A phylogeny of Onobrychis and its relationships with allied genera of Hedysareae.

TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2013) 37: 981-992

doi:10.3906/bot-1210-32

A phylogenetic analysis of genus *Onobrychis* and its relationships within the tribe *Hedysareae* (Fabaceae)

Nadeesha LEWKE BANDARA[1,*], Alessio PAPINI[1,*,], Stefano MOSTI[2], Terence BROWN[3], Lydia Mary Josephine SMITH[1]**

[1]The John Bingham Laboratory, National Institute of Agricultural Botany, Cambridge, UK
[2]Department of Evolutionary Biology, University of Florence, Florence, Italy
[3]Faculty of Life Sciences, Manchester Interdisciplinary Biocentre, University of Manchester, Manchester, UK

Received: 19.10.2012 • **Accepted:** 28.07.2013 • **Published Online:** 30.10.2013 • **Printed:** 25.11.2013

Abstract: Results about a phylogenetic analysis of the genus *Onobrychis* Mill., tribe *Hedysareae* DC. are presented. The systematic knowledge of tribe *Hedysareae* is still incomplete, with difficult circumscription of genera and species. Analyses were undertaken using both nuclear (ITS) and chloroplast (matK) markers for a set of 78 accessions covering 41 *Onobrychis* species, besides previously sequenced *Hedysareae* accessions. The phylogenetic methods used were maximum parsimony, maximum likelihood, and Bayesian analyses to produce phylogenetic trees and robustness indices. The genus *Onobrychis* was resolved as paraphyletic, with species of the genera *Eversmannia* Bunge and *Hedysarum* L. nested within it. The position of the section *Membranacea* of genus *Hedysarum* was as a sister group to *Onobrychis* and *Eversmannia*, separated from other accessions of *Hedysarum*. Variation in the 2 markers was sufficient to resolve infrageneric groups in *Onobrychis* and *Hedysarum*, but we were unable to completely resolve certain species in *Onobrychis*, particularly those within the sect. *Onobrychis*. The cause of this difficult species delimitation may be related to recent speciation, hybridization, and introgression events, particularly between cultivated species and their wild relatives, and the presence of cryptospecies as suggested by intraspecific polyploid series.

Key words: ITS sequences, Leguminosae, matK sequences, molecular phylogenetic

1. Introduction

The tribe *Hedysareae* DC. comprises a group of genera of family Fabaceae currently circumscribed to: *Taverniera* DC., *Stracheya* Benth., *Eversmannia* Bunge, *Hedysarum* L., *Corethrodendron* Basiner, *Alhagi* Adans., *Ebenus* L., *Onobrychis* Mill. (Polhill, 1981; Thulin, 1985), and *Sartoria* Boiss. (Arslan et al., 2012). The genera *Calophaca* Fisch., *Caragana* Lam., and *Halimondendron* Fisch. ex DC., which were previously treated in tribe Galegeae, were transferred to tribe *Hedysareae* by Lock (2005). Members of *Hedysareae* are commonly found in dry open habitats with a continental, temperate, or Mediterranean climate, including Eurasia, North America, and the Horn of Africa (Ahangarian et al., 2007). Some taxa of the tribe are economically important as fodder legumes due to their high protein content (Hayot Carbonero et al., 2011).

Molecular analyses by Wojciechowski et al. (2004) and Lavin et al. (2005) showed that *Caragana* Fabr. was the most closely related sister group to the rest of the tribe *Hedysareae*.

Hedysareae is included in the Inverted Repeat Lacking Clade (IRLC) group sensu Wojciechowski et al. (2000, 2004) and Wojciechowski (2003, 2005). In more recent studies, it has been suggested that *Hedysareae* sensu Lock (2005) is a sister group to the Astragalean clade, which includes genera such as *Astragalus* L., *Oxytropis* DC., and *Colutea* L., in addition to *Chesneya* Bertol. and its close relatives (Lock and Schrire, 2005). According to Lavin et al. (2005) the most recent common ancestor of the *Hedysareae* and the Astragalean clade originated between 25.0 and 39.2 million years ago.

The genus *Onobrychis* is divided into 2 subgenera: *Onobrychis* and *Sisyrosema* Bunge (Schischkin and Bobrov, 1971; Rechinger, 1984; Ahangarian et al., 2007). These 2 subgenera are characterized by different karyotype features and geographical origins (Rechinger, 1984; Hejazi et al., 2010). The main genera of the tribe are *Hedysarum*, with about 160 species (Ahangarian et al., 2007), and *Onobrychis*, with at least 162 species (Yildiz et al., 1999).

* These 2 authors contributed equally to this work.
** Correspondence: alpapini@unifi.it

Hedysarum and *Onobrychis* were separated taxonomically on the basis of fruit morphology, in addition to pollen structure, chromosome number, and biochemical features (Polhill, 1981; Yildiz et al., 1999).

Different approaches that have been used to define the taxonomy of *Onobrychis* in terms of species and infrageneric taxa circumscription have led to contradictions and uncertainty. This is probably due to the fact that only a limited number of characters have been considered in any one of the available taxonomic descriptions (Boissier, 1872; Ball, 1968; Hedge, 1970; Rechinger, 1984). We used the sectional treatment of Schischkin and Bobrov (1972), with updates by Yildiz et al. (1999) and Ahangarian et al. (2007).

The most frequently used characters are: annual or perennial habit, number of ovules, adnate or free stipules, size, the proportion or character of the indumentum, fruit morphology, and seed number. Yildiz et al. (1999), for example, outlined a classification based mainly on fruit morphology using a sample of 40 species for 5 sections of a total of 162 species classified into 2 subgenera and 8 sections. In addition to the other morphological data, Dolya and Vasilissa (2000) and Avcı et al. (2013) used pollen morphology, while Irfan et al. (2007) used electrophoretic analysis of total seed proteins to study the systematics of *Onobrychis*. Unfortunately, the number of species included in these last 2 studies was too low to draw clear general conclusions on the genus.

A detailed taxonomic investigation of the genus *Onobrychis* based on molecular markers is still lacking. More recently, a molecular investigation using rDNA internal transcribed spacers (ITS) molecular data (Ahangarian et al., 2007) considered the tribe *Hedysareae*. The sample set included 11 species of *Onobrychis*.

The ITS sequences have been shown to elucidate phylogenetic relationships, especially at the species and genus levels (Baldwin et al., 1995; Gültepe et al., 2010; İkinci et al., 2011). Important results in Leguminosae have been obtained with this marker (for instance, Wojciechowski et al., 1999) such that it hence appeared appropriate for our investigation. The *matK* gene is one of the most rapidly evolving plastid-coding regions; it consistently showed high levels of discrimination capability among angiosperm species and was used in many studies, and also in Leguminosae (e.g., Wojciechowski et al., 2004; Terzioğlu et al., 2012 in other angiosperms). A phylogenetic analysis of Leguminosae with the plastid *matK* gene sequences supported many well-resolved subclades within the Leguminosae (Wojciechowski et al., 2004). The results obtained with the *matK* sequences are generally consistent with those obtained from other plastid sequence data (*rbcL* and *trnL*), with higher resolution and clade support in Leguminosae (Hu et al., 2000; Wojciechowski et al., 2004). In our molecular phylogenetic study we used both nuclear (ITS) and the chloroplast matK (partial sequence) markers on a sample set of 78 accessions from 41 *Onobrychis* species. The choice of the plastid matK marker was due also to the fact that this marker, like the rbcL marker, has been chosen as a plant barcoding marker by the Consortium for the Barcode of Life (CBOL Plant Working Group, 2009).

2. Materials and methods

2.1. Sampling material and total DNA extraction

Seed samples were collected from different locations, including the Mediterranean area, North America, Iran, and other areas of Asia. The seeds were stored at the National Institute of Agricultural Botany (NIAB) Gene Bank (Cambridge, UK). Additional samples were obtained from leaves of dried specimens of the Bu-Ali Sina University Herbarium, Iran (for all specimens used in the analysis, see Table 1S in the supplementary material at http://www.unifi.it/caryologia/tjb).

