PHYLOGENY OF ASTRAGALUS SECTION DISSITIFLORI BASED ON nrDNA ITS AND MORPHOLOGICAL DATA IN IRAN

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Twenty four species belonging mainly to *Astragalus* L. section *Dissitiflori* DC. and some related sections were analyzed using maximum parsimony and Bayesian methods on the basis of nrDNA ITS sequences and morphological data. Trees resulted from two datasets were in agreement on their overall topologies. Based on our results, members of the section *Dissitiflori* didn't constitute a monophyletic group. However, the section with inclusion of the species from the other related ones, considered to be a monophyletic group. Since *A. virgatus* as the lectotype of the section *Dissitiflori* was not included in the present study; it is difficult to evaluate the monophyly of the section and to delimit it explicitly. *Astragalus juladakensis*, shows a basal position in both nrDNA ITS and the combined nrDNA ITS-morphology trees, as a sister to the remaining species of the sect. *Dissitiflori*. However, according to the present study, the affinity of this species to the section appeared to be questionable. Based on our molecular data, three species belonging to the section *Corethrum* in Iran, are pertained to the sect. *Dissitiflori*. In addition, our results revealed that *A. pravitzii*, which had been transferred to sect. *Ornithopodium* Bunge, belongs to the section *Dissitiflori*.

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فیلوژنی بخشه Dissitiflori از جنس گون در ایران براساس داده‌های ریخت شناختی و nrDNA ITS

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در مطالعه حاضر فیلوژنی بیست و دو گونه از گونه‌های کرک دوشاخه‌ای متعلق به بخشه دیسیتی فلوری و چند بخشه وابسته و نزدیک به آن، با استفاده از داده‌های ریخت‌شناختی و مولکولی مورد ارزیابی قرار گرفت. براساس نتایج بدست آمده، بخشه *Dissitiflori* در تمام آنالیزهای انجام شده، به تنهایی گروهی منوفیلتیک را تشکیل نمی‌دهد. البته اعضای این بخشه بهمراه گونه‌های مربوط به دیگر بخشه‌های نزدیک به آن را می‌توان یک گروه تک‌تبار در نظر گرفت. بنظر می‌رسد برای تعیین دقیق وضعیت فیلوژنتیکی بخشه *Dissitiflori* بایستی مطالعات بیشتر با استفاده از قطعات دیگری از ژنوم و همچنین وارد کردن گونه *A. virgatus.* (بعنوان لکتوتیپ بخشه *Dissitiflori*) در آنالیزها، انجام پذیرد. براساس نتایج حاصل از داده‌های دی ان اِی ریبوزومی، گونه *A. juladakensis* یک گروه خواهری را نسبت به بقیه گونه‌های مطالعه‌شده تشکیل داد. بنظر می‌رسد جایگاه این گونه بایستی در بخشه دیگری از جنس ارزیابی شود. همچنین نتایج مطالعه مولکولی (براساس دی اِی ریبوزومی) و ریخت شناختی نشان دادند، گونه *A. pravitzii* که به بخشه *Ornithopodium* منتقل شده بود به *Dissitiflori* تعلق دارد.

INTRODUCTION

Astragalus L. is the largest genus of flowering plants, comprising about 3000 species. The main centers of biodiversity of the Old World *Astragalus* are in southwestern and central Asia (Lock & Simpson 1991). There are over 800 species in Iran alone (Podlech 1999, Maassoumi 1998, Maassoumi 2005). *Astragalus* sect. *Dissitiflori* DC. is one of the largest sections of the genus with about 20 species in Iran. The recent molecular phylogenetic study of the genus based on nrDNA ITS data revealed that this section with only two sampled species, nested in a polytomic assemblage along with several related medifixed-hair sections such as *Erioceras* Bunge, *Cytosides* Bunge, etc. (Kazempour & al. 2003, 2005). The classical classification of *Astragalus* mainly depends on morphological characteristics (Bunge 1868-69, Podlech 1990). These characteristics are mainly affected by environmental factors during plant growth (Cai & al. 1999). Although identification of some sections within the genus *Astragalus* is relatively simple, however some sections pose much more complex situations. The positioning of the species within each section is the most challenging task facing the taxonomists. Section *Dissitiflori* is one of the most complex sections within this genus. The objectives of the present study are to test the monophyly of the section and to evaluate the relationships within it.

