Frahm <i>et al.</i> , 9	Algeria	<i>Natrix / Natrix</i>	-	+
20	<i>O. nuristanica</i> Podlech	GQ488500- GQ488575	Podlech, 727	Afghanistan	<i>Ononis / Ononis</i>	+	+
21	<i>Ononis alba</i> Poir. subsp. <i>monophylla</i> (Desf.) Murb.	GQ488469- GQ488547	Podlech, 39084	Algeria	<i>Ononis / Villosissimae</i> Širj.	+	+
22	<i>O. antennata</i> Pomel subsp. <i>antennata</i>	GQ488473- GQ488551	Deil, 1023	Morocco	<i>Natrix / Viscosae</i> Širj.	+	+
23	<i>O. aursiaca</i> Förther & Podlech	GQ488475- GQ488553	Podlech, 38634	Algeria	<i>Natrix / Natrix</i>	+	+
24	<i>O. alopecuroides</i> L. subsp. <i>alopecuroides</i>	GQ488548	Erben, <i>s.n.</i>	Sicily	<i>Ononis / Salzmannianae</i> Širj.	-	+
25	<i>O. rotundifolia</i> L.	GQ488515- GQ488588	Weimer, <i>s.n.</i>	Switzerland	<i>Natrix / Antiquae</i> Širj.	+	+
26	<i>O. atlantica</i> Ball	GQ488552	Podlech, 47466	Morocco	<i>Natrix / Natrix</i>		+
27	<i>O. dentata</i> Sol. ex Lowe	GQ488484- GQ488559	Gaisberg, 162	Canary Islands	<i>Natrix / Reclinatae</i> Širj.	+	+
28	<i>O. filicaulis</i> Salzm. ex Boiss.	GQ488486- GQ488561	Lobin, 321a	Spain	<i>Ononis / Villosissimae</i> Širj.	+	+
29	<i>O. hesperia</i> (Maire) Förther & Podlech	GQ488489- GQ488564	Podlech, 48769	Morocco	<i>Natrix / Natrix</i>	+	+
30	<i>O. laxiflora</i> Desf.	GQ488492- GQ488567	Breitfeld, <i>s.n.</i>	Gran Canaria	<i>Natrix / Reclinatae</i> Širj.	+	+
31	<i>O. biflora</i> Desf.	GQ488555	Liston, <i>s.n.</i>	Israel	<i>Natrix / Biflorae</i> Širj.	-	+
32	<i>O. macrosperma</i> Hub.-Mor.	GQ488477- GQ488554	Hertel, 34.138	Turkey	<i>Natrix / Reclinatae</i> Širj.	+	+
33	<i>O. maweana</i> Ball	GQ488494- GQ488569	Lambinon & Lewalle, 84/M/90	Morocco	<i>Natrix / Pilosae</i> Širj.	+	+
34	<i>O. megalostachys</i> Munby	GQ488495- GQ488570	Faure, <i>s.n.</i>	Algeria	<i>Ononis / Verae</i> Širj.	+	+
35	<i>O. minutissima</i> L.	GQ488496- GQ488571	Grau, <i>et al.</i> , <i>s.n.</i>	Spain	<i>Ononis / Bugranoides</i> Willk.	+	+
36	<i>O. mogadorensis</i> Förther & Podlech	GQ488498- GQ488573	Podlech, 46441	Morocco	<i>Natrix / Natrix</i>	+	+
37	<i>O. ornithopodioides</i> L.	GQ488502- GQ488576	Zohary & Liston, 7-82-53	Israel	<i>Natrix / Torulosae</i> Širj.	+	+
38	<i>O. paralias</i> Förther & Podlech	GQ488503- GQ488577	Podlech, 48353	Morocco	<i>Natrix / Natrix</i>	+	+
39	<i>O. pendula</i> Desf. subsp. <i>pendula</i>	GQ488504- GQ488578	Förther, 1297	Sicily	<i>Natrix / Reclinatae</i> Širj.	+	+
40	<i>O. pinnata</i> Brot.	GQ488506- GQ488579	Segura Zubizarreta, 27464	Spain	<i>Ononis / Pinnatae</i> Širj.	+	+
41	<i>O. polyphylla</i> Ball	GQ488519- GQ488592	Podlech, 41542	Morocco	<i>Natrix / Reclinatae</i> Širj.	+	+
42	<i>O. polysperma</i> Barratte & Murb.	GQ488507- GQ488580	Podlech, 43002	Morocco	<i>Natrix / Biflorae</i> Širj.	+	+
43	<i>O. pseudoserotina</i> Batt. & Pit.	GQ488508- GQ488581	Krach & Koepff, 3784	Morocco	<i>Natrix / Natrix</i>	+	+
44	<i>O. pubescens</i> L.	GQ488509- GQ488582	Kramer & Kürschner, 4258	Turkey	<i>Natrix / Viscosae</i> Širj.	+	+
45	<i>O. ramosissima</i> Desf.	GQ488511- GQ488585	Erben, <i>s.n.</i>	Sardinia	<i>Natrix / Natrix</i>	+	+

Appendix 1 (contd.)