Genomic DNA was isolated either from approximately 40 mg of fresh leaves or from herbarium sample leaves, using the modified Tanksley method (Fulton et al., 1995). Plant tissues were stored at –80 °C until DNA extraction. The microprep buffer was prepared by mixing DNA extraction buffer (0.35 M sorbitol, 0.1 M Tris, 5 mM EDTA), nuclei lysis buffer (0.2 M Tris, 0.5 M EDTA, 2 M NaCl, 2% CTAB), 5% sarkosyl, sodium bisulfite, and RNAse. This microprep buffer was incubated at 65 °C. Frozen dried leaf samples were milled using the QIAGEN Geno/Grinder with 500 µL of microprep buffer. Milled samples were incubated at 65 °C for 30 min and then DNA purification continued using chloroform:isoamylalcohol, isopropanol, and 70% ethanol steps. DNA concentrations were estimated by gel electrophoresis on 1% agarose. We used 1 DNA sample of more than 10 ng/µL for each accession.

2.2. Amplification of ITS and matK region

DNA fragments were amplified as follows: the nuclear ribosomal RNA internal transcribed spacer regions, which includes ITS1 spacer – 5.8S rRNA gene – ITS2 spacer, were amplified and later sequenced using 4 primers according to White et al. (1990). The primers trnK685F GTATCGCACTATGTATCATTTGA and trnK2R* CCCGGAACTAGTCGGATGG were used for the amplification of the matK sequence as forward and reverse primers, respectively, as suggested by Wojciechowski et al. (2004) for Fabaceae. For sequencing, we used only trnK685F for about 700 bp for the run, corresponding to about half of the matK DNA fragment. The set of matK sequences was much smaller than the ITS set.

The ITS amplification was performed as follows: 180 s at 95 °C; followed by 28 cycles of 30 s at 95 °C, 60 s at 42

°C, and 120 s at 72 °C; then a final extension for 180 s at 72 °C. For the matK amplification, PCR conditions were: 180 s at 95 °C; followed by 35 cycles of 30 s at 95 °C, 60 s at 53 °C, and 120 s at 72 °C; with a final extension for 180 s at 72 °C. Clear-cut, single-banded fragments were purified and directly sequenced in both directions by using the amplification primers. Cycle sequencing and the BigDye Terminator Ready Reaction Kit (Applied Biosystems) were used. Data were collected by the ABI automated sequencer 3730x gel at the NIAB. Resulting sequences were further checked with the software CHROMAS 2.3 (www.technelysium.com.au). A BLAST (Altschul et al., 1997) search was performed to exclude sequences from contaminant organisms.

2.3. Sequence alignment and phylogenetic analysis

The boundaries of the fragments (about 700 bp for matK and 560 bp for ITS sequences) were determined by comparison with previously published sequences. All new accessions with a corresponding GenBank accession number are reported online in Table 1S (supplementary material: http://www.unifi.it/caryologia/tjb/).

Optimal multiple alignment was obtained with CLUSTALW 1.81 (Thompson et al., 1994) and checked by eye. The matrices were combined with the Python (Python version 2.6.4; Biopython 1.57) program combinex1_0.py, written by one of the authors, A Papini, which was released under GPL license and is available at www.unifi.it/caryologia/PapiniPrograms.html. The matrices are available by the authors as Table 2S for the combined matrix with matk+ITS+indels-derived characters and Table 3S with only ITS sequences+indels-derived characters (supplied as supplementary material at http://www.unifi.it/caryologia/tjb/Tab2Hedysaroid_comb.nex).

Three representatives of genus *Caragana* were used as outgroups for the phylogenetic analysis: *Caragana korshinskii*, *Caragana microphylla*, and *Caragana arborescens*. These outgroups were chosen according to the relationships of *Onobrychis* and allied genera outlined in recent molecular studies by Wojciechowski (2003), Wojciechowski et al. (2004), Lavin et al. (2005), and Ahangarian et al. (2007). Sequences described in these studies (75 ITS and 7 matK sequences) were also used in the analysis and not directly produced by us (GenBank accession numbers are supplied in Table 1S).

Parsimony analysis was performed with PAUP* version 4 (Swofford, 2002). A preliminary heuristic search was performed with multrees off and 100 replicates with random addition. The obtained trees were used as a start for a successive analysis with multrees on and 10 replicates (default settings in PAUP for hs command).

All characters were weighted equally, and character state transitions were treated as unordered. Gaps were treated as "simple indel coding" after Simmons and Ochoterena (2000), coding them with the software Gapcoder (Young and Healy, 2003). This process codes indels as separate characters at the end of the same DNA sequences data matrix (see Table 2S, supplementary material).

A maximum likelihood (Felsenstein, 1981) search was conducted as follows: MrModeltest 2.0 (Nylander, 2004) was used to test the best model of sequence evolution (based on the Akaike information criterion, Akaike, 1974). The model with the best score was used for settings in a maximum likelihood (ML) phylogenetic analysis in PAUP. The model obtained was used to calculate the likelihood value of the maximum parsimony trees.

The analysis was executed with the GARLI package, which is based on a stochastic genetic algorithm-like approach to simultaneously find the topology, branch lengths, and substitution model parameters that maximize the log-likelihood (lnL). The package was used on a server provided by the Cipres portal (Miller et al., 2009 for the site address). For maximum likelihood analysis, indel-derived characters were excluded.

Bootstrap (Felsenstein, 1985) resampling was performed setting search = faststep (with no TBR branch-swapping because of computational time limits) with 10 random taxon entries per replicate and the multrees option in effect (with 10,000 replicates) under parsimony criterion.

A decay analysis was performed for Bremer support (Bremer, 1988) with AutoDecay version 5.0 (Eriksson, 2001) to assess the internal support for relationships obtained in the maximum parsimony heuristic analyses.

MrModeltest 2.0 results were also used as an evolutionary model for the Bayesian analysis with MrBayes (Hulsenbeck and Ronquist, 2001). We used the same model for the indel-coded characters of the matrix as we did for restriction sites (coded as binary character states), as implemented in MrBayes. Bayesian analysis is particularly useful to treat mixed character sets (Nylander et al., 2004).

The Bayesian phylogenetic analysis was used to assess the robustness of tree topology and the support for clades. The posterior probability of the phylogenetic model was estimated using Markov chain Monte Carlo sampling with the Metropolis–Hastings–Green algorithm. Four chains were run, 3 heated and 1 cold, for 10^6 generations and were sampled every 100 generations. Following the analysis, the posterior probabilities were checked in the output of MrBayes (in the file .p produced by the software) to estimate the number of trees that should be discarded as "burn-in" when the values reached stationarity (that is, it did not vary anymore out of a range). When stationarity was reached (quite stable values of the log likelihood scores), it was possible to evaluate how many of the beginning trees to discard as "burn-in." After the "burn-in" trees were

removed from the data set, the remaining trees were used to produce a 50% majority-rule consensus tree with PAUP, in which the percentage support indicated a measure of the Bayesian posterior probabilities. The stationarity was reached at approximately generation 30,000, and so the first 300 trees (or the "burn-in" period of the chain) were discarded. Phylogenetic inferences are therefore based on those trees sampled after generation 30,000 for both the combined data set and the data set for only ITS.

The Templeton (Wilcoxon signed-ranks) test (Templeton, 1983), implemented in PAUP, was used to test the alternative less parsimonious topologies with respect to the most parsimonious tree. This test was used to evaluate the significance of an alternative position of taxa of *Onobrychis* s.l.

A partition homogeneity test was performed to check compatibility between the plastid sequence matK and the ITS sequences with PAUP version 4 (Swofford, 2002), with heuristic search, 100 replicates, and swap=none to reduce the computational effort.