MATERIALS AND METHODS
Taxon sampling
A total of 24 species of *Astragalus* (15 from section *Dissitiflori*) plus seven other related taxa from sections: *Corethrum* Bunge, *Cystisodes* Bunge and *Erioceras* Bunge (Maassoumi 1998) and two species of *Astragalus* sections: *Incani* DC. and *Caraganella* Bunge as outgroups (based on previous studies, Kazempour & al. 2003, 2005), were included in phylogenetic analyses based on both nrDNA ITS sequence and morphological data (Table 1). The nrDNA ITS was newly sequenced for all *Astragalus* species in this study except for the outgroups adopted from Kazempour Osaloo & al. (2003) and downloaded from GenBank.

Morphological study
Characters used in the cladistic analysis were obtained through examination of fresh materials in the field and herbarium specimens deposited at Central Herbarium of Iran (TARI), and Herbarium of Ferdowsi University (FUMH). Thirty six vegetative and reproductive characters with relevant character states used in present analyses are given in Table 2. The polarity of characters was determined using the outgroup method (Maddison & al. 1984).

Molecular study
DNA extraction, PCR and Sequencing
Total genomic DNA was extracted from dry leaves of individual plants deposited in Central Herbarium of Iran (TARI) and FUMH following the modified CTAB procedure (did not use PVP along with extraction buffer and using 5X CTAB buffer instead of 2X one) of Doyle and Doyle (1987). The complete nrDNA ITS+5.8S region was amplified using primers ITS4 of White & al. (1990) and ITS5m of Sang & al. (1995). The total volume of amplification reactions was 25 µl, made up of 18 µl deionized water, 2.5 µl of 10× PCR buffer, 2.5 µl of 2.5 mM dNTPs, 0.5 µl of each primer (5 pmol µl^{-1}), 0.25 µl (5 units) of Taq polymerase and 0.75 µl of template DNA. The PCR cycles consisted of 2.5 min at 95°C for predenaturation followed by 27 cycles of 1 min at 95°C for denaturation, 45 sec at 53.7°C for primer annealing and 50 sec at 72°C for primer extension, followed by a final primer extension of 7 min at 72°C. PCR products were used for sequencing reactions. Sequencing of the nrDNA ITS fragments was performed in an ABI Genetic Analyzer 3130 using ITS5m primer.

Phylogenetic analyses
Sequences of nrDNA were aligned using ClustalX (Larkin & al. 2007) and indel positions were treated as missing data. Phylogenetic analyses were performed on the aligned nrDNA ITS dataset and morphological dataset separately and in combination, using maximum parsimony (MP) and Bayesian approaches.

Maximum parsimony
Initially, phylogenies were inferred from two datasets using maximum parsimony method (MP) as implemented in the version 4.0b10 of PAUP* (Swofford 2002). Multiple tree searches were conducted using heuristic search options that included random addition sequences (100 replicates) holding five trees per replicate, and tree bisection-reconnection (TBR) branch swapping, with retention of multiple parsimonious trees (Maxtrees = 10000). Bootstrap (BP) support (Felsenstein 1985) was determined with 1000 replicates using heuristic search options and TBR branch swapping. For morphological analysis, initially all characters were used as unweighed. Multistate taxa were defined as polymorphism. For improving the tree indices and decreasing the effect of characters showing high homoplasy on tree topologies, a successive weighing process based on character's best fits for rescaled consistency index (Farris 1989) was carried out. After three rounds of reweighing no change in tree indices was observed. To assess combinability of two

Table 1. List of analyzed *Astragalus* taxa and their voucher specimens. Sections are based on Maassoumi (2005) and Podlech et al. (2010), separately.

