PHYLOGENY OF IRANIAN ASTRAGALUS SECT. ONOBRYCHOIDEI DC. (FABACEAE) BASED ON NUCLEAR RIBOSOMAL DNA ITS SEQUENCES AND MORPHOLOGICAL DATA

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Phylogenetic relationships among 30 species of sect. *Onobrychoidei* and some related sections are investigated using nrDNA ITS and morphological data. Based on Maximum Parsimony and Bayesian analyses the morphological characters are useful at the sectional level but not at lower taxonomic ranks. *Astragalus* sect. *Onobrychoidei* at the current status is not monophyletic. Some members of the section are well intermixed with members of related sections. Another species of the section, *A. oligoflorus,* is well allied with species of the sect *Erioceras* at the base of the resulting phylogenetic trees. Analyses confirmed that debatable species *A. goktschaicus* and *A. scapiger* are belonging to the sect. *Onobrychoidei*. *A. huthianus* (of sect. *Craccina*) was also placed within sect. *Onobrychoidei* and it should be classified as a member of lat er section.

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Key words. Asrtagalus, Section *Onobrychoidei*, Phylogeny, nrDNA ITS, Iran.

مطالعه فیلوژنتیکی گونه‌های ایرانی بخش *Onobrychoidei* از جنس گون (تیره بقولات) بر اساس داده‌های ریخت شناسی و توالی‌های ITS هسته‌ای

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با استفاده از داده‌های حاصل از توالی‌های ITS ژنوم هسته‌ای و داده‌های ریخت‌شناسی، مطالعه فیلوژنتیکی گونه‌های بخش *Onobrychoidei* به همراه گونه‌هایی از بخش‌های نزدیک مرتبط با آن انجام پذیرفت. نتایج آنالیزهای Bayesian و بیشینه صرفه‌جویی نشان داد که صفات ریخت‌شناسی برای تمایزات سطح بخش مناسب هستند و در سطوح پائین‌تر کاربردی ندارند. در این مطالعه نشان داده شد که بخش *Onobrychoidei* یک بخش تک‌تبار نمی‌باشد. برخی اعضای این بخش بطور گسترده‌ای با اعضای بخش‌های مجاور آمیخته شده‌اند. در نتایج فیلوژنتیکی مولکولی نشان داده شد که گونه *A.oligoflorus* از بخش مطالعه شده در مجاورت اعضای بخش *Erioceras* قرار می‌گیرد، در حالی که در این نتایج تعلق گونه‌های بحث برانگیز *A. goktschaicus* و *A. scapiger* به بخش *Onobrychoidei* تائید گردید. همچنین با قرارگیری گونه *A. huthianus* (که قبلا از بخش *Craccina* گزارش شده بود) در میان گونه‌های این بخش به نظر می‌رسد که این گونه باید به بخش *Onobrychoidei* انتقال بیابد.

INTRODUCTION

The genus *Astragalus* L., is the largest genus of flowering plants with 2500-3000 species distributed in semi-arid steppe regions of Eurasia, North Africa, North and South America. The Old World *Astragalus* species has been classified into ca. 150 sections, of which 65-70 sections were documented to occur in Iran (Maassoumi 1998, 2003, 2005, Podlech 1998).

Sect. *Onobrychoidei* DC. is one of large sections of the Old World *Astragalus* with more than 80 species of which 23-26 ones are in Iran and ca. 8-15 are endemics (Podlech & al. 2010, Maassoumi 1998, 2005). The section was treated in various regional Floras including Flora of U.S.S.R (Gontscharov & al. 1946), Flora of Turkey (Chamberlain & Matthews 1970), Flora of Iraq (Townsend & Guest 1974), (Maassoumi 2005), and Flora Iranica (Podlech & al. 2010). Although some revisionary works have been carried out on section *Onobrychoidei* (Ekici & al. 2011, Ghahremani-Nejad 2004, Podlech & Sytin 2002, Ranjbar & Maassoumi 1998), status of the section and evolutionary relationships among its species are unknown.

Recent molecular systematic studies of *Astragalus* revealed that few sampled species of the sect. *Onobrychoidei* were nested in an unresolved clade with members of sections *Ornithopodium* Bunge and *Hololeuce* Bunge (Kazempour Osaloo & al. 2003, 2005). Whereas these results and such similar phylogenetic analysis of different sections are not concordant with the traditional subgeneric classification of the genus (Riahi & al. 2011, Kazemi & al. 2009, Podlech & al. 2010, Maassoumi 2005).