The trees were edited for better readability with the program FigTree v1.3.1 by Andrew Rambaut, Institute of Evolutionary Biology, University of Edinburgh: http://tree.bio.ed.ac.uk/software/figtree/.

Supplementary materials (Figures S1–S4 and Tables S1–S3, with their legends, are in the file SupplOnobrychis.html) are available at www.unifi.it/caryologia/tjb/.

3. Results

3.1. Sequence analysis

The total alignment with both markers consisted of 67 taxa and 1501 characters, of which 717 resulted from nucleotide sequence alignment of matK, 643 from the ITS sequences (ITS1+5.8SrDNA+ITS2), and another 140 characters as a result of indel coding (36 for the matK and 103 for the ITS). The partition homogeneity test in PAUP (Swofford, 2002) showed that the matK (plastid genome-encoded) and the ITS gene set were congruent at P = 0.01 (just P-value = 1 – (99/100) = 0.010).

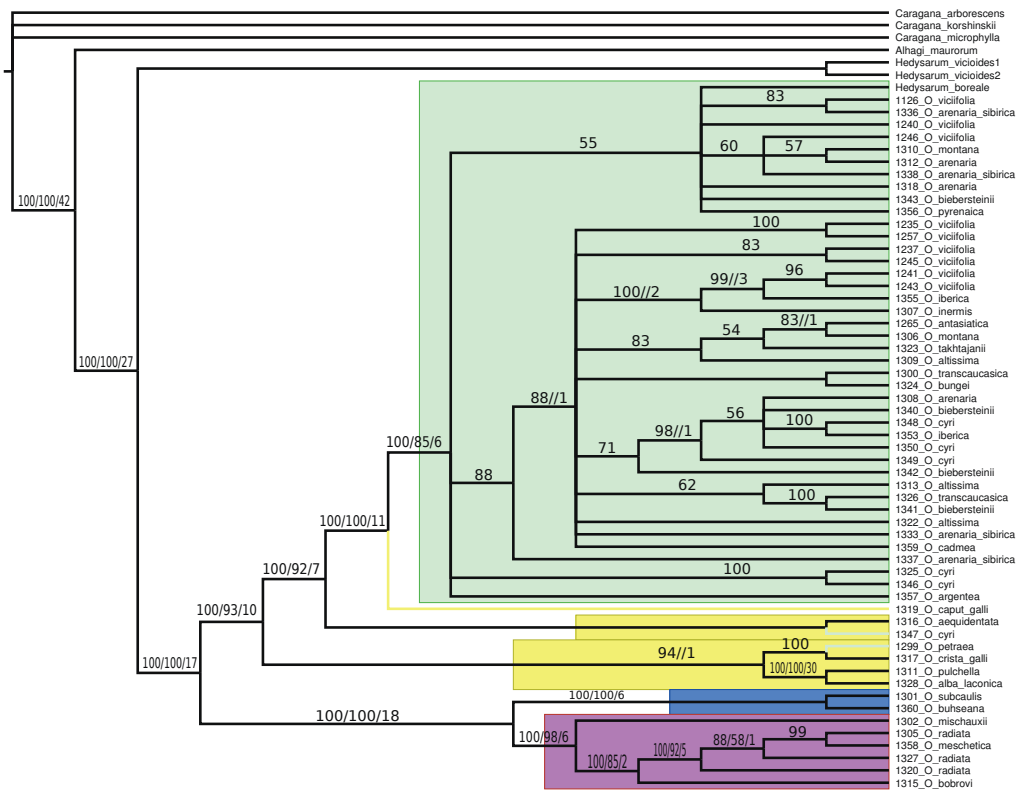

Figure 1. Majority rule consensus tree obtained from the Bayesian trees (excluding the "burn-in" trees) from the total evidence matrix formed by matK+ITS1+5.8SrDNA+ITS2 and indels coded as simple gaps. Robustness is indicated above branches: the first number corresponds to the Bayesian support, the second to the bootstrap (maximum parsimony) support, and the third to the decay values. The value is empty for values lower than 50% for Bayesian and bootstrap support and lower than 1 for the decay values. If only one number is present, it corresponds to the Bayesian support. In green, *Onobrychis* subgenus *Onobrychis* section *Onobrychis*; in yellow, *O.* subgenus *O.* section *Lophobrychis*; in pink, *O.* subgenus *Sisyrosema* section *Hymenobrychis;* in blue, *O.* subgenus *Sisyrosema* section *Heliobrychis*.

3.2. MatK/ITS phylogenetic tree

The phylogenetic analysis, on the basis of the total evidence (matK+ITS) with the heuristic search, produced 109 trees 1111 steps long. Three of these trees were those with the best maximum likelihood value (calculated without considering indels) on the basis of the evolutionary models found with MrModeltest. One of these 3 trees is supplied as supplementary material (Figure S1).

The tree obtained as majority rule consensus trees of the Bayesian analysis trees (obtained with MrBayes) is shown in Figure 1.

Genus *Onobrychis* plus *Hedysarum boreale* (apparently inserted in *Onobrychis* subgenus *Onobrychis*) was supported with 100% Bayesian and bootstrap support, value of decay = 17. In fact, the analysis with matK alone (Figure S4, supplementary material) resulted in *H. boreale* clustering together with the other 2 accessions of *Hedysarum* considered in the analysis and not within *Onobrychis*.

Genus *Onobrychis* subgenus *Onobrychis* section *Onobrychis* plus *Hedysarum boreale* (in green color in Figure 1) had 100% Bayesian and bootstrap support and decay value = 11. *O.* subgenus *Onobrychis* section *Lophobrychis* Hand.-Mazz. was not monophyletic, since *O. pulchella*, *O. alba* subsp. *laconica*, and *O. crista-galli* formed a clade with *O. petraea*, while *O. aequidentata* and *O. caput-galli* were sister groups to section *Onobrychis*.

O. subgenus *Sysirosema* Bunge was supported as monophyletic, with 100% Bayesian and bootstrap support and autodecay value = 18. *O.* subgenus *Sisyrosema* section *Hymenobrychis* DC. (in pink in Figure 1) was supported as monophyletic with 100% Bayesian support, 98% bootstrap support, and autodecay index = 11. *O.* subgenus *Sisyrosema* section *Heliobrychis* Bunge (in blue in Figure 1) was a sister group to section *Hymenobrychis* and monophyletic with 100% Bayesian and bootstrap support and autodecay index = 6, even though only 2 accessions were sampled. The interspecific relationships in *Onobrychis*, especially within the subgenus *Onobrychis*, were not resolved. In fact, in some cases different accessions of the same species, such as *O. viciifolia*, clustered in a different point of the tree without forming monophyletic groups.

The maximum likelihood tree obtained with GARLI was very similar to that shown in Figure 1 (data not shown). A strict consensus tree of maximum parsimony for 1,014,420 trees (search stopped after 90 min) obtained only with matK sequences (including indel-derived characters) is supplied in the supplementary material as Figure S4 (www.unifi.it/caryologia/tjb/FigS4.pdf). In this tree, the accession of *Hedysarum boreale* clustered together with the other 2 accession of *Hedysarum* used in the analysis and not together with genus *Onobrychis*.

3.3. ITS phylogenetic tree

The analysis of the ITS data set showed that the genus *Onobrychis* was not monophyletic because of the presence, within *Onobrychis*, of 1 accession of *Eversmannia subspinosa* and 2 accessions of *Hedysarum*, *H. boreale* and *H. candidissimum* (Figure 2). The so-formed clade had 93% Bayesian support. *H. membranaceum* was a sister group to *Onobrychis* + *Eversmannia* with 93% Bayesian and 62% bootstrap support and autodecay index = 2 (Figure 2). A Templeton test was then performed with PAUP to test an alternative position of *H. membranaceum*, inserting this last species within *Hedysarum* s. s. The alternative tree was significantly different and 10 steps longer with respect to the maximum parsimony tree.