Species	Voucher no.	Section (Maassoumi)	Section (Podlech et al.)	GenBank accession no.
A. argyroides	Mozaffarian & Freitag, 28538(TARI)	*Dissitiflori*	*Dissitiflori*	AB721936
A. aucheri	Mottaghi, 1061(TARI)	*Dissitiflori*	*Dissitiflori*	AB721937
A. eburneus	Mozaffarian, 44936(TARI)	*Dissitiflori*	*Dissitiflori*	AB721938
A. husseinovii	Maassoumi & Safavi, 8721(TARI)	*Dissitiflori*	*Dissitiflori*	AB721939
A. juratzkanus	Maassoumi & Pakravan 72351(TARI)	*Dissitiflori*	*Dissitiflori*	AB721940
A. melanocalyx	Noruzi & Feizi, 5860(TARI)	*Dissitiflori*	*Dissitiflori*	AB721941
A. baraftabensis	Tayebi, 4458(TARI)	*Dissitiflori*	*Dissitiflori*	AB721942
A. nigrolineatus	Faghihnia & Zangooee, 29042(FMUH)	*Dissitiflori*	*Dissitiflori*	AB721943
A. pravitzii	Foroughi, 2183(TARI)	*Dissitiflori*	*Ornithopodium*	AB721944
A. ruscifolius	Mozaffarian & Freitag, 28640(TARI)	*Dissitiflori*	*Dissitiflori*	AB721945
A. saadatabadensis	Grant, 15784(TARI)	*Dissitiflori*	*Dissitiflori*	AB721946
A. sitiens	Wendelbo & Foroughi, 11270(TARI)	*Dissitiflori*	*Dissitiflori*	AB721947
A. sumbari	Wendelbo & Foroughi, 11063(TARI)	*Dissitiflori*	*Dissitiflori*	AB721948
A. xiphidium	Youssefi, 7611(TARI)	*Dissitiflori*	*Dissitiflori*	AB721949
A. juladakensis	Maassoumi, S.N. (TARI)	*Dissitiflori*	-	AB721950
A. aestimabilis	Dehshiri, 38523(TARI)	*Corethrum*	*Dissitiflori*	AB721951
A. dendroproselius	Dehshiri, 30231(TARI)	*Corethrum*	*Dissitiflori*	AB721952
A. viridis	Moussavi, 1152(TARI)	*Corethrum*	*Dissitiflori*	AB721953
A. zoshkensis	Mozaffarian, 77059(TARI)	*Cytisodes*	*Dissitiflori*	AB721954
A. gigantirostratus	Maassoumi & al., 72339(TARI)	*Cytisodes*	*Cytisodes*	AB721955
A. anacamptus	Emadzadeh & al., 35908(FMUH)	*Erioceras*	*Erioceras*	AB721956
A. djenarensis	Joharchi & Zangooee, 1100(TARI)	*Erioceras*	*Erioceras*	AB721957
A. supervisus	Wendelbo et al., 10844(TARI)	*Incani*	*Incani*	AB231116
A. stocksii	Foroughi, 10802(TARI)	*Caraganella*	*Caraganella*	AB051966

Table 2. Characters and character states used in the cladistic analysis.