The present study deals with phylogenetic analysis of the sect. *Onobrychoidei* using nrDNA ITS sequences and morphology to address the following questions:

1. Is this section monophyletic?
2. What are the species relationships within this section?

MATERIALS AND METHODS

Taxon sampling

The sampling included thirty species including 20 species of the sect. *Onobrychoidei*, three species of the sect. *Hololeuce*, one species of the sect. *Craccina* (Steven) Bunge and four species of the sect. *Ornithopodium* and sect. *Erioceras* Bunge as closely related groups to the mentioned section. Finally, two species belonging to sect. *Caraganella* Bunge (*A. stocksii* Benth. ex Bunge) and sect. *Incani* DC. (*A. supervisus* Sheld.) were chosen as outgroups based on previous studies (Kazempour Osaloo & al. 2003, 2005). The complete nrDNA ITS region for the majority of taxa were produced for the first time in the present study. Some sequences were obtained from GenBank for outgroups and representatives of sect. *Ornithopodium* or were obtained by personal communication with other researchers (Sheikh Akbari Mehr 2012; Sheikh Akbari Mehr et al. 2012). List of species with their voucher information and Genbank accession numbers are given in Table 1.

Morphological data

Morphological characters used in this study were obtained through examination of fresh materials in the field and herbarium specimens deposited at Central Herbarium of Iran (TARI) and adopted from appropriate references (Podlech & al. 2010, Maassoumi 2005). Thirty five studied characters and their relevant character states are shown in table 2. The polarity of characters was determined using the outgroup comparison method (Maddison & al. 1984).

DNA extraction, amplification and sequencing

Leaf materials were sampled from herbarium specimens deposited in the Herbarium of Research Institute of Forests and Rangelands (TARI) and then the total genomic DNA were extracted following the modified CTAB method of Doyle and Doyle (1987). In the next step, the internal transcribed spacer region (ITS) of nuclear ribosomal DNA was amplified using primers introduced by White & al. (1990) and Kazempour Osaloo & al. (2003). PCR products were used for sequencing process and finally the results were analyzed under sequencer program using ABI Genetic Analyzer.

Phylogenetic analysis

The nrDNA ITS region for 30 species were aligned using Clustal X software (Larkin & al. 2007). On the other hand, morphological dataset was formed and then Phylogenetic analyses were performed on both morphological and aligned molecular data matrices. For this propose, the datasets were analyzed by Maximum Parsimony and Bayesian methods using PAUP* ver. 4.0b10 (Swofford 2002) and MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck, 2003) softwares respectively.

Maximum parsimony method

Maximum Parsimony (MP) analyses were conducted using the PAUP* program for phylogenetic analyses. All 35 scored characters were parsimony informative. The heuristic search option was employed for each of the datasets, using tree bisection-reconnection (TBR) branch swapping, with 1000 replications of random addition sequence without an automatic increase in the maximum number of trees with MulTrees on and steepest descent off. Supports for branches were evaluated by bootstrapping analysis (Felsenstein 1985) using 10000 replications with the heuristic search option, simple addition sequence and TBR branch swapping. Initially all characters were used as

Table 1. *Astragalus* species, their voucher information and Genbank accession number included in the present study.