Within the genus *Onobrychis*, the subgenus *Onobrychis* was also monophyletic (98% Bayesian support, 86% bootstrap support, and decay index = 7) (Figure 2). Subgenus *Sisyrosema* was monophyletic with 100% Bayesian support and decay index = 13. *Eversmannia subspinosa* was supported as a sister group to subgenus *Onobrychis* (88% Bayesian support and decay index = 2). An alternative hypothesis with the *Eversmannia* sister group to the whole genus *Onobrychis* produced a 2-step-longer tree. The difference was not statistically significant after the Templeton test.

3.4. Relationships within *Onobrychis*

Section *Onobrychis* plus 1 accession of *O. cyri* (shown in light green in Figure 2) formed a monophyletic group (89% Bayesian support). Section *Lophobrichis* (shown in yellow in Figure 2) was not monophyletic, since *O. caput-galli* and *O. aequidentata* were not included in it, but were sisters to section *Onobrychis*. Section *Dendrobrychis* DC. (in dark green) was divided into 2, with 3 accessions of *O. cornuta* clustered within the main part of section *Lophobrychis* and *O. arnacantha* (considered as belonging to subgenus *Sysirosema*) in an unresolved position with respect to the recognized sections of this subgenus. *O. petraea* clustered together with *Lophobrychis* + part of *Dendrobrychis*.

O. subgenus *Sysirosema* (Figure 2: fuchsia, blue, gray, red, and a basal dark green branch) was supported as monophyletic with 100% Bayesian and bootstrap support and autodecay value = 13, with the exception of *O. arnacantha* (section *Dendrobrychis*, in dark green), taxonomically assigned to subgenus *Onobrychis*. Subgenus *Sysirosema* was formed by sections *Hymenobrychis* (in fuchsia) + *Heliobrychis* (in blue) + *Laxiflorae* (Širj.) Rech.f. (in red) + *Afghanicae* Širj. (in gray). *O.* subgenus *Sisyrosema* section *Hymenobrychis* (in pink in Figure 2) was supported as monophyletic with 100% Bayesian support and decay index = 4, provided that we consider *O. acaulis* (taxonomically, this is considered to belong to section *Anthyllium* Nábělek) inserted in *Hymenobrychis*. *O.* subgenus *Sisyrosema* section *Heliobrychis* (in blue in

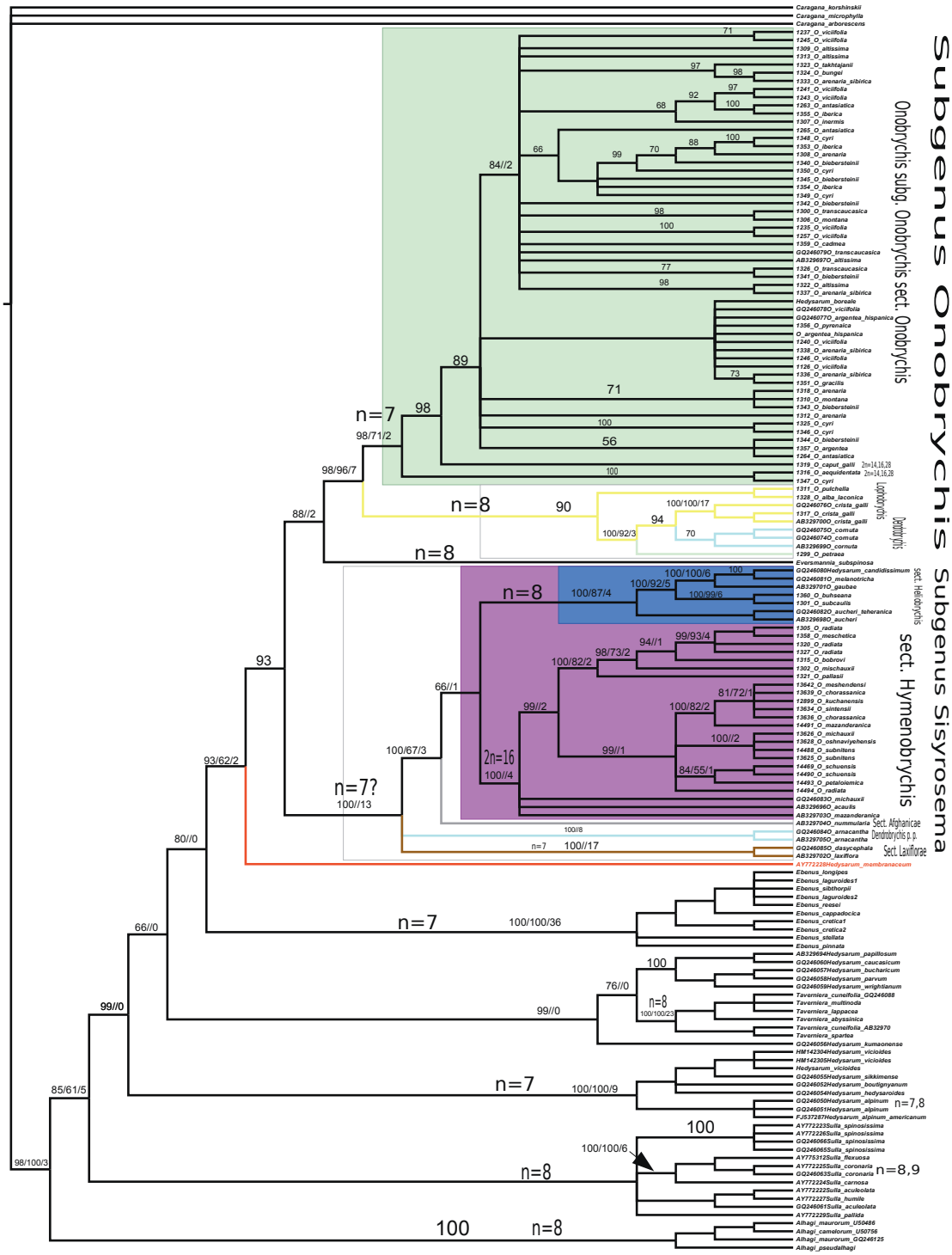

Figure 2. Majority rule consensus tree obtained from the Bayesian trees (excluding the "burn-in" trees) from the ITS matrix formed by ITS1+5.8SrDNA+ITS2 and indels coded as simple gaps. Robustness is indicated above branches: the first number corresponds to the Bayesian support, the second to the bootstrap (maximum parsimony) support, and the third to the decay values. The value is empty for values lower than 50% for Bayesian and bootstrap support and lower than 1 for the decay values. If only one number is present, it corresponds to the Bayesian support. N= corresponds to the available data about the chromosome number. In green, *Onobrychis* subgenus *Onobrychis* section *Onobrychis*; in yellow, *O.* subgenus *O.* section *Lophobrychis*; in light blue, *O.* subgenus *O.* section *Dendrobrychis;* in fuchsia, *O.* subgenus *Sisyrosema* section *Hymenobrychis*; in blue, *O.* subgenus *Sisyrosema* section *Heliobrychis*; in brown, *O.* subgenus *Sisyrosema* section *Laxiflorae*; in gray, *O.* subgenus *Sisyrosema* section *Afghanicae*; in red, *Hedysarum membranaceum*. For karyological data references see the text (Section 4.2).

Figure 2) was supported as monophyletic (100% Bayesian support, 87% Bootstrap support, and decay index = 4) and sister group to section *Hymenobrychis*. Section *Laxiflorae* was in the basal position of the subgenus in an unresolved position with respect to *O. arnacantha* and the clade formed by sections *Hymenobrychis* + *Heliobrychis* + *Afghanicae*. Section *Afghanicae* (represented here by a single accession, *O. nummularia*) was an outgroup to *Hymenobrychis* + *Heliobrychis*.