46	<i>O. reuteri</i> Boiss. & Reut.	GQ488513- GQ488587	Huter, 34484	Spain	<i>Ononis / Chrysanthae</i> (Willk.) Širj.	+	+
47	<i>O. serotina</i> Pomel	GQ488516- GQ488589	Faure, <i>s.n.</i>	Algeria	<i>Natrix / Natrix</i>	+	+
48	<i>O. speciosa</i> Lag.	GQ488520- GQ488593	Nydegger, 33772	Spain	<i>Ononis / Chrysanthae</i> (Willk.) Širj.	+	+
49	<i>O. cephalotes</i> Boiss.	GQ488479- GQ488556;	Lippert, W. & B. 9914	Spain	<i>Ononis / Bugranoides</i> Willk.	+	+
50	<i>O. tazaensis</i> Förther & Podlech	GQ488527- GQ488600	Podlech, 46441	Morocco	<i>Natrix / Natrix</i>	+	+
51	<i>O. thomsonii</i> Oliv.	GQ488528- GQ488601	Podlech, 47638	Morocco	<i>Natrix / Mauritanicae</i> Širj.	+	+
52	<i>O. tournefortii</i> Coss.	GQ488529- GQ488602	Podlech, 53259a	Morocco	<i>Ononis / Diffusae</i> Širj.	+	+
53	<i>O. tridentata</i> L. subsp. <i>tridentata</i>	GQ488530- GQ488603	Nydegger, 35832	Spain	<i>Natrix / Rhodanthae</i> Willk.	+	+
54	<i>O. fruticosa</i> L.	GQ488487- GQ488562	Lambinon, 00/F/250	France	<i>Natrix / Rhodanthae</i> Willk.	+	+
55	<i>O. baetica</i> Clemente	GQ488476	Merxmüller & Lippert, 23326	Spain	<i>Ononis / Diffusae</i> Širj.	+	-
56	<i>O. diffusa</i> Ten.	GQ488485- GQ488560	Baumann, 0/306	Rhodes	<i>Ononis / Diffusae</i> Širj.	+	+
57	<i>O. mitissima</i> L.	GQ488497- GQ488572	Baumann, <i>s.n.</i>	Tenerife	<i>Ononis / Mitissimae</i> Širj.	+	+
58	<i>O. cintrana</i> Brot.	GQ488557	Nydegger, 33874	Spain	<i>Ononis / Intermediae</i> Širj.	-	+
59	<i>O. vilosissima</i> Desf.	GQ488535- GQ488608	Podlech, 45394	Morocco	<i>Ononis / Villosissimae</i> Širj.	+	+
62	<i>O. verae</i> Širj.	GQ488534- GQ488607	Širjaev, 12192	Crete	<i>Natrix / Reclinatae</i> Širj.	+	+
63	<i>O. christii</i> Bolle	GQ488480	Bramwell, 10362	Fuerteventura	<i>Natrix / Canariensis</i> Širj.	+	-
64	<i>O. cossoniana</i> Boiss. & Reut.	GQ488482	Conert <i>et al.</i> , 682	Spain	<i>Ononis / Diffusae</i> Širj.	+	-
65	<i>O. leucotricha</i> Coss.	GQ488493	Segura Zubizarreta, 42580	Spain	<i>Ononis / Pinnatae</i> Širj.	+	-
66	<i>O. striata</i> Gouan	GQ488526	Dubuis, 7547	France	<i>Ononis / Bugranoides</i> Willk.	+	-
67	<i>O. adenotricha</i> Boiss.	GQ488466	Nydegger, 42160	Turkey	<i>Natrix / Natrix</i>	+	-
68	<i>Trigonella caerulea</i> Ser.	GQ488542- GQ488615	Podlech, 37947	Austria	-	+	+