≠1 Habit: spiny lignified (0); non-spiny lignified (1); herbaceous (2).
≠2 Plant height: ≤ 10 cm (0); 10-50 cm (1); > 50 cm (2).
≠3 Shoot branching: low (0); high (1).
≠4 Stem state: stem absent (0); standing (1); prostrate (2).
≠5 Stipule length: ≤ 2 mm (0); > 2 mm (1).
≠6 Stipule color: greenish (0); membranous white (1).
≠7 Stipule hair color: only white (0); white mixed with black (1).
≠8 Leaf type: paripinnate (0); imparipinnate (1); single leaflet (2).
≠9 Leaf length: ≤ 2 cm (0); 2-7 cm (1); > 7 cm (2).
≠10 Leaflet pairs number: ≤ 3 (0); 3-10 (1); > 10 (2).
≠11 Leaflet L/W ratio: ≤ 1.5 (0); > 1.5 (1).
≠12 Leaflet shape: linear (0); oblong elliptic (1); elliptic (2); obovate (3).
≠13 Leaflet hair type: dense on both sides (0); disperse on both sides (1); one side dense and other side disperse (2).
≠14 Black hair on peduncle: absent (0); present (1).
≠15 Inflorescence: sparse raceme (0); dense raceme (1).
≠16 Bract length: ≤ 0.5 mm (0); > 0.5 mm (1).
≠17 Calyx type: campanulate (0); tubular (1); gibbose tubular (2).
≠18 Calyx hair state: appressed hair (0); standing hair (1).
≠19 Calyx hair symmetry: symmetrical (0); asymmetrical (1).
≠20 Calyx length: ≤ 5 mm (0); 5-15 mm (1); > 15 mm (2).
≠21 Calyx teeth length: ≤ 0.5 mm (0); 0.5-3 mm (1); > 3 mm (2).
≠22 Calyx teeth internal surface hair: absent (0); present (1).
≠23 Corolla color: yellow (0); purple (1); blue (2).
≠24 Standard L/W ratio: ≤ 2.5 (0); > 2.5 (1).
≠25 Standard shape: elliptic (0); obovate (1); rhomboid (2).
≠26 Standard tip: obtuse (0); acute (1); emarginated (2).
≠27 Wing L/W ratio: ≤ 3.5 (0); > 3.5 (1).
≠28 Keel L/W ratio: ≤ 2 (0); > 2 (1).
≠29 Ovary stalk: absent (0); present (1).
≠30 Style hair: absent (0); present (1).
≠31 Pod shape: linear (0); oblong elliptic (1).
≠32 Pod cross section: orbicular (0); triangular (1).
≠33 Pod L/W ratio: ≤ 3 (0); 3-15 (1); > 15 (2).
≠34 Pod hair type: hair absent (0); long and asymmetrical (1); short and symmetrical (2).
≠35 Hair compression on pod: dispersed (0); dense (1).
≠36 Black hair on pod: absent (0); present (1).

datasets, the incongruent length difference (ILD, Farris & al. 1995) test was conducted using PAUP.

Bayesian analyses

ITS and combined ITS-morphology datasets were analyzed using Bayesian inference as implemented in MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003). Models of sequence evolution were selected using the program MrModeltest2 (Nylander 2004) based on the Akaike information criterion (AIC) (Posada & Buckley 2004). On the basis of this analysis, nrDNA ITS dataset was analyzed using the SYM+I+G lonely. In combined dataset ITS sequences were included as a separate partition along with morphology character states as a second partition. A standard (morphology) discrete state model (lset coding=variable, (nst=1+G) was applied to the latter partition. Both analyses were run for two million generations, using 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. Once reaching the stationary phase, trees were collected and after burning in one fourth of them, used to build a 50% majority rule consensus tree accompanied with posterior probability (PP) values and showed using Treeview (Page 1996).

RESULTS

Morphological data analysis

Morphological analysis based on equally weighted characters resulted in three most parsimonious trees with length (L) 151 steps, Consistency Index (CI) = 0.364, and Retention Index (RI) = 0.532. All characters used in analysis were parsimony informative. After three rounds of reweighing no changes in tree indices were observed (CI = 0.553 and RI = 0.802). The single most parsimonious tree resulting from the successive reweighting is almost the same as that of unweighting analysis except that the resolution and bootstrap support is higher (Fig. 1). Based on the reweighting analysis of morphological data, *Astragalus husseinovii* Rzazade positioned at the base of the tree followed by a clade of six species from *A. saadatabadensis* Podl. through *A. argyroides* G. Beck., sister to a larger clade of the remaining species.

nrDNA ITS sequence data

The length of the aligned nrDNA ITS dataset was 600 nucleotide sites, of which 26 sites were parsimony informative characters. The Bayesian tree with posterior probabilities (PP) and bootstrap values is presented in Fig. 2. This tree was the same as the MP tree. Based on these analyses, *A. juladakensis* Maass. was placed at the base of tree as a sister group to a large assemblage of three subclades. Relationships among these three subclades not resolved but each is supported moderately to high bootstrap or PP values.

The combined nrDNA ITS and Morphological data

ILD test suggested that the nrDNA ITS and morphological datasets were incongruent (p=0.01). Following the suggestions of several authors (Seelanan & al. 1997, Wiens 1998, Yoder & al. 2001) that the ILD test may be unreliable, we decided to combine these datasets directly. The topology of the resulting tree (Fig. 3) was roughly the same as that of nrDNA ITS tree than to morphology-based tree, with the exceptions that the resolution, bootstrap and PP values are higher. Again, *A. juladakensis* formed the most basal branch sister to the remainder species.

