

MORPHOLOGICAL CHARACTER EVOLUTION OF ASTRAGALUS SECTION INCANI DC. (FABACEAE) BASED ON NUCLEAR AND PLASTID DNA SEQUENCES

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Section *Incani* is one of the most species-rich section of *Astragalus*, ranging throughout Southern Europe, North Africa, Turkey, Iran, central Asia and Caucasus. Ninety-two of 140 species in the section were analyzed using nuclear ITS and chloroplast *rpl32-trnL^(UAG)* markers representing the full range of morphological variation and geographical distribution. The NeighborNet analysis revealed two major lineages, reflecting the biogeographical distribution of sect. *Incani*. This analysis postulates an east-west pattern of dispersal revealing a major phylogenetic split between eastern and western lineages. A large assemblage (lineage II) comprises the great majority of species of section *Incani* s.s. from west and northwestern Iran and Turkey along with *A. incanus* and *A. monspessulanus*, both restricted to southern Europe and exhibits the bulk of species diversity. We used Mesquite software to reconstruct patterns of evolution of morphological characters within the section. Six morphological diagnostic characters (life form, corolla color, leaflet number, presence or absence of bracteole, hair color of calyx and the bracts color) were mapped on the concatenated tree using Mesquite software. The reliance on morphological characters alone in the delimitation of species in *Astragalus* sect. *Incani* may well lead to erroneous phylogenetic results and thus to incorrect taxonomic conclusions.

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Key words: Character evolution; *Astragalus*; section *Incani*; NeighborNet analysis; nrDNA ITS

تکامل صفات *Astragalus* بخشه *Incani* DC. (تیره باقلائیان) بر اساس نشانگرهای هسته‌ای و کلروپلاستی الهام امینی: دانش‌آموخته دکتری، گروه زیست‌گیاهی، دانشکده علوم زیستی، دانشگاه تربیت مدرس، تهران، ایران
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بخشه *Incani* یکی از بخشه‌های غنی از گونه‌ی *Astragalus* است که در جنوب اروپا، شمال آفریقا، ترکیه، ایران، آسیای مرکزی و قفقاز پراکنش دارد. ۹۲ گونه از ۱۴۰ گونه این بخشه با استفاده از نشانگرهای هسته‌ای ITS و کلروپلاستی *rpl32-trnL^(UAG)*، معرف تنوع ریخت‌شناسی و توزیع جغرافیایی گسترده مورد آنالیز قرار گرفتند. آنالیز شبکه همسایه، دو دودمان اصلی نشان دهنده پراکنش بیوجغرافیایی این بخشه را نشان داد. این آنالیز الگوی پراکنش شرقی-غربی، که معرف یک شکاف تبارزایشی بین دودمانهای شرقی و غربی است را آشکار کرد. اجتماع بزرگی (دودمان II)

شامل اکثریت گونه‌های بخشه *Incani* از غرب و شمالغرب ایران و ترکیه بعلاوه *A. monspessulanus* و *A. incanus* که هر دو محدود به جنوب اروپا هستند و تنوع زیادی از گونه‌ها را نشان می‌دهند، مشخص شد. از نرم‌افزار مسکیت برای بازسازی تکامل ویژگی‌های ریخت‌شناسی صفات برای ارزیابی روند تکامل در بخشه استفاده شد. شش صفت مهم ریختی (فرم رویشی، رنگ جام، تعداد برگچه، حضور یا غیاب براکتول، رنگ کرک کاسه و رنگ براکنه) روی درخت ترکیبی با استفاده از نرم‌افزار مسکیت ترسیم شدند. تکیه بر صفات ریختی به تنهایی در تعیین مرز گونه‌های *Incani* منجر به نتایج تبارزایشی نادرست و استنتاج‌های نادرست آرایه شناختی می‌شود.