Species	Section	Herbarium number and collectors	Genbank accession numbers
A. aduncus Willd.	*Onobrychoidei*	TARI – 84104: Safavi, Alizadeh & Nikchehreh	AB727507
A. arguricus Bunge	*Onobrychoidei*	TARI – 84091 : Safavi & Nikchehreh	AB727508
A. asciacalyx Bunge	*Onobrychoidei*	TARI – 84173: Safavi , Alizadeh & Nikchehreh	AB727509
A. bijarensis Podlech & Sytin	*Onobrychoidei*	TARI – 1662: unknown collector	AB727510
A. brevidens Freyn & Sint.	*Onobrychoidei*	TARI – 21366: Assadi & Maassoumi	AB727511
A. brevipes Bunge	*Onobrychoidei*	TARI – 80638: Maassoumi & Shahsavari	AB727512
A. cancellatus Bunge	*Onobrychoidei*	TARI – 84074: Safavi & Nikchehreh	AB727513
A. effuses Bunge	*Onobrychoidei*	TARI – 69398: Jamzad & Taheri	AB727514
A. goktschaicus Grossh.	*Onobrychoidei*	TARI – 13756: Foroughi & Assadi	AB727515
A. lilacinus Boiss.	*Onobrychoidei*	TARI – 58962: Mozaffarian	AB727516
A. oligoflorus Maassoumi & Javadi	*Onobrychoidei*	TARI – 84036: Safavi & Nikchehreh	AB727517
A. onobrychis L.	*Onobrychoidei*	TARI – 84011: Safavi & Nikchehreh	AB727518
A. parvarensis Podlech & Sytin	*Onobrychoidei*	TARI – 80598: Safavi	AB727519
A. scapiger Ranjbar & Maassoumi	*Onobrychoidei*	TARI – 47545: Maassoumi	AB727520
A. sevangensis Grossh.	*Onobrychoidei*	TARI – 82555: Maassoumi & Safavi	AB727521
A. suffianicus Podlech & Sytin	*Onobrychoidei*	TARI – 84022: Safavi & Nikchehreh	AB727522
A. tehranicus Boiss & Hohen.	*Onobrychoidei*	TARI – 15069: Babakhanlou & Amin	AB727523
A. trifoliolatus Boiss.	*Onobrychoidei*	TARI – 757: Fattahi & Hamzei	AB727524
A. vegetus Bunge	*Onobrychoidei*	TARI – 80138: Maassoumi	AB727525
A. xerophilus Ledb.	*Onobrychoidei*	TARI – 1667: Hekmatjou	AB727526
A. alyssoides Lam.	*Hololeuce*	TARI – 80175: Maassoumi & Nikchehre	AB727527
A. neochaldoranicus Podlech & Maassoumi	*Hololeuce*	TARI – 84054: Safavi & Nikchehreh	AB727528
A. psoraloides Lam.	*Hololeuce*	TARI – 47407: Akbarzadeh	AB727529
A. huthianus Freyn & Bornm.	*Craccina*	TARI – 24: Khosravi	AB727506
A. ornithopodioides Lam.	*Ornithopodium*	TARI – 34629 Mozaffarian & Nowroozi	AB051975
A. shelkovnikovii Grossh.	*Ornithopodium*	TARI – 6032: Foroghi	AB051971
A. kerejensis Podlech	*Erioceras*	TARI – 82404: Maassoumi & Jalili	AB749819
A. djenarensis Sirj. & Rech. f.	*Erioceras*	TARI – 16786: Jouharchi & Zangoii	AB721957
A. stocksii Benth. ex Bunge	*Caraganella*	TARI – 10802: Foroughi	AB051966.1
A. supervisus Sheld.	*Incani*	TARI – 10844: Wendelbo	AB231116.1

unweighted and then in order to improve the trees indices and decrease the effect of characters showing high homoplasy on tree topologies, the analyses were conducted using a successive reweighting (SW) strategy (Farris 1969) based on the rescaled consistency (RC) index (Farris 1989) with a base weight of 1. When the tree length, consistency index (CI), retention index (RI) and RC remained unchanged in successive rounds, these trees were accepted as the SW trees. To assess combinability of datasets, the incongruent length difference test (ILD; Farris & al. 1995) was conducted using PAUP* (Swofford 2002).

Bayesian method

Models of sequence evolution were selected using the program MrModeltest version 2.3 (Nylander 2004) based on Akaike information criterion (AIC) (Posada & Buckley 2004). On the basis of this analysis, datasets were analyzed using the GTR+I+G for nrDNA ITS sequences and standard (morphology) discrete state (lset coding = variable, (nst =1) +G) for morphological data partition, respectively. Finally the combined dataset for 26 taxa were analyzed as separate partitions

with their assumed model. Posteriors on the model parameters were estimated from the data, using the default priors. The analysis was carried out with 3 million generations, using the Markov Chain Monte Carlo search. MrBayes performed two simultaneous analyses starting from different random trees (Nruns=2) each with four Markov chains and trees sampled at every 100 generations were performed. The first 25% of trees were discarded as the burn in. The remaining trees were summarized in a 50% majority rule consensus tree, using Posterior Probabilities (PP) as a measure of clade support. Tree visualization was carried out using Tree View version 1.6.6 (Page 2001).

RESULTS

Morphological analysis

The data matrix consisted of 17 binary and 18 multi-state characters (table 2). The phylogenetic analysis based on equally weighted characters resulted in 7 most parsimonious trees with length (L) 175 steps, Consistency Index (CI) = 0.337, and Retention Index (RI) = 0.457. The same analysis based on the successive reweighing analysis using rescaled consistency index (RC), generated 9 most parsimonious trees (after five rounds of reweighing) with length = 25.84 steps, consistency index (CI) = 0.588 and retention index (RI) = 0.723. The strict consensus tree and also posterior probability values of Bayesian analysis in this dataset with more than two million generations were shown in Fig. 1. As indicated in cladogram, a few branches are showing bootstrap (BS) values more than 50%.