Even in the ITS data set (with more taxa than the combined set), the interspecific relationships in *Onobrychis* and particularly within subgenus *Onobrychis* were not easily resolved. The ITS marker does not produce trees that keep all the accessions of the same species together, such as *O. viciifolia*, *O. cyri*, *O. iberica*, *O. biebersteinii*, *O. transcaucasica*, and *O. altissima* in subgenus *Onobrychis* section *Onobrychis*, and *O. michauxii* and *O. mazanderanica* in *O.* subgenus *Sysirosema* section *Hymenobrychis*. Hence, the phylogenetic analysis of the ITS sequence variation did not insert all the accessions of the same species into monophyletic groups.

The maximum likelihood tree obtained with GARLI is supplied as supplementary material (Figure S2) together with one of the maximum parsimony trees with the best maximum likelihood score obtained with PAUP on the basis of the MrModeltest settings (Figure S3). These trees supported *H. membranaceum* as a sister group to genus *Onobrychis*+*Eversmannia* and the position of *Eversmannia* as a sister group to *Onobrychis* subgenus *Onobrychis* and of *O. petraea* within section *Lophobrychis*. Some of the maximum parsimony trees (as in Figure S3) positioned genus *Ebenus* as a sister group to *O.* subgenus *Sysirosema*. This alternative topology had Bayesian and bootstrap support lower than 50% and decay index of <1, but the Templeton test showed that, at least with the parsimony criterion, the difference was not statistically significant (data not shown).

3.5. Indels in the combined matK and ITS matrix

The combined matrix is provided as Table S2 (supplementary material: http://www.unifi.it/caryologia/tjb/): the indicated indel positions are referred to in Table S2. Three indels were shared by the outgroups and *Alhagi* (1153, 1157, and 1297 of the combined alignment). Three indels were shared by the outgroups, *Alhagi*, and 2 accessions of *Hedysarum* (919, 947–949, and 976 of the combined alignment).The indel in position 1146–1153 was shared by *Onobrychis* sect. *Lophobrychis* plus *O. petraea*. An indel in 1197 was shared only by the 2 accessions of *Hedysarum viciifolia*.

The ITS matrix was composed of 153 accessions for 897 positions, of which 1–285 belong to the ITS1, 286–463 to the 5.8S rDNA, 464–711 to the ITS2, and 712–897 to the indels, coded as simple gaps.

3.6. Indels found only in the ITS matrix

Relative only to the ITS alignment, an indel in 35–36 was shared by *Alhagi* plus *Eversmannia*. Five indels in 71–73, 108–112, 208, 299–300, and 473 characterized the whole genus *Ebenus*. An indel in 87–88 characterized *Hedysarum membranaceum*+*Onobrychis* subgenus *Sysirosema*. An insertion in position 80 was shared by *Eversmannia*, *Onobrychis* subgenus *Sysirosema*, and *Hedysarum membranaceum*. An insertion in 471–477 was shared by *O. petraea* + section *Lophobrychis*.

4. Discussion

4.1. Phylogenetic relationships

The results of the partition homogeneity test showed that the 2 data sets, the plastid partial matK sequence and the nucleus encoded sequence ITS, were congruent only at P = 0.01 (P-value = 1 – (99/100) = 0.010). This P-value is just the threshold at which combining 2 data sets would improve phylogenetic confidence after Cunningham (1997). After Rokas et al. (2003), concatenating more sequences in a single matrix would reduce the total number of maximum parsimony trees. For these reasons an initial analysis was performed with the combined data set, followed by a focus on the larger ITS data matrix. We also supplied the only matK strict consensus tree of 1,014,420 trees (PAUP maximum parsimony heuristic search with outgroup *Caragana*, search stopped after 90 min), as in Figure S4 in the supplementary material.

The combined (matK+ITS) phylogenetic analysis showed that an accession of *Hedysarum boreale* clustered together with genus *Onobrychis*, as already observed by Ahangarian et al. (2007). However, the analysis using matK only (Figure S4, supplementary material) positioned *H. boreale* together with the other 2 accessions of *Hedysarum*. This matK sequence (AY386892 by Wojciechowski, Lavin, and Sanderson from a sample from Arizona: Wojciechowski 259) was obtained from a different sample with respect to the ITS sequence. This last was the same used by Ahangarian et al. (2007) in his study, that is U50482 for the ITS1 and U50483 for the ITS2, both by Sanderson and Wojciechowski from the same sample "Wojciechowski and Sanderson 131". The sample of *H. boreale* may be of hybrid (intergeneric!) origin, since the maternally inherited plastid sequence resulted in a different phylogenetic position with respect to the nuclear ITS. Alternatively, the original samples (or at least one of them) of *H. boreale* may have been wrongly identified.

The accession of *Hedysarum candidissimum* (within *Onobrychis* in our results) was published in GenBank by Ahlquist and Wojciechowski, voucher M.Nyedegger 42636 (MSB). Since this sequence was not yet employed in other phylogenetic analysis, it was not discussed further in this study. As *H. boreale*, *H. candidissimum* may also be

really more related to genus *Onobrychis* than to the rest of *Hedysarum* and further DNA markers may be necessary to clarify their positions (Martin F Wojciechowski, personal communication).

After Martin F. Wojciechowski (personal communication), *Hedysarum* and *Onobrychis*, both containing well over 100 species, are probably paraphyletic and relationships are fluid. In view of this uncertainty, these species were omitted from the following discussion.

In the total evidence matrix, *O*. subgenus *Onobrychis* section *Lophobrychis* was not monophyletic since *O. pulchella*, *O. alba* subsp. *laconica*, and *O. crista-galli* formed a clade with *O. petraea*, while *O. aequidentata* and *O. caput-galli*, belonging to this section after most treatments, were sister groups to section *Onobrychis*. However, very variable chromosome numbers have been documented for *O. aequidentata*: 2n = 14, 16, and 28 (De Montmollin, 1984; Romano et al., 1987; Baltisberger, 1991; Abou-El Enain, 2002). The same values were documented for *O. caput-galli* (Heyn, 1962; Slavivk et al., 1993; Abou-El Enain, 2002). Such variation (even apparently with different base chromosome numbers: 7 and 8) may suggest the presence of a different species poorly characterized from a morphological point of view, or even the presence of hybrids or species of hybrid origin, under the names *O. aequidentata* and *O. caput-galli*. The not "orthodox" position within the section *Lophobrychis* may be justified by such variability. The difficult phylogenetic reconstruction due to species of hybrid origin is well known in "difficult" genera such as *Quercus* (Fagaceae) (Simeone et al., 2013) or *Rebutia* (Cactaceae) (Mosti et al., 2011).

On the basis of the molecular data here presented, genus *Onobrychis* cannot be considered monophyletic without inserting in it at least the genus *Eversmannia* (which was nested within *Onobrychis*). Conversely, the Templeton test showed that an alternative tree topology with *Eversmannia* as sister group to *Onobrychis* was 2 steps longer (with parsimony), but not significantly different. *Eversmannia* may be seen as a specialized version of *Onobrychis* with many autapomorphies, even at the molecular level. This uncertainty suggests that a larger sampling of *Eversmannia* in particular and *Hedysareae* in general is necessary to clarify the position of this genus before suggesting its eventual transfer into *Onobrychis*.

Ahangarian et al. (2007) separated *Hedysarum membranaceum* as sister group of the genus *Sulla* (88% bootstrap support), while *Eversmannia* was put into a sister group of the genus *Onobrychis* (see maximum parsimony trees). However, these analyses were undertaken on a much smaller sample with respect to the data presented here and only under the maximum parsimony criterion. The position of *H. membranaceum* as a sister group of *Onobrychis* was confirmed by the Templeton test (alternative positions resulted in trees with a statistically significant difference).