INTRODUCTION

Section *Incani* DC., with about 140 species worldwide, is the largest and most common bifurcate hairy section of *Astragalus* L. in Iran. This section is distributed throughout Southern Europe, North Africa, Turkey, Iran, central Asia and Caucasus (Podlech & Zarre 2013). Considerable topographic and climatic heterogeneity make Iran (ca. 90 species) and Turkey (ca. 30 species) as hot spots of diversity for the section *Incani*, of which 85% (ca. 120 species) of species occur in these regions (Podlech & Zarre 2013). Species of *Incani*s. have diverged from other sections in having a reduced stem, bifurcate hairs, free stipules, sometimes shortly adnate to the petioles and mostly imparipinnate leaves, but in a few species tri- or unifoliolate leaves, tubular (rarely inflated at fruiting stage) calyx and bracteoles (Podlech & Zarre 2013; Maassoumi & al. 2016) are observed. The original circumscription of *Incani* dates to De Candolle (1825), who described 12 species in this section. Bunge (1868) published a new section, namely *Proselius* Bunge, for the *Astragalus* group with bifurcate hairs, unaware that this had already been done by De Candolle (1825). The first species from sect. *Incani* was established as *A. incanus* by Linnaeus (1753) and Podlech (1990) chose this species, as the lectotype of section. Some regional floras (USSR: Gontcharov & al. 1965; Turkey: Chamberlain & Matthews 1970; Iraq: Townsend & Guest 1974) accepted Bunge's treatment, but, Podlech (1990) adopted the section *Incani* as the credible name.

In the past, several morphological studies including hair micromorphology in leaf (Ghahremaninejad 2004 a, b), petiolar anatomy (Mehrabian & al. 2007; Al-Joboury 2016), embryology (Riahi & al. 2003), cytology (Ranjbar & al. 2010; 2011) have been conducted on some members of *Incani*. Lately, some new species of the section *Incani* have been identified from Iran (Podlech & Maassoumi 2003; Ranjbar & Karamian 2003; Maassoumi & Kazempour-Osaloo 2006; Ranjbar 2007; Bagheri & al. 2011; Ranjbar &

al. 2011; Ghahremaninejad & Bagheri 2009; 2012). Kazempour-Osaloo & al. (2003, 2005) examined a few species of the section *Incani* using nuclear ribosomal internal transcribed spacer (nrDNA ITS) sequences, indicated *Incani* along with *A. subsecundus* Boiss. & Hohen. (Section *Laguropsis* Bunge) is monophyletic. Dizkirici & al. (2014) presented the first molecular phylogenetic analysis of *Incani* based on 30 species from Turkey, exclusively based on ribosomal DNA (nrDNA ITS) and revealed this section is monophyletic and evolutionarily separated from sections *Hypoglottidei* DC. and *Dissitiflori* DC. Recently, Amini & al. (2018) examined *Incani* in greater detail using a cpDNA (*rpl32-trnL^{UAG}*) and a nuclear (ITS) genomic regions.

Section *Incani* is well known for its taxonomic complication as a result of overlapping morphological characters (Amini & al. 2018). The species of this section are not distinguishable in any available keys because of the morphological variation. Based on these diversities, the classification of its species has always been controversial. There has not been conducted any characters evolution study of the section *Incani* with such a broad sampling covering its whole geographical range.

Therefore, the main goals of the present study are: 1) to evaluate the evolutionary trends of several diagnostic morphological characters in the context of molecular phylogeny; 2) to assess a procedure for the phylogenetic network and interpretation of splits graphs in the context of plant biogeography and species radiations.

MATERIALS AND METHODS

Taxon Sampling, DNA extraction, PCR and sequencing, sequence alignment and phylogenetic inferences of this work is based on the previous study by authors (E. Amini & al. 2018).

Phylogenetic Networks

NeighborNet (NN), a distance-based network construction method is main implement for describing the phylogenetic complexity in molecular sequence

data (Bryant & Moulton 2004). To visualize phylogenetic structure and possible reticulating relationships within the *Incani*, the Neighbor Net algorithm (Bryant & Moulton 2004), implemented in the program Splits Tree 4 version 4.10 (Huson & Bryant 2006). The application of networks, rather than a bifurcating tree, is clearly necessary when the evolutionary process is complicated and not well defined by a bifurcating model. Indeed to apply NeighborNet is suitable for analysis of larger data sets and this method is informative and fast (Bryant & Moulton 2004). In order to reconstruct un-rooted topology our networks, both ITS and the concatenated matrices were modified by excluding the outgroupspecies of the related sections and further removal of extra gaps.