Two species of sect. *Erioceras* were formed a moderately supported clade (BS = 77% and PP = 0.52) at the base of cladogram. Twenty sampled species of sect. *Onobrychoidei* plus other studied species constructs weekly supported clade (PP = 0.56); whereas, species of sect. *Hololeuce* and *Ornithopodium* formed strongly supported subclades (BS = 86% and PP = 0.97 for *Hololeuce* and BS = 82% and PP = 0.94 for *Ornithopodium* respectively). These subclades were placed at the base of other species in a distinct part of cladogram. Nonetheless, species of sect. *Onobrychoidei* made a polytomic clade and species relationships within this section were not resolved.

On the other hand, *A. huthianus* Freyn & Bornm. (sect. *Craccina*) was not isolated from *Onobrychoidei* and the relationships among these species remained unresolved. Thus it seems that morphological characters are inadequate to resolve the main complexity of this group.

Analysis of nrDNA ITS sequence data

The length of the nrDNA ITS dataset for 30 taxa was 674 nucleotide sites, of which 46 sites were potentially parsimony informative characters. The phylogenetic analysis based on equally weighted characters resulted in 12 most parsimonious trees with length (L) 69 steps, Consistency Index (CI) = 0.839, and Retention Index (RI) = 0.889. The same analysis based on the successive reweighing analysis using rescaled consistency index (RC), generated 12 most parsimonious trees (after two reweighing rounds) with length = 48 steps, consistency index (CI) = 0.932 and retention index (RI) = 0.959. The strict consensus tree and also posterior probability values of Bayesian analysis of this dataset with more than two million generations were shown in Fig. 2. In this tree, two species of sect. *Erioceras* along with *A. oligoflorus* Maassoumi (sect. *Onobrychoidei*) formed a strongly supported clade at the base of cladograms (BS = 100%, PP = 1.00). The next is the two well supported subclades of sect. *Onobrychoidei* as successive grades being sister to an assemblage of the remaining species of the section plus a dozen of related sections (sects. *Hololeuce* , *Ornitopodium* and *Craccina*). The first grade is composed of *A. xerophilus* Ledeb, *A. onobrychis* L and *A. bijarensis* Podlech & Sytin (BS = 100 %, PP = 1.00). The second one comprises *A. scapiger* Ranjbar & Maassoumi, *A. goktschaicus* Grossh and *A. suffianicus* Podlech & Sytin (BS = 51 %, PP = 0.95). Within the large assemblage, three well supported subclades were appeared. The first subclade includes *A. ornithopodioides* Lam. (sect. *Ornithopodium*) and *A. tehranicus* Boiss. & Hohen. (sect. *Onobrychoidei*). The second subclade contains *A. psoraloides* Lam. (sect. *Hololeuce*) and *A. sevangensis* Grossh (sect. *Onobrychoidei*). The third one as a trichotomy, comprises *A. trifoliolatus* Boiss., *A. effuses* Bunge and *A. arguricus* Bunge (sect. *Onobrychoidei*).

Analysis of the combined data

ILD test suggested that the morphological and nrDNA ITS datasets are incongruent ($p<0.01$). Following the suggestions of several authors (Yoder 2001, Wiens 1998, Seelanan 1997) that the ILD test may be unreliable, we still decided to combine these datasets directly. Bayesian analysis of the combined dataset, as two separate partitions, with four million generations generated a 50% majority rule consensus tree along with posterior probability (Fig. 3).

Again, the two members of sect. *Erioceras* along with *A. oligoflorus* were positioned at the base of the tree (PP = 0.96). The next is a subclade of *A. goktschaicus* and *A. scapiger* sister to a larger clade. This clade is, in turn, composed of a small subclade of four species of the sect. *Onobrychoidei* and a large polytomy of most of the remaining species of the

Table 2. Morphological characters and character states used in the analysis.