DISCUSSION

Phylogenetic analysis of the present data revealed that the members of sections *Corethrum*, *Erioceras* and *Cytisodes* were well nested within section *Dissitiflori* (Fig. 3). Since *A. virgatus* Pallas as the lectotype of the sect. *Dissitiflori* was not included in the present study; it is difficult to evaluate the monophyly of the section and to delimit it explicitly. However, bulk members of the section *Dissitiflori* constitute a paraphyletic group

and are distributed among three main subclades (Fig. 2). Hence, with keeping these in mind, section *Dissitiflori* with the inclusion of those three sections considered to be a monophyletic group (Fig. 3). On the other hand, *A. juladakensis*, which was recently established as a new species belonging to the section, from the alpine area of Qazvin city, northern Iran (Maassoumi 2007), was positioned at the base of the nrDNA ITS and the combined nrDNA ITS-morphology trees, as a sister to the remaining species (see Figs 2, 3). However, on the morphology-based tree, *A. juladakensis* has a derived position within the tree as weakly allied with *A. zoshkensis* F. Ghahrem. and in turn, sister to a clade of six species (Fig. 1). This taxon was morphologically considered to be similar to *A. aestimabilis* Podl., *A. viridis* Bge. and *A. dendroproselius* Rech. f., but differs from them by having linear leaflets and lacking black hair on the pod (Maassoumi 2007). According to the present data and Kazempour Osaloo & al. study (unpub. data), the affinity of this species to the section appeared to be questionable. Hence, to assess the exact position of this enigmatic species, additional molecular markers and more taxon sampling are absolutely needed. A recent classical taxonomic work assumed that *A. viridis* and *A. dendroproselius* (plus *A. kharvanensis* Ranjbar, not analyzed here) are closely related to each other, so called the viridis group, within the section *Dissitiflori* (Ranjbar, 2004). This is consistent with our morphology-based cladistic analysis that the first two species plus *A. aestimabilis* are closely related (see Fig. 1). On the other hand, these three species were separated from the section and moved to the section *Corethrum*, based on having ovate-elliptic pods and asymmetrical standing indumentum on calyx (Maassoumi 2005). However, our nrDNA ITS and combined dataset revealed that these taxa neither closely related to each other nor nested in a single clade, indicating these features were evolved independently between the pair species *A. viridis* and *A. dendroproselius*, and *A.aestimabilis*. Therefore, these taxa are best to be treated as the members of *Dissitiflori*. Indeed, Podlech (2010), in Flora Iranica, treated them within the section.