Analysis of Morphological Data

Patterns of morphological evolution are assessed for 12 characters considered important diagnostic features in taxonomic treatments of *Astragalus* section

Incani (Maassoumi 1998; 2005; Podlech & Zarre 2013). To infer ancestral states, we used stochastic character mapping as implemented in Mesquite v. 2.75 (Maddison & Maddison 2011). The features were coded in a binary matrix and traced on the concatenated topology. The characters (table 1) were compiled from original observations on field and herbarium material. The polarity of character states was determined following Maddison & al. (1984). To analyze life history evolution, we used a pruned dataset of 63 species of *Incani* that excluded identical and incompatible species or that were widespread. Among the 12 characters studied, the optimization of six taxonomically important characters onto the Bayesian tree is shown in figs. 3-8 and further discussed below. These characters include growth habit, leaflet number, the presence or absence of bracteole, corolla color, hair color of calyx and the bract color.

Table 1. Morphological characters traced on the molecular tree. Characters 1, 2, 3, 4, 5 and 6 are mapped onto the Bayesian tree inferred from the concatenated nr DNA ITS and plastid *rpl32-trnL^(UAG)* sequences.

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1. Life form: erect= (0); caespitose = (1)
 2. Leaflet number: multi-leaved= (0); single-leaved= (1)
 3. Bracteole: present= (0); absent= (1)
 4. Corolla color: Yellow= (0); Yellow-Violet= (1); Violet = (2)
 5. Hair color of calyx: Black= (0); Black and white= (1); White= (2)
 6. Bract color: white= (0); brown= (1); red- white= (2); red= (3)
 7. Bract length: > 2mm= (0); < and =2 mm= (1)
 8. Bracteole length: > 0.5mm= (0); < and =0.5 mm= (1)
 9. Inflorescence: densely flowered (>5) = (0); remotely flowered (1-5) = (1)
 10. Ovary hair: absent= (0); present= (1)
 11. Legume shape: erect= (0); pendulous= (1)
 12. Stipe in legume: sessile= (0); stipitate= (1)
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RESULTS

In a Bayesian consensus tree based on concatenated sequences it was shown that all members of section *Incani*, (with the inclusion of *A. subsecundus* of section *Laguroopsis*), except for *A. platyphyllus* Kar. & Kir. formed a well-supported clade (PP = 1, ML/BS = 100), (E. Amini & al. 2018). The *Incani* s.s. clade is composed of two groups. One group (I) includes three lineages which are restricted to East Iran, Pakistan and Central Asia. The next group (II) comprises the rest of the species which are confined to northwest, west of Iran, Turkey, southern Europe and North Africa (E. Amini & al. 2018).

Phylogenetic Networks

The ITS splits graph determined two main groups. One of these, lineage II is composed of many species

is distributed in northwest, west of Iran and Turkey. The rest of the NNet tree, (fig. 1) revealed lineage I including Ia, Ib and Ic. lineage Ia is composed only of eastern taxa while Ib and Ic includes several species from northwestern Iran, Turkey and southern Europe. In the concatenated splits graph, there is obvious geographical grouping of samples within the NNet splits graph (fig. 2). NeighborNet analysis of concatenated data set revealed two genetic clusters (Lineage A and B). It is interesting to note that in the splits graph, all eastern samples cluster together (lineages A). Lineage B comprises the rest of the species (including *A. incanus*, as the lectotype of the section) and are confined to northwest, west of Iran and Turkey and southern Europe/North Africa.