	Character	**Character states**
1	Habit	Spiny Shrub = 0, Herbaceous without spine = 1, Shrub without spine = 2
2	Plant height	Less than 10 cm = 0, between 10 to 50 cm = 1, more than 50 cm = 2
3	Stem state	Erect = 0, Procumbent = 1, Erect or Procumbent = 2, Sulcate = 3
4	Stem hair density	Sparse= 0, Dense = 1
5	Stem black hair	Absent = 0, Present = 1
6	Stipule length	Less than 2 mm = 0, between 2 to 5 mm = 1, more than 5 mm = 2
7	Stipule hair color	Only white = 0, White mixed with black = 1
8	Leaf type	Pinnate = 0, Odd pinnate = 1 Single leaflet = 2
9	Leaflet pair number	Less than 3 = 0, between 3 to 10 = 1, more than 10 = 2
10	Leaflet l/w ratio	Less than 1.5 = 0, between 1.5 to 15 = 1, more than 15 = 2
11	Leaflet shape	Linear = 0, Narrowly Elliptic = 1, Elliptic = 2, ovate = 3
12	Leaflet hair state and density	Equal = 0, Unequal and Sparse =1, Unequal and Dense = 2
13	Peduncle black hair	Absent = 0, Present = 1
14	Inflorescence	Sparse raceme =0, Dense raceme =1,Ovate or elliptic = 2,Globular = 3, Cylindrical=4
15	Bract length	Less than 0.5 mm = 0, between 0.5 to 3 mm = 1, more than 3 mm = 2
16	Calyx hair state	Procumbent = 0, Erect = 1
17	Calyx hair symmetry	Symmetrical = 0, Asymmetrical = 1
18	Calyx length	Less than 5.5 mm = 0, between 5.5 to 15 mm = 1, more than 15 mm = 2
19	Calyx symmetry	Teeth equal = 0, Teeth unequal = 1
20	Calyx tooth length	Less than 0.5 mm = 0, between 0.5 to 3 mm = 1, more than 3 mm = 2
21	Calyx tooth surface	Without hairs = 0, densely hairy = 1, sparsely hairy = 2
22	Corolla color	Yellow = 0, Pink = 1, Violet = 2, Blue =3, White =4
23	Standard l/w ratio	Less than 2.5 = 0, more than 2.5 = 1
24	Standard shape	Elliptic = 0, Ovate = 1, Narrowly elliptic =2, Rhombic = 3
25	Standard tip	Obtuse = 0, Acute = 1, Emarginate = 2, Truncate = 3
26	Wing l/w ratio	Less than 3.5 = 0, more than 3.5 = 1
27	Keel l/w ratio	Less than 2 = 0, more than 2 = 1
28	Ovary stalk	Absent = 0, Present = 1
29	Style hair	Absent = 0, Present = 1
30	Fruit shape	Elliptic or Narrowly elliptic = 0, Ovate = 1
31	Fruit section	Complete bilocular= 0, Incomplete bilocular= 1
32	Fruit l/w ratio	Less than 3 = 0, between 3 to 15 = 1, more than 15 = 2
33	Fruit hair	Absent = 0, Present = 1
34	Hair state on fruit	Procumbent = 0, Erect or Semi-erect = 1
35	Fruit black hair	Absent= 0, Present = 1

section and allies.

DISCUSSION

It has been already noted that the delimitation of the sections in non-spiny *Astragalus* (especially sections studied herein) is very difficult due to some uncertain characters (Ekici & al. 2011, Podlech & al. 2010, Maassoumi 2005, Ghahremani-Nejad 2004). Various molecular systematic analysis using nuclear and plastid gene sequences have shown that the sectional classification of the genus is artificial, and thus, monophyly of various sections were not confirmed (Riahi & al. 2011, Kazemi & al. 2009, Kazempour Osaloo & al. 2003, 2005). As mentioned in the result, it seems that sect. *Onobrychoidei* at the current status is not monophyletic. One species, *A. oligoflorus* is well allied with the two species of sect. *Ere oceras* at the base of nrDNA ITS and combined phylogenies (see Figs. 2 and 3). The remaining species of the section with the inclusion of other sections formed a moderately to well supported clade.

Maassoumi (2005) postulated that the sectional assignment of *A. scapiger*, *A. goktschaicus* and in particular *A. oligoflorus* (characterized by 3- leaflet pairs) to sect. *Onobrychoidei* is problematic. Nevertheless, he placed these species in sect.