The section *Cytisodes* is distinguished among bifurcate hairy sections with having the stem of short internodes, calyx with standing hairs and long beak on the pod (Bunge 1868-69). Maassoumi (2005) moved the newly established species *A. zoshkensis* by Ghahremani-nejad (2003), from the section *Dissitiflori* to the *Cytisodes* based on calyx hairs and pod features. Ghahremani-nejad (2003) noted this species is closely
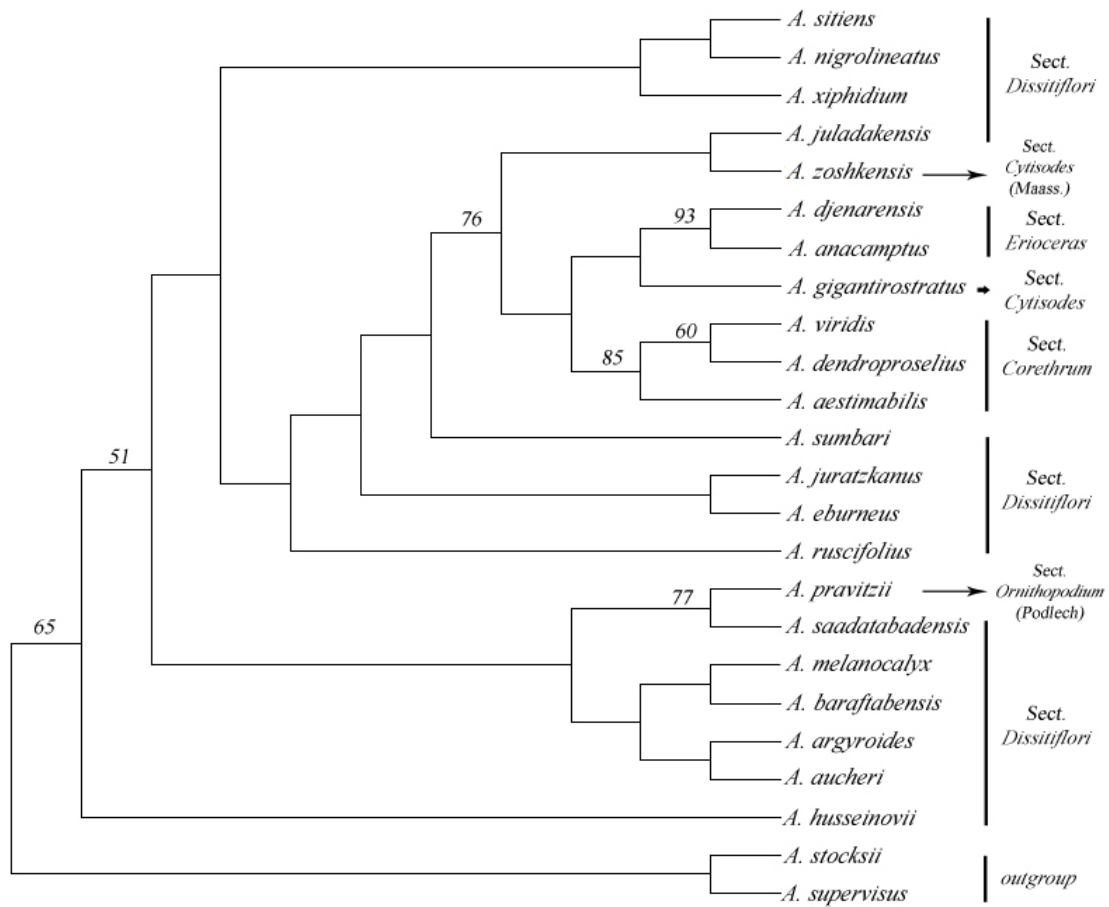
Fig. 1. The single most parsimonious tree obtained from morphological cladistic analysis after successive reweighting with RC (Length = 23.072 steps, CI= 0.553, RI= 0.802). Bootstrap values are given above branches (Values below 50% were not shown).

related to *A. sumbari* M. Pop (= *A. tolgorensis*, see Podlech 2010). This is well corroborated with our nr DNA tree, on which the two species are sister taxa nested in a clade with the other *Dissitiflori* species (Fig. 2). It is noteworthy that the other species of the *Cytisodes* (*A. gigantirostratus* Maassoumi & al.) studied here was placed beside the members of the *Dissitiflori* (Fig. 3).

The two morphologically similar species *A. pravitzii* Podl. and *A. saadatabadensis* are sister taxa based on the present analyses (see Fig. 1-3). *A. pravitzii* was established as a new species from the sect. *Dissitiflori* by Podlech (2001). Later on, Podlech and Sytin (2010) transferred it to the sect. *Ornithopodium* Bunge. Our results revealed that *A. pravitzii* belongs to the sect. *Dissitiflori*. Although *A. husseinovii* placed as a sister group to the other studied species based on morphology analysis (Fig. 1), our nrDNA ITS and combined dataset trees showed that this species is allied to *A. xiphidium*