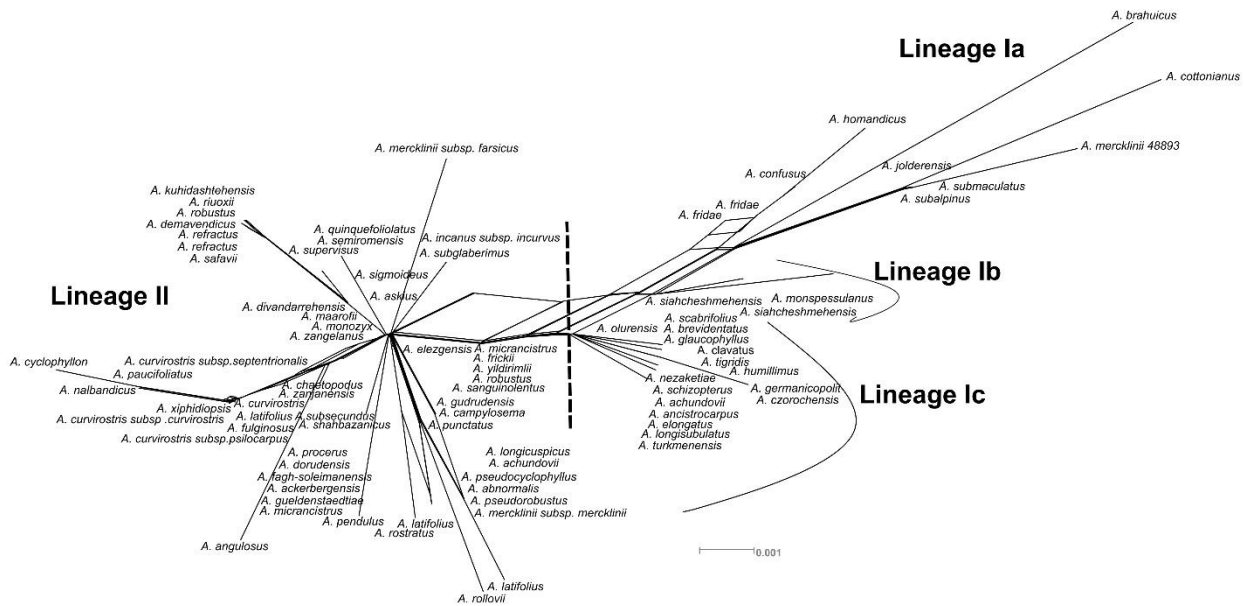


Fig. 1. Splits graph for ITS sequences of *Incanis. s*. Two major branches were recovered (i. e., lineage I and lineages II).

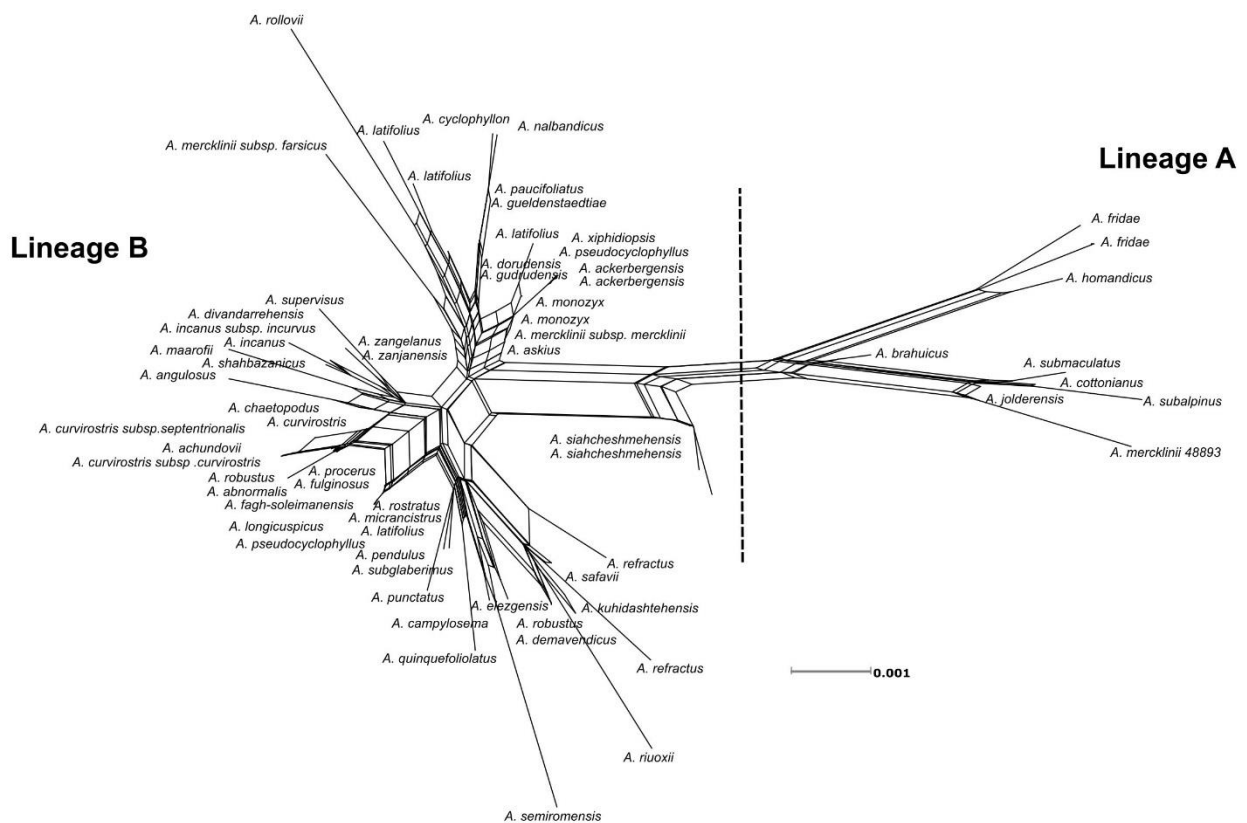


Fig. 2. Splits graph for the concatenated nrDNA ITS and *rpl32-trnL^(UAG)* sequences of *Incanis. s*. Two major branches were recovered (i.e., lineage A and lineages B).