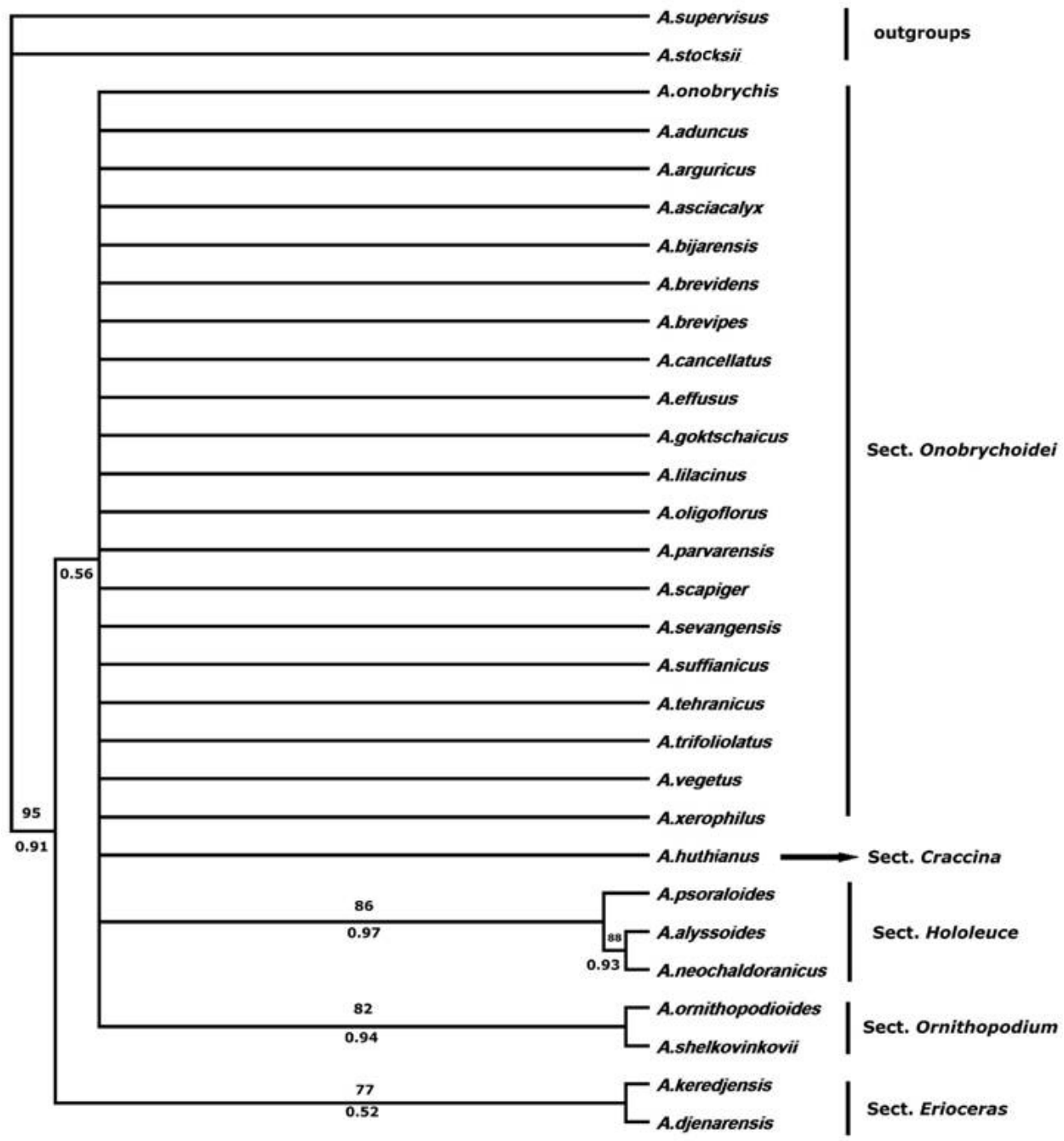

Fig. 1. Strict consensus tree of nine most-parsimonious trees obtained from a morphological cladistic analysis after successive reweighing. Bootstrap values greater than 50% and Bayesian posterior probabilities were shown above and below the branches, respectively.

Onobrychoidei. It is worth to note that the latter species was not treated in the Flora Iranica (Podlech & al. 2010). Our data clearly demonstrated that *A. oligoflorus* is not belonging to this section and should be transferred to sect. *Erioceras*. But, the derived position of *A. goktschaicus* and *A. scapiger* indicates that they are definitely members of sect. *Onobrychoidei*. As mentioned above, the studied