H. membranaceum is the only species within the monotypic section *Membranacea* B.Feldtsch. of the genus *Hedysarum*. This species is restricted to north Africa and is morphologically distinct from the other *Hedysarum* species and the other genera of *Hedysareae* in that it has pods with wide wings up to 3 mm wide, short inflorescences, ovate standard, wings with a short auricle, a keel with a short claw, and a protruded hilum in the seed (Choi and Ohashi, 2003). Choi and Ohashi (1996) noted that *H. mebranaceum* Coss. et Bal. is intermediate between sect. *Fruticosa* and other species of *Hedysarum* in terms of pollen morphology, petal shape, and a suffrutescent habit. *H. membranaceum* turned out to be the most divergent species among the Mediterranean representatives of the genus *Hedysarum* on the basis of ISSR data (Chennaoui-Kourda et al., 2007) and in a previous analysis with ITS data restricted to 8 species of *Hedysarum* s. l. (Chennaoui et al., 2007). The molecular data, together with the results of the Templeton test and the morphological features (Choi and Ohashi, 2003), indicate that this taxon may be recognized as a separate genus with respect to *Hedysarum* if further morphological and/or molecular characters confirm the here-observed phylogenetic position.

The position of *O. petraea*, previously positioned in *O*. section *Onobrychis* subsection *Macropterae* Hand.-Mazz., was nested within *Lophobrychis*+*Dendrobrychis*. On the basis of these data, we suppose that the sectional treatment may deserve some changes. On the basis of the molecular data alone, in order to form a monophyletic clade corresponding to a section, part of *Lophobrychis*, *Dendrobrychis*, and subsection *Macropterae* of section *Onobrychis* (*O. petraea*) should be kept together. Further molecular data may be useful to further clarify the relationships of these species.

Onobrychis subgenus *Sysirosema* was resolved as monophyletic with high support and should therefore be maintained. At the sectional level, *O*. subgenus *Sisyrosema* section *Heliobrychis* was monophyletic and a sister group of section *Hymenobrychis*. This result confirmed that of Arslan and Ertuğrul (2010), who used seed storage proteins as molecular markers. The monophyly of sections *Laxiflorae* and *Afghanicae* is not excluded by the here-presented results, even if the relationships of section *Laxiflorae* were not completely clear. A gametophytic chromosome count of n = 7 for *O. laxiflora* (Kathoon and Ali, 1991) may suggest this number as the basal number for the subgenus *Sisyrosema*. Section *Afghanicae* is positioned as a sister group to *Heliobrychis* + *Hymenobrychis*.

The presence of *O. arnacantha* (section *Dendrobrychis*, in dark green in Figure 2), taxonomically assigned to subgenus *Onobrychis*, in a basal position close to subgenus

Sysirosema was quite unexpected. The same result was obtained by Ahangarian et al. (2007), who suggested that some morphological features, similar those of other species of section *Dendrobrychis* (as *O. cornuta*, inserted in the analysis), were due to parallelism.

O. acaulis was nested within section *Hymenobrychis* despite it currently being classified as belonging to section *Anthyllium*. Such incongruence may be due to erroneous assignment of *O. acaulis* to section *Anthyllium*, or a wrong assignment of the status of the section to this last group of species. Section *Anthyllium* did not appear to be sufficiently separated from section *Hymenobrychis*, but better sampling within this section is necessary before eventual definitive taxonomic rearrangements.

An alternative tree topology with genus *Ebenus* as a sister group to *O*. subgenus *Sysirosema* appeared in some maximum parsimony trees. This position had Bayesian and bootstrap support lower than 50% and decay index of <1, but the Templeton test showed that, at least with the parsimony criterion, the difference was not statistically significant. For this reason it was also decided that the current status of *Eversmannia* should not be changed (together with a current insufficient sampling of species within this genus and the lack of data for other small genera within *Hedysareae*).

Results from this study showed that the genus *Hedysarum* was polyphyletic, and to a greater extent than was already proposed by Choi and Ohashi (2003). Phylogenetic analysis showed that it was resolved into 4 different clades. One clade separated from *Hedysarum*, corresponding to genus *Sulla* (already separated from the rest of *Hedysarum* by Choi and Ohashi, 2003), *Hedysarum* s. s. with the type species *H. alpinum*, and a last group resulted in a sister group to *Taverniera* and *H. kumaononense*. We did not make further taxonomic decisions in relation to *Hedysarum* (apart from *H. membranaceum*) since sampling in the here-presented data favored *Onobrychis*. Moreover, some important genera related to *Hedysarum*, such as *Sartoria* and *Corethodendron*, are not represented here.

The key to the genera of *Hedysarum* and allied genera as proposed by Choi and Ohashi (2003) still remains valid even though taxonomic rearrangements have been proposed as a result of this study.

4.2. Karyotype evolution in *Hedysareae*

The known chromosome numbers are indicated in Figure 2. On the basis of the data available from the IPCN chromosome numbers databank (tropicos.org, Missouri Botanical Garden), genera *Alhagi*, *Sulla*, and *Taverniera* have a basic number of n = 8. These 3 genera are those resulting sister groups to the rest of *Hedysareae* after our phylogenetic analysis (Figure 2). *Astragalus* and *Chesneya* of the related Astragalean clade (Sepet et al., 2011) also have a basic number of n = 8. A count of 2n = 18 in *Sulla coronaria* (as *Hedysarum coronarium*) is known (Issolah et al., 2006), such that Arslan et al. (2012) also consider n = 9 to be a possible basis chromosome number in *Hedysarum* s. l. The count 2n = 16 was also found in the genus *Sartoria* (not sampled here; Arslan et al., 2012). *Hedysarum* s. s. (the clade containing the genus type *H. alpinum*) has n = 7, apart from 1 count of n = 8 for an accession of *H. alpinum*. The same number (7) was also documented for the genus *Ebenus*. This last genus was the sister group of the clade containing *Hedysarum membranaceum+Onobrychis+Eversmannia*. A count of n = 8 is available for *Eversmannia*. The situation is more complex in *Onobrychis*. Within the subgenus *Sysirosema*, the chromosome number is always n = 7 in section *Hymenobrychis*, apart from a count of 2n = 16 for *Onobrychis galegifolia*, not sampled here and which would deserve further testing, and some counts of 2n = 16 for *O. subnitens* (Ranjbar et al. 2012), while n = 8 would be the base chromosome number in section *Heliobrychis*. This last section appears to be homogeneous regarding chromosome number and DNA sequence evidence, while it appeared quite variable in morphological characters (Karamian et al. 2012). Within the subgenus *Onobrychis*, n = 8 is known for sections *Dendrobrychis* and *Lophobrychis*. These 2 sections clustered together in the phylogenetic analysis and were the sister groups to section *Onobrychis*, which only had known chromosome counts of n = 7. Comparing the phylogenetic analysis with the karyotype data, we can assume a base number n = 8 for the tribe (present in the more basal genera), changing to 7 in *Hedysarum* s. s. and *Ebenus*. This number would be maintained in *Onobrychis* subgenus *Sysirosema*, while n = 8 in sect. *Heliobrychis* would be a derived condition. The change to n = 8 would occur also in *Eversmannia*, a possible sister group to subgenus *Onobrychis* in some of the presented trees based on the ITS (Figure 2). The same number is maintained in sect. *Dendrobrychis* and sect. *Lophobrychis*, while n = 7 in section *Onobrychis* would be again a derived condition.

A variation in chromosome number and ploidy level is known for some species, particularly for *Onobrychis* subgenus *Onobrychis* section *Onobrychis*. Accessions of *O. altissima*, for instance, have 2n = 14 (Arslan et al., 2012) and others have 2n = 28 (Hejazi et al., 2010), while a variation of 2n = 22, 27, 28, 29 was found in *O. viciifolia*. After Ranjbar et al. (2010), *O. altissima* is considered to be closely related to the cultivated sainfoin (*O. viciifolia*) and may be a progenitor of it, while, based on morphological similarity, a close relationship between the 2 species was postulated by Hedge (1970). Gömürgen (1996) found also cases of meiotic chromosome instability in *O. armena*. This chromosome number variation even within species may suggest the presence of cryptospecies with similar morphology but a different chromosome number, at

least in some of the species of this section. The presence of cryptospecies may partly explain the failure of the ITS sequence to distinguish between species within section *Onobrychis*. Moreover, polyploidy within some taxa may be due to ITS polymorphism, even with the possible formation of paralogues. This phenomenon may cause difficult phylogenetic reconstruction and possible conflicts between nuclear and chloroplast sequences, as in the genus *Quercus* (Bellarosa et al., 2005; Simeone et al., 2009; Papini et al., 2011).