Patterns of Morphological Evolution

The results from the 12 diagnostic morphological characters on the Bayesian tree gained from the concatenated dataset are shown in table 1. In order to decrease terminal polytomies, identical sequences indicating more than one accession of the same species were removed from the analysis. The patterns of homoplasy are shown using various colors. Among the 12 characters studied, the optimization of six taxonomically important characters onto the Bayesian tree is presented in figs. 3-8 and further discussed below. These characters include life form (fig. 3), leaflet numbers (fig. 4), the presence or absence of bracteole (fig. 5), corolla color (fig. 6), hair color of calyx (fig. 7) and the bract color (fig. 8). The tracing of character evolution for these morphological characters (i.e. bract color, hair color of calyx and corolla color) indicated multiple independent origins of their states. Among these characters, corolla color, is synapomorphy for the clade I comprising taxa of Eastern Iran, Pakistan and Central Asia and are valuable characters in the delimitation of the clades of *Incani* spp. The most common corolla color among the species of the section *Incani* is violet. The reconstruction of character evolution for the rest of the morphological characters (i.e. life form, leaflet numbers, the presence or absence of bracteole and.....) indicated two-state characters.

DISCUSSION

In this study, we carried out NeighborNet analysis based on two markers information. It is based on a comprehensive taxon sampling that covers the full spectrum of morphological variation and geographical range. Furthermore, the phylogenetic tree inferred in the present study coupled with evidence from morphology represents a significant step towards a better understanding of character evolution and circumscription of *Incani*. Section *Incani* is known for its complicated taxonomy and rapid speciation, which causes challenges in molecular phylogenetics as molecular evolution interpretations..

Phylogenetic Network

The split-graph constructed by the NeighborNet method exhibits reticulation. The groups formed in the NNet networks were almost correlated to the clades recovered in the phylogenetic tree (E. Amini & al. 2018). In the present study, we use the term "lineage" to refer to groups of specimens in the NNet trees (figs. 1 & 2), and "clade" to refer to groups in the phylogeny (Amini & al. 2018).

The NeighborNet analysis does show a compatible

clustering defined clades (figs. 1 & 2) and also presented similar patterns with the Bayesian analysis (Amini & al. 2018) and indicates a network composed of two major lineages (figs. 1 & 2). The Bayesian analysis is supported by a strong split in the NeighborNet analysis (figs. 1 & 2).

Morphological Character Evolution

Most of the morphological traits that have been used in delimitation of species of *Astragalus* present high levels of homoplasy. Our analysis displays among 12 morphological characters selected, (table1) six characters including life form, corolla color, leaflet number and the presence or absence of bracteole show few homoplasy and represent some synapomorphy for the monophyletic groups on the molecular trees. Most of the characters traced diverged several times in the section *Incani*. The remaining six characters provided some support for grouping of taxa. These are bract length, bracteole length, inflorescence, ovary hair, legume shape, stipe in legume. Our results proposed that morphology cannot clarify relationships in *Astragalus* section *Incani* exactly. Evolutionary trends of six diagnostic morphological characters are discussed below (figs. 3-8).

Life form: The erect character is ancestral and caespitose character shows parallel evolution at least 7 times. Most *Incani* spp. are erect, and a few species are caespitose. Ten species (included in this study) are caespitose that appear throughout the concatenated phylogenetic tree, suggesting independent evolution of the caespitose habit in *Incani* (fig. 3). Four members of the clade I, including *A. jolderensis*, *A. brahuicus*, *A. cottonianus* and *A. subalpinus* show caespitose habit.