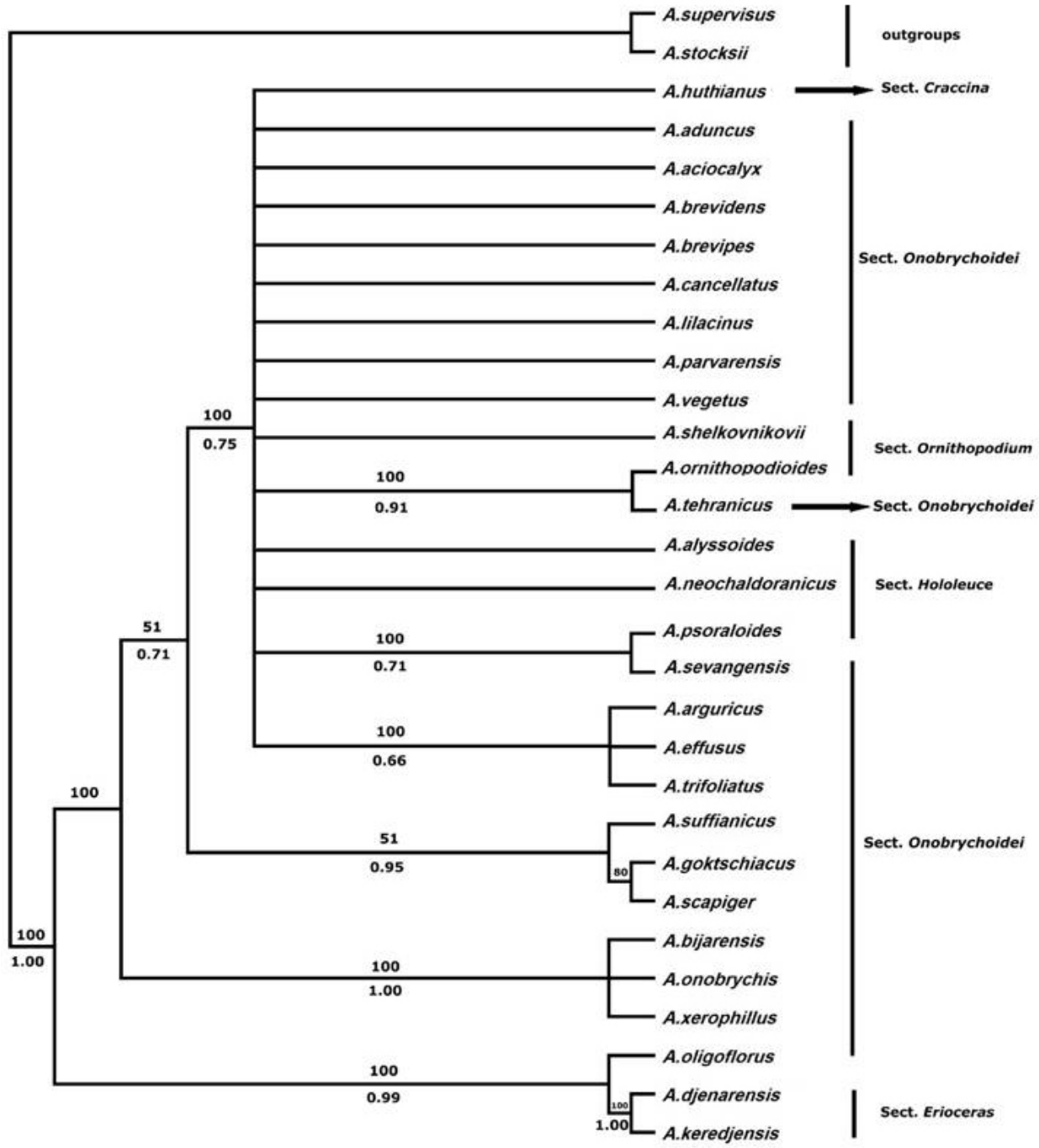

Fig. 2. Strict consensus tree of twelve most-parsimonious trees resulting from analyses of the nrDNA ITS data set after successive reweighing. Numbers below branches are posterior probabilities and MP bootstrap values are above them, values less than 50% were not shown.