Bge. (a typical species of the sect. *Dissitiflori*), with high BP & PP values (see Figs 2, 3). In addition, two widespread species of the *Dissitiflori* (*A. ruscifolius* Boiss. and *A. argyroides*), revealed a high affinity based on nrDNA ITS sequence and placed beside two species sampled from the sect. *Erioceras* (Fig. 2). These results showed that delimitation of sect. *Dissitiflori* needs to be revised using other fast evolving genic regions including non-coding cpDNA fragments and single copy nuclear genes.
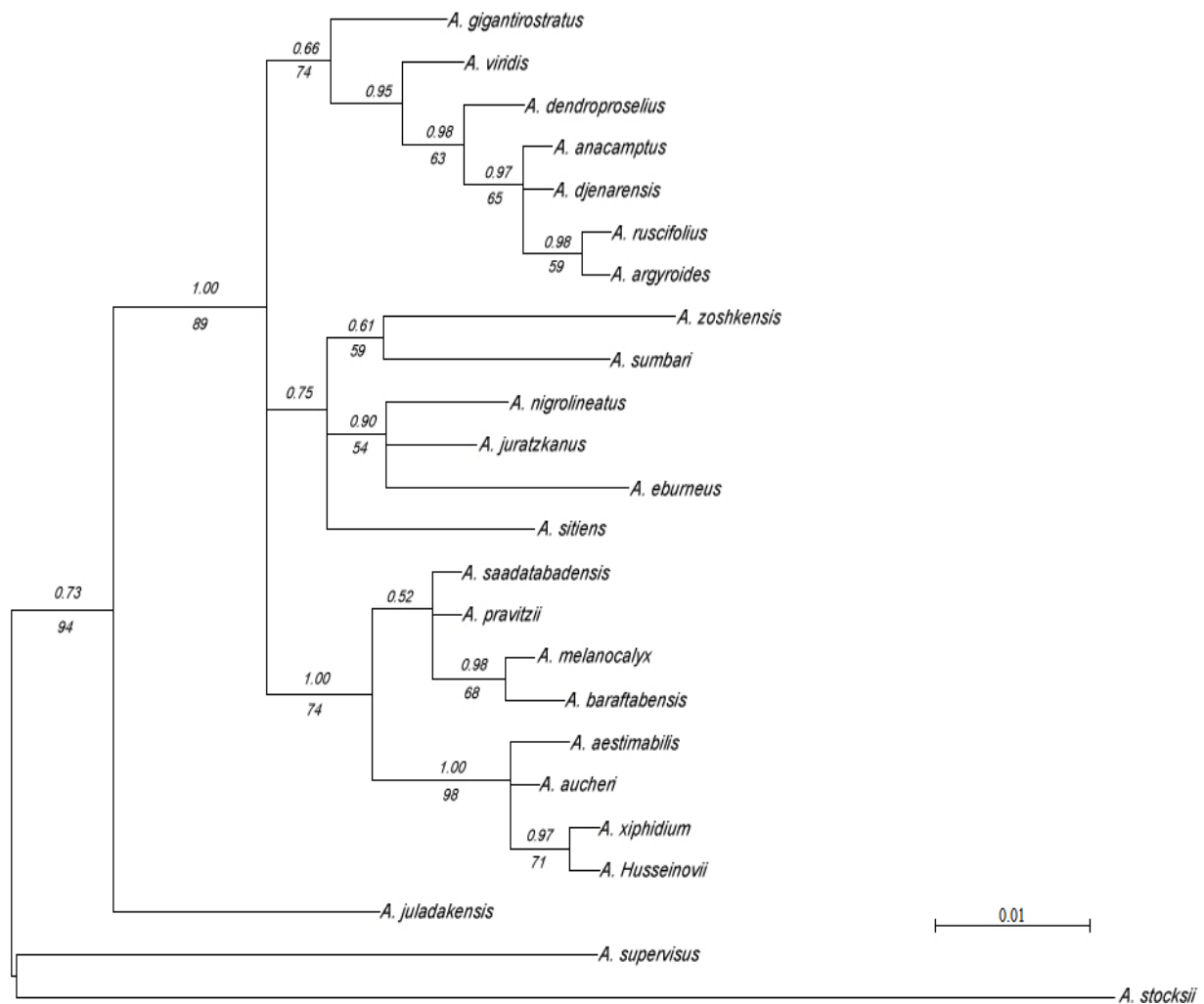
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Fig. 2. Fifty percent majority rule consensus tree resulting from Bayesian analyses of the nrDNA ITS data set. Numbers above branches are posterior probabilities (PP) and the numbers below them indicate MP bootstrap (BP) values.