Leaflet Number: The multi-leaved character is ancestral and this state has been observed in outgroups and single-leaved character shows parallel evolution 5 times in *A. supervisus*, *A. latifolius*, *A. abnormalis*, *A. gueldenstadiae* and *Astragalus fridae*. Although multi-leaved appears to be plesiomorphic (fig. 4), it shows a reversal in the clades I and II. Within sect. *Incani* this trait (single-leaved) occurs in the both clade I and II. However, four members of the clade II, including *A. supervisus*, *A. latifolius*, *A. abnormalis* and *A. gueldenstadiae* show single-leaved. *Astragalus fridae* in clade I is another species of single-leaved in the section (fig. 4). It is noteworthy that all four single-leaved species examined (*A. abnormalis* Rech.f., *A. latifolius*, *A. gueldenstaedtia* and *A. supervisus* (Kuntze) Sheld. are scattered throughout the larger clade of section *Incani* s.s., indicating independent evolutionary origins of this trait.

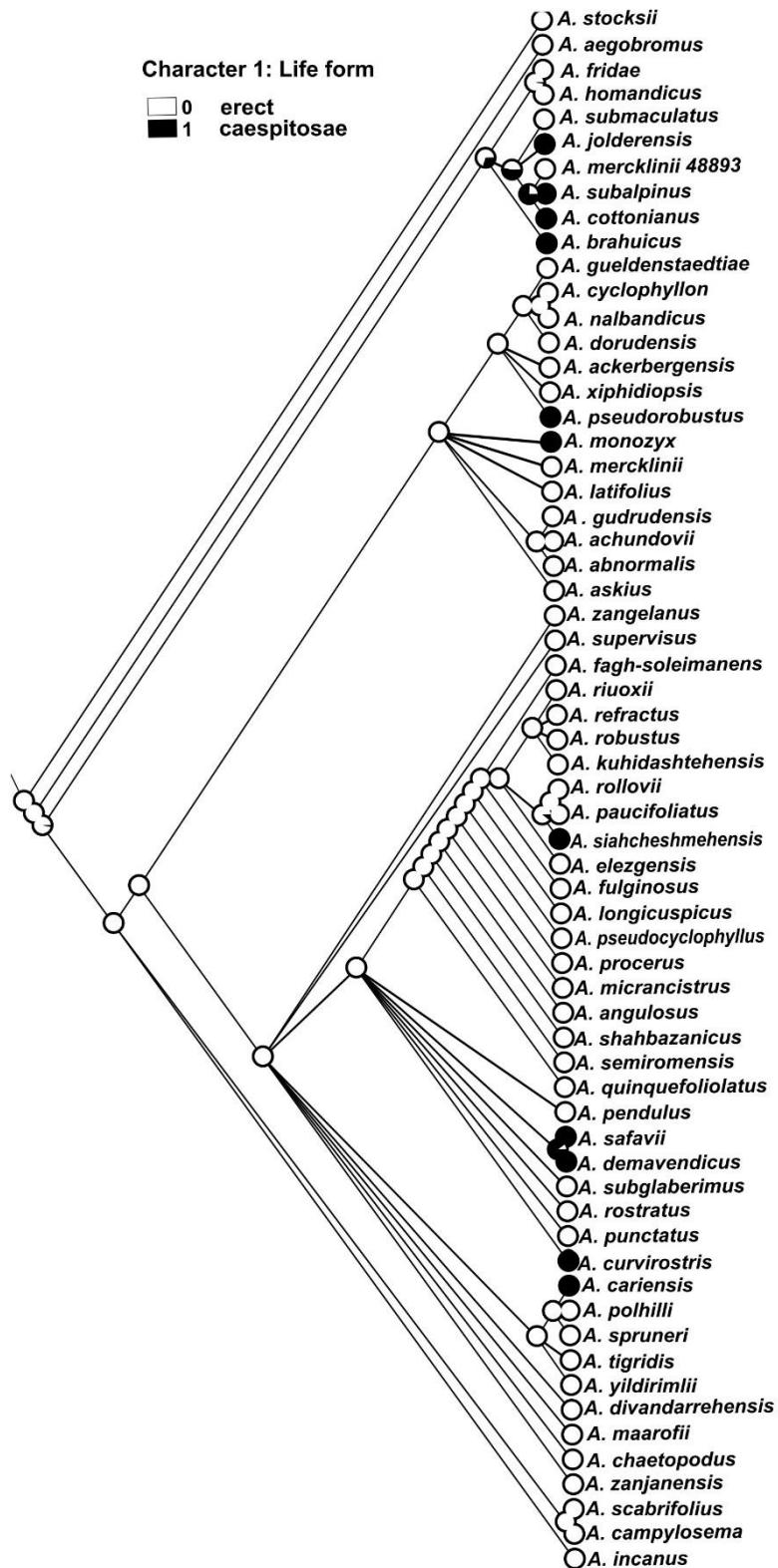


Fig. 3. Evolutionary history of character 1 (Life form) mapped on the Bayesian tree obtained from the concatenated nr DNA ITS and plastid *rpl32-trnL*^(UAG) sequences.