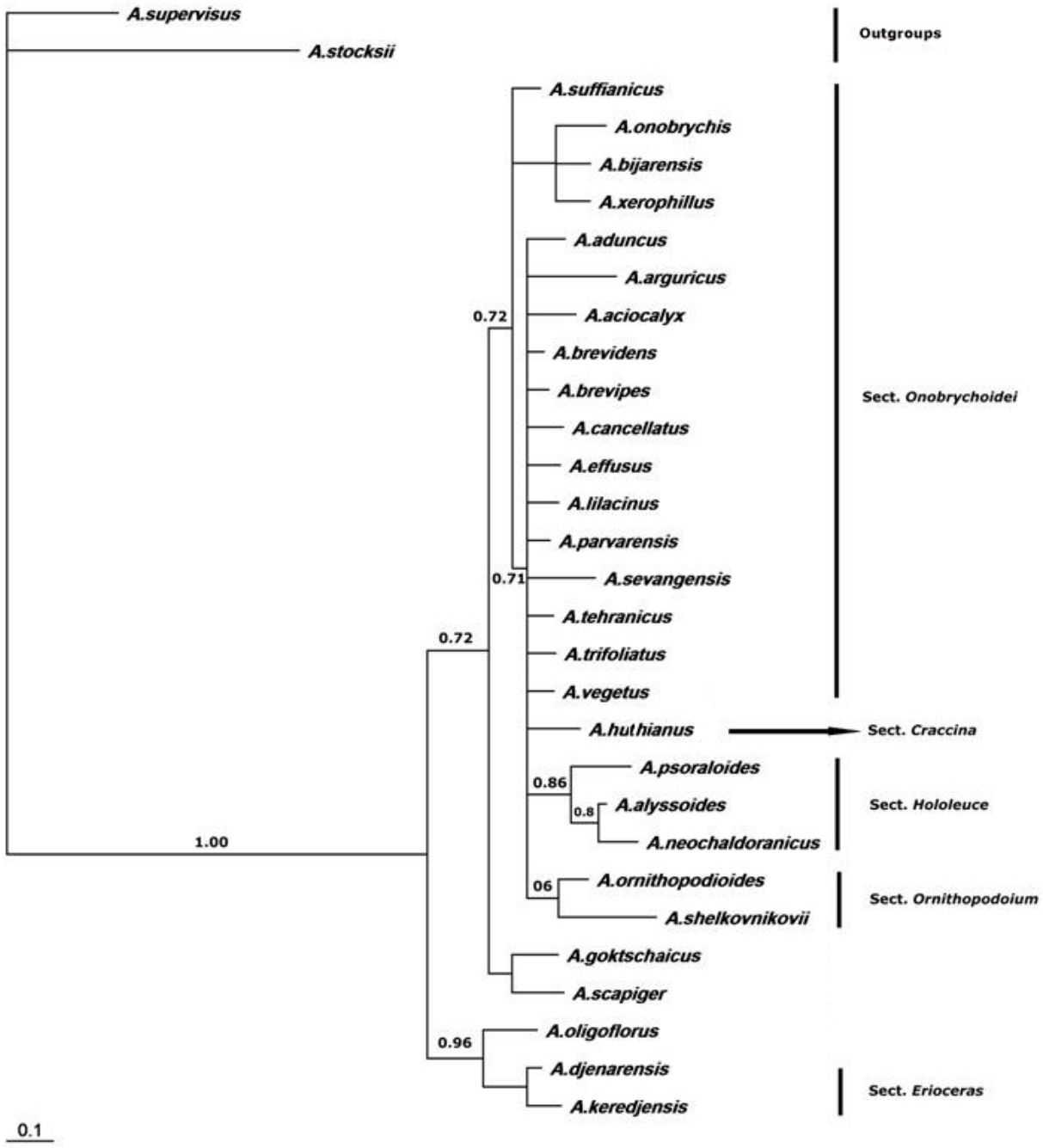

Fig. 3. Fifty percent majority rule consensus tree resulting from Bayesian analyses of combined datasets. Numbers of the branches are posterior probabilities, values less than 0.5 were not shown.

members of sections *Craccina*, *Hololeuce* and *Ornithopodium* were nested among the species of sect. *Onobrychoidei*. According to different treatments, sect. *Craccina* is represented by 1-3 species in Iran (Podlech & al. 2010, Maassoumi 1998, 2005), of which, *A. huthianus* was analyzed here. Due to some differences in pod and calyx features, *A. huthianus* was placed in this section (Podlech & al. 2010, Maassoumi 1998, 2005). Some authors (Ghahremani-Nejad 2004, Maassoumi 2005) noted, however, that placement of

the species in sect. *Craccina* is doubtful, and thus might be transferred to sect. *Onobrychoidei*. This is well consistent with our finding. Sections *Hololeuce* and *Ornithopodium* formed their own clades among the members of sect. *Onobrychoidei.* Affinity of these two sections with the latter one was indicated by previous studies (Ekici & al. 2011, Maassoumi 2005, Ghahremani-Nejad 2004, Kazempour Osaloo 2003, Ranjbar & Karamian 2002). The present study is well corroborated with these hypotheses. However, to give an accurate assessment of classification of these sections, more taxon sampling and more DNA sequences are definitely necessary.

CONCLUSION

The studied species of sect. *Onobrychoidei* did not comprise a single monophyletic group. Due to the paucity of informative nucleotide sites in nrDNA, relationships among the bulk of sect. *Onobrychoidei* remains unresolved. Therefore, fast evolving genic regions including non-coding cpDNA fragments and single copy nuclear genes are clearly required to resolve phylogenetic relationships between and within these related sections.

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