5. Conclusion

The main results of this analysis are: genus *Onobrychis* may be not considered monophyletic, since *Eversmannia* was nested within it, while the position of the monotypic section *Membranacea* B.Feldtsch. of genus *Hedysarum* (containing only one species, *H. membranaceum* Coss. & Bal.) was supported as a sister group of *Onobrychis+Eversmannia.* The 2 markers that were employed (ITS and matK) were able to distinguish between genera within the tribe *Hedysareae* and intrageneric relationships in *Onobrychis* and *Hedysarum*. Nevertheless, these markers were unable to fully distinguish between species of *Onobrychis*, particularly in sect. *Onobrychis*. Published cytogenetic data may suggest that this result is linked to difficult species circumscription (various chromosome numbers in polyploid series are present within the same species). The phylogenetic analysis showed that the most basal clades of the tribe have n = 8 as a basic chromosome number, with n = 7 appearing in a clade corresponding to *Hedysarum* s. s. and *Ebenus*. These last groups were clustered as a sister group to *Onobrychis+Eversmannia+Hedysarum membranaceum*. The chromosome numbers followed a variable pattern in *Onobrychis*. Subgenus *Sysirosema* section *Hymenobrychis* has a chromosome number of n = 7 while section *Heliobrychis* has n = 8. This chromosomal switch may be the basis of the separation of these sections. In subgenus *Onobrychis*, the sections *Dendrobrychis* and *Lophobrychis* have n = 8, while the more derived section *Onobrychis* has again n = 7. A count of n = 8 was reported for *Eversmannia*, in agreement with the molecular phylogenetic analysis (indicating a sister group position with respect to subgenus *Onobrychis*).

We did not take taxonomic decisions in relation to *Hedysarum*, since sampling in the here-presented data favored *Onobrychis*. Moreover, some important genera related to *Hedysarum*, such as *Sartoria* and *Corethodendron*, are not represented here.

Acknowledgments

We acknowledge the support from the EU (MRTN-CT-2006-035805; 'Healthy Hay' project) and from Dr Massoud Ranjbar, renowned expert and taxonomist defining new species of *Onobrychis*, Department of Systematic Botany, Bu-Ali Sina University, Iran, for support and supply of herbarium samples for analysis. We also thank the anonymous reviewers who helped us to improve the quality of the manuscript.

References

Abou-El-Enain MM (2002). Chromosomal criteria and their phylogenetic implications in the genus *Onobrychis* Mill. sect. *Lophobrychis* (Leguminosae), with special reference to Egyptian species. Bot J Linn Soc 139: 409–414.

Ahangarian S, Kazempour Osaloo S, Maassoumi AA (2007). Molecular phylogeny of the tribe *Hedysareae* with special reference to *Onobrychis* (Fabaceae) as inferred from nrDNA ITS sequences. Iran J Bot 13: 64–74.

Akaike H (1974). A new look at the statistical model identification. IEEE Trans Automat Cont 19: 716–723.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402.

Arslan E, Ertuğrul K (2010). Genetic relationships of the genera *Onobrychis*, *Hedysarum*, and *Sartoria* using seed storage proteins. Turk J Biol 34: 67–73.

Arslan E, Ertuğrul K, Tugay O, Dural H (2012). Karyological studies of the genus *Onobrychis* Mill. and related genera *Hedysarum* L. and *Sartoria* Boiss. & Heldr. (Fabaceae, *Hedysareae*) from Turkey. Caryologia 65: 11–17.

Avcı S, Sancak C, Can A, Acar A, Pınar NM (2013). Pollen morphology of the genus *Onobrychis* (Fabaceae) in Turkey. Turk J Bot 37: 669–681.

Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS, Donoghue MJ (1995). The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann Mo Bot Gar 82: 247–277.

Ball PW (1968). *Onobrychis* Miller. In: Tutin TG, Heywood VH, editors. Flora Europaea, Vol. 2. Cambridge, UK: Cambridge University Press, pp. 187–191.

Baltisberger M (1991). Cytological investigations of some Greek plants. Flora Med 1: 157–173.

Bellarosa R, Simeone MC, Papini A, Schirone B (2005). Utility of ITS sequence data for phylogenetic reconstruction of Italian *Quercus* spp. Mol Phylogenet Evol 34: 355–370.

Boissier PE (1872). Flora orientalis sive enumeratio plantarum in oriente: A Graecia et Aegypto ad Indiae fines hucusque observatarum. Vol 2. Geneva, Switzerland.

Bremer K (1988). The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. Evolution 42: 795–803.

CBOL Plant Working Group (2009). A DNA barcode for land plants. P Natl Acad Sci USA 31: 12795–12797.

Chennaoui H, Marghali S, Marrakchi M, Trifi-Farah N (2007). Phylogenetic relationships in the North African genus *Hedysarum* as inferred from ITS sequences of nuclear ribosomal DNA. Genet Resour Crop Ev 54: 389–397.

Chennaoui-Kourda H, Marghalia S, Marrakchia M, Trifi-Farah N (2007). Genetic diversity of *Sulla* genus (*Hedysareae*) and related species using inter-simple sequence repeat (ISSR) markers. Biochem Syst Ecol 35: 682–688.

Choi B, Ohashi H (1996). Pollen morphology and taxonomy of *Hedysarum* and its related genera of the tribe *Hedysareae* (Leguminosae-Papilionoideae). J Jpn Bot 71: 191–213.

Choi BH, Ohashi H (2003). Generic criteria and an infrageneric system for *Hedysarum* and related genera (Papilionoideae-Leguminosae). Taxon 52: 567–576.

Cunningham CW (1997). Can three incongruence tests predict when data should be combined? Mol Biol Evol 14: 733–740.

De Montmollin B (1984). Etude cytotaxonomique de la flore de la Crete. II. Nombres chromosomiques. Bot Helv 94: 261–267.

Dolya K, Vasilissa I (2000). Pollen morphology of the genera *Onobrychis* and *Hedysarum* (*Hedysareae*, Fabaceae) in Bulgaria. Ann Bot Fenn 37: 207–217.

Eriksson T (2001). AutoDecay Ver. 5.0 (program distributed by the author). Stockholm, Sweden: Bergius Foundation, Royal Swedish Academy of Sciences.

Felsenstein J (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol 17: 368–376.

Felsenstein J (1985). Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791.

Fulton TM, Chunzoongse J, Tanksley SD (1995). Microprep protocol for extraction of DNA from tomato and other herbaceous plants. Plant Mol Biol Rep 13: 207–209.

Gömürgen AN (1996). Meiotic analysis of selected material of sainfoin and its progeny with branched and unbranched peduncles. Turk J Bot 20: 399–411.

Gültepe M, Uzuner U, Coşkunçelebi K, Beldüz AO, Terzioğlu S (2010). Internal transcribed spacer (ITS) polymorphism in the wild *Primula* (Primulaceae) taxa of Turkey. Turk J Bot 34: 147–157.

Hayot Carbonero C, Mueller-Harvey I, Brown TA, Smith L (2011). Sainfoin (*Onobrychis viciifolia*): a beneficial forage legume. Plant Gen Res 9: 70–85.

Hedge IC (1970). *Onobrychis*. In: Davis P, editor. Flora of Turkey and the East Aegean Islands, Vol. 3. Edinburgh, UK: Edinburgh University Press, pp. 549–590.

Hejazi H, Mohsen S, Nasab MZ (2010). Cytotaxonomy of some *Onobrychis* (Fabaceae) species and populations in Iran. Caryologia 63: 18–31.

Heyn CC (1962). On the cytotaxonomy of *Onobrychis cristagalli* (L.) Lam. and *O. squarrosa* Vivi. B Res Counc Israel 3: 177–182.