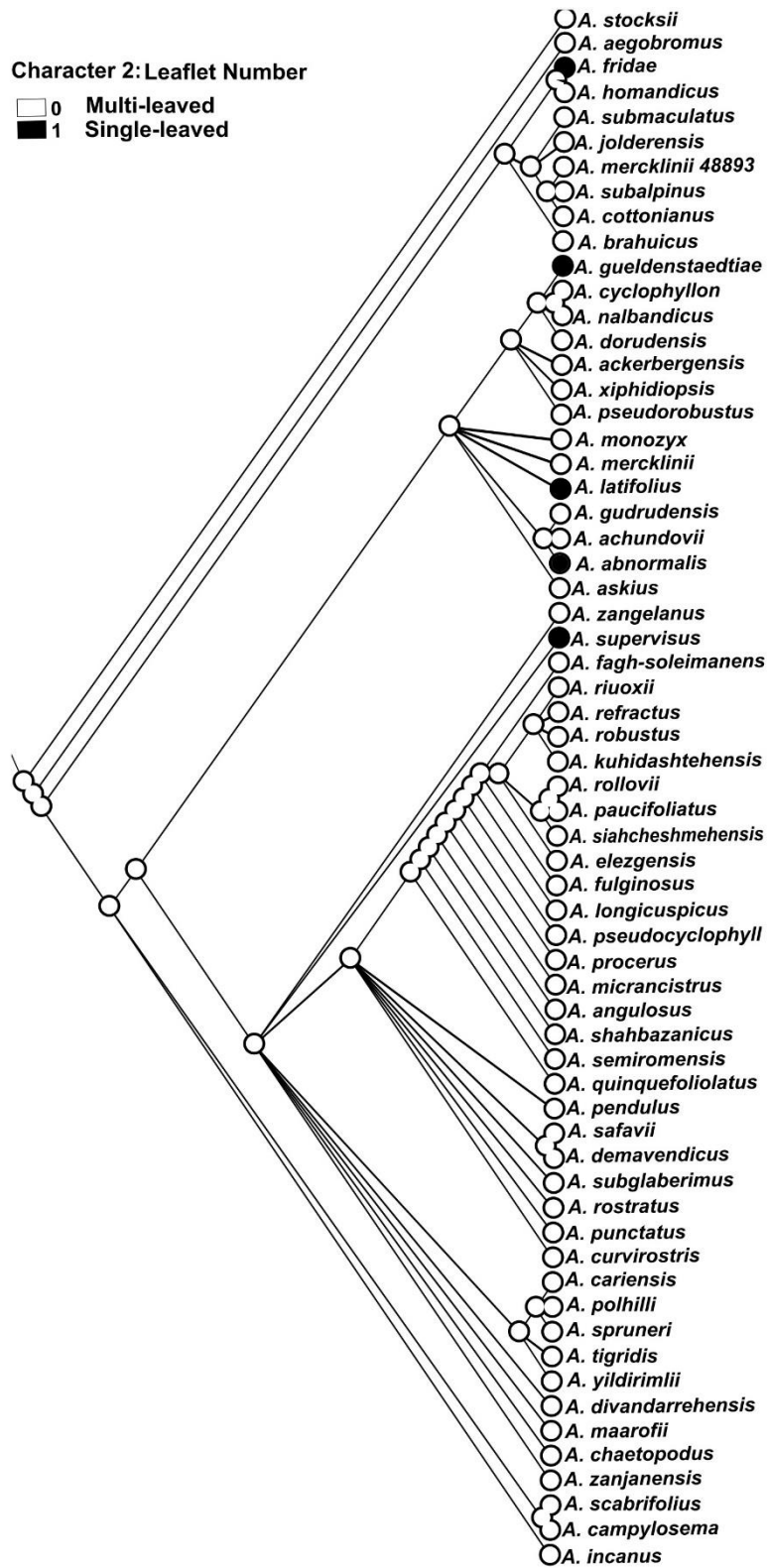


Fig. 4. Evolutionary history of character 2 (Leaflet number) mapped on the Bayesian tree obtained from the concatenated nr DNA ITS and plastid *rpl32-trnL^(UAG)* sequences.

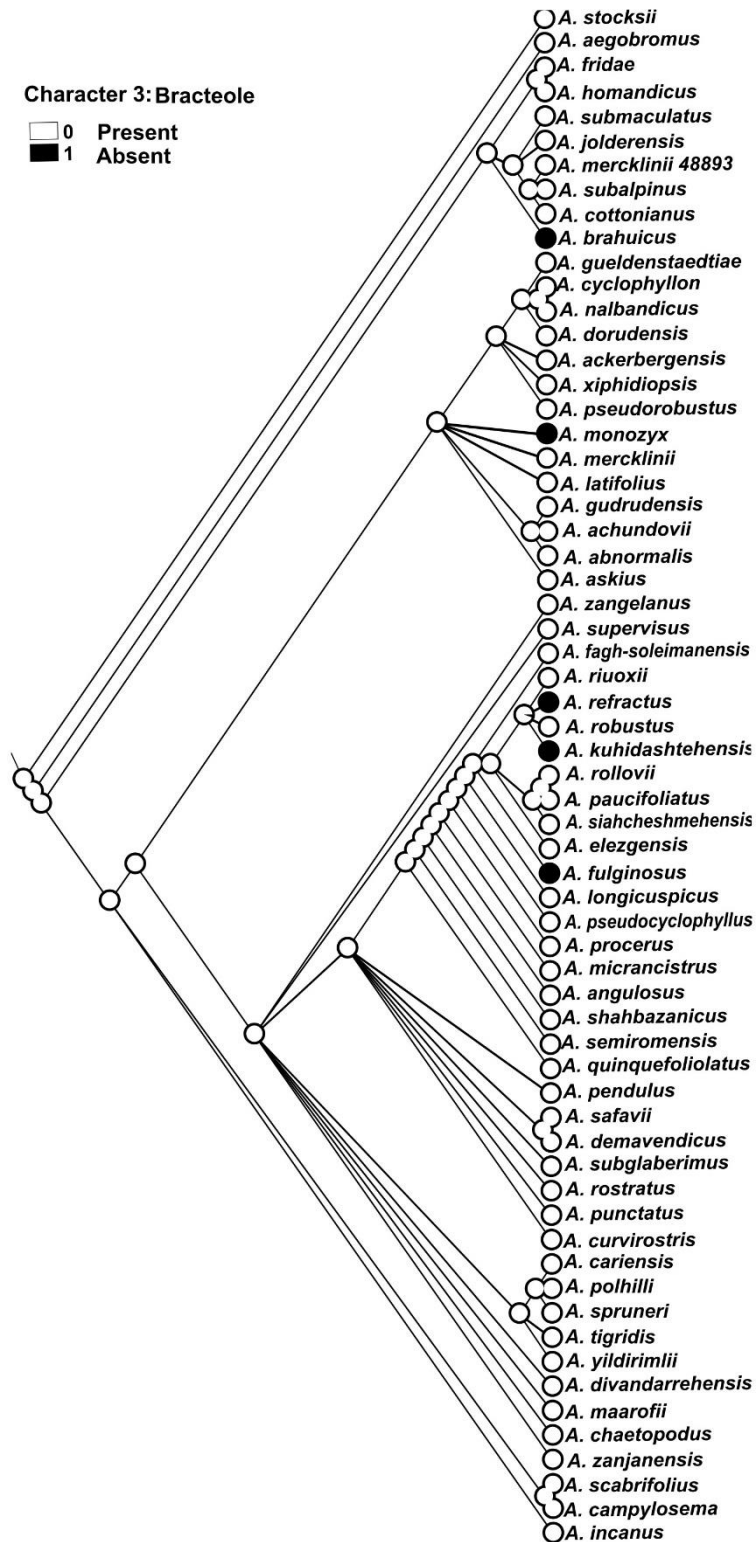


Fig. 5. Evolutionary history of character 3 (Bracteole) mapped on the Bayesian tree obtained from the concatenated nr DNA ITS and plastid *rpl32-trnL*^(UAG) sequences.