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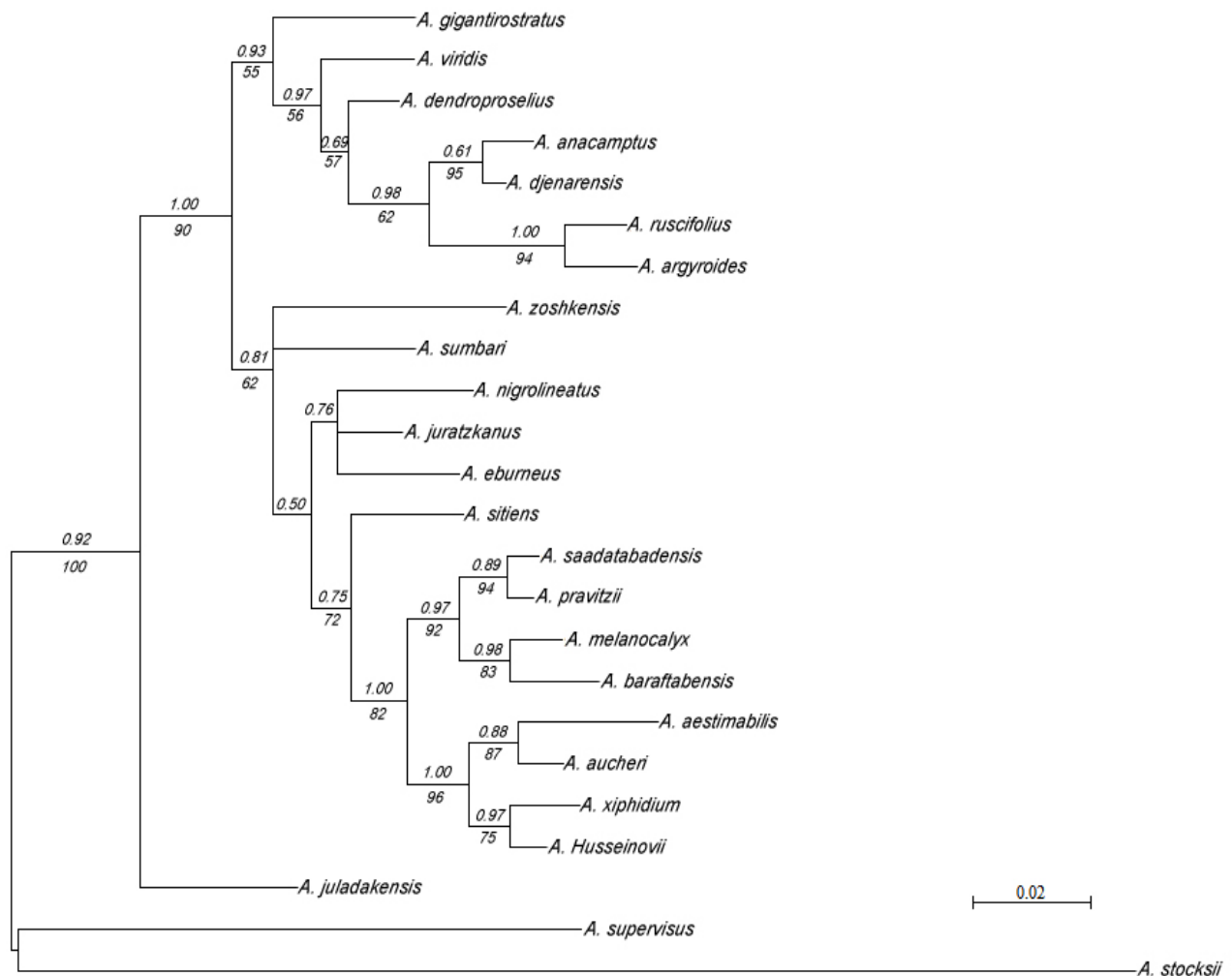
Fig. 3. Fifty percent majority rule consensus tree resulting from Bayesian analyses of the combined dataset (nrDNA ITS and morphology). Posterior probabilities are above branches and MP bootstrap values are presented below them.

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