Hu JM, Lavin M, Wojciechowski MF, Sanderson MJ (2000). Phylogenetic systematics of the tribe Millettieae (Leguminosae) based on chloroplast *trnK/matK* sequences and its implications for evolutionary patterns in Papilionoideae. Am J Bot 87: 418–430.

Huelsenbeck JP, Ronquist F (2001). MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754–755.

İkinci N (2011). Molecular phylogeny and divergence times estimates of *Lilium* section *Liriotypus* (Liliaceae) based on plastid and nuclear ribosomal ITS DNA sequence data. Turk J Bot 35: 319–330.

Irfan E, Turgut-Balik D, Sahin A, Kursat M (2007). Total electrophoretic band patterns of some *Onobrychis* species growing in turkey. J Agr Env Sci 2: 123–126.

Issolah R, Benhizia H, Khalfallah N (2006). Karyotype variation within some natural populations of sulla (*Hedysarum coronarium* L., Fabaceae) in Algeria. Genet Resour Crop Ev 53: 1653–1664.

Karamian R, Behjou AM, Ranjbar M (2012). Anatomical findings of *Onobrychis* sect. *Heliobrychis* (Fabaceae) in Iran and their taxonomic implications. Turk J Bot 36: 27–37.

Khatoon S, Ali SI (1991). Chromosome numbers in subfamily Papilionoideae (Leguminosae) from Pakistan. Willdenowia 20: 159–165.

Lavin M, Herendeen P, Wojciechowski MF (2005). Evolutionary rates analysis of Leguminosae implicates a rapid diversification of lineages during the Tertiary. Syst Biol 54: 575–594.

Lock JM (2005). Tribe *Hedysareae*. In: Lewis G, Schrire B, Mackinder B, Lock M, editors. Legumes of the World. Kew, UK: Royal Botanical Gardens, pp. 489–495.

Lock JM, Schrire BD (2005). Tribe Galegeae. In: Lewis G, Shrive B, Mackinder B, Lock M, editors. Legumes of the World. Kew, UK: Royal Botanical Gardens, pp. 475–488.

Mabberley DJ (1990). The Plant-Book: A Portable Dictionary of the Higher Plants. Cambridge, UK: Cambridge University Press.

Miller MA, Holder MT, Vos R, Midford PE, Liebowitz T, Chan L, Hoover P, Warnow T (2009). The CIPRES Portals. CIPRES. 4 August 2009. URL: http://www.phylo.org/sub_sections/portal [accessed 18 October 2012].

Mosti S, Lewka Bandara N, Papini A (2011). Further insights and new combinations in *Aylostera* (Cactaceae) based on molecular and morphological data. Pak J Bot 43: 2769–2785.

Nylander JAA (2004). Mr Modeltest, version 1.0b. Uppsala, Sweden: Department of Systematic Zoology, EBC, Uppsala University.

Nylander JAA, Ronquist F, Huelsenbeck JP, Nieves-Aldre JL (2004). Bayesian phylogenetic analysis of combined data. Syst Biol 53: 47–67.

Papini A, Simeone MC, Bellarosa R, Spada F, Schirone B (2011). *Quercus macranthera* Fisch. & Mey. ex Hohen. and *Quercus iberica* M.Bieb.: taxonomic definition and systematic relationships with European oaks inferred from nuclear ITS data. Plant Biosyst 145: 37–49.

Polhill RM (1981). Papilionoideae. In: Polhill RM, Raven PH, editors. Advances in Legume Systematics. Part 1. Kew, UK: Royal Botanic Gardens, pp. 367–370.

Ranjbar M, Hajmoradi F, Karamian R (2012). An overview on cytogenetics of the genus *Onobrychis* (Fabaceae) with special reference to *O.* sect. *Hymenobrychis* from Iran. Caryologia 65: 187–198.

Ranjbar M, Karamian R, Hadadi A (2010). Cytosystematics of three *Onobrychis* species (Fabaceae) in Iran. Caryologia 63: 237–249.

Rechinger KH (1984). *Onobrychis*. In: Rechinger KH, editor. Flora Iranica. Graz, Austria: Akad. Druck. und Verl., 157: 387–464.

Rokas A, Williams BL, King N, Carroll SB (2003). Genome-scale approaches to resolving incongruence in molecular phylogenies. Nature 425: 798–804.

Romano S, Mazzola P, Raimondo FM (1987). Numeri cromosomici per la flora Italiana. Inform Bot Ital 19: 173–180.

Schischkin BK, Bobrov EG (1972). Flora of U.S.R.R. Vol. 13. Jerusalem, Israel: Israel Program for Scientific Translations, pp. 244–281.

Sepet H, Ozdemir C, Bozdag B (2011). Cytological study the genus *Chesneya* Lindl. (Fabaceae) in Turkey. Caryologia 64: 184–188.

Simeone MC, Papini A, Vessella F, Bellarosa R, Spada F, Schirone B (2009). Multiple genome relationships and a complex biogeographic history in the eastern range of *Quercus suber* L. (Fagaceae) implied by nuclear and chloroplast DNA variation. Caryologia 62: 236–252.

Simeone MC, Piredda R, Papini A, Vessella F, Schirone B (2013). Application of plastid and nuclear markers to DNA barcoding of Euro-Mediterranean oaks (Quercus, Fagaceae): problems, prospects and phylogenetic implications. Bot J Linn Soc 172: 478–499.

Simmons MP, Ochoterena H (2000). Gaps as characters in sequence-based phylogenetic analyses. Syst Biol 49: 369–381.

Slavivk B, Jarolivmovav V, Chrtek J (1993). Chromosome counts of some plants from Cyprus. Candollea 48: 221–230.

Swofford DL (2002). PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sunderland, MA, USA: Sinauer Associates.

Templeton AR (1983). Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the evolution of humans and the apes. Evolution 37: 221–244.

Terzioğlu S, Coşkunçelebi K, Gültepe M (2012). *Primula* × *uzungolensis* Primulaceae): a new natural hybrid from NE Anatolia. Turk J Bot 36: 9–19.

Thompson JD, Higgins DG, Gibson TJ (1994). ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680.

White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR Protocols: A Guide to Methods and Applications. New York, NY, USA: Academic Press, pp. 315–322.

Wojciechowski MF (2003). Reconstructing the phylogeny of legumes (Leguminosae): an early 21st century perspective In: Klitgaard BB, Bruneau A, editors. Advances in Legume Systematics. Part 10. Higher Level Systematics. Kew, UK: Royal Botanic Gardens, pp. 5–35.

Wojciechowski MF (2005). *Astragalus* (Fabaceae): a molecular phylogenetic perspective. Brittonia 57: 382–399.

Wojciechowski MF, Lavin M, Sanderson MJ (2004). A phylogeny of legumes (Leguminosae) based on analysis of the plastid MATK gene resolves many well-supported subclades within the family. Am J Bot 91: 1846–1862.

Wojciechowski MF, Sanderson MJ, Hu JM (1999). Evidence on the monophyly of *Astragalus* (Fabaceae) and its major subgroups based on nuclear ribosomal DNA ITS and chloroplast DNA trnL intron data. Syst Bot 24: 409–437.

Wojciechowski MF, Sanderson MJ, Steele KP, Liston A (2000). Molecular phylogeny of the "temperate herbaceous tribes" of Papilionoid legumes: a supertree approach. In: Herendeen PS, Bruneau A, editors. Advances in Legume Systematics. Part 9. Kew, UK: Royal Botanic Gardens, pp. 277–298.

Yildiz B, Ciplak B, Aktoklu E (1999). Fruit morphology of sections of the genus *Onobrychis* Miller (Fabaceae) and its phylogenetic implications. Isr J Plant Sci 47: 269–282.

Young ND, Healy J (2003). GapCoder automates the use of indel characters in phylogenetic analysis. BMC Bioinformatics 4: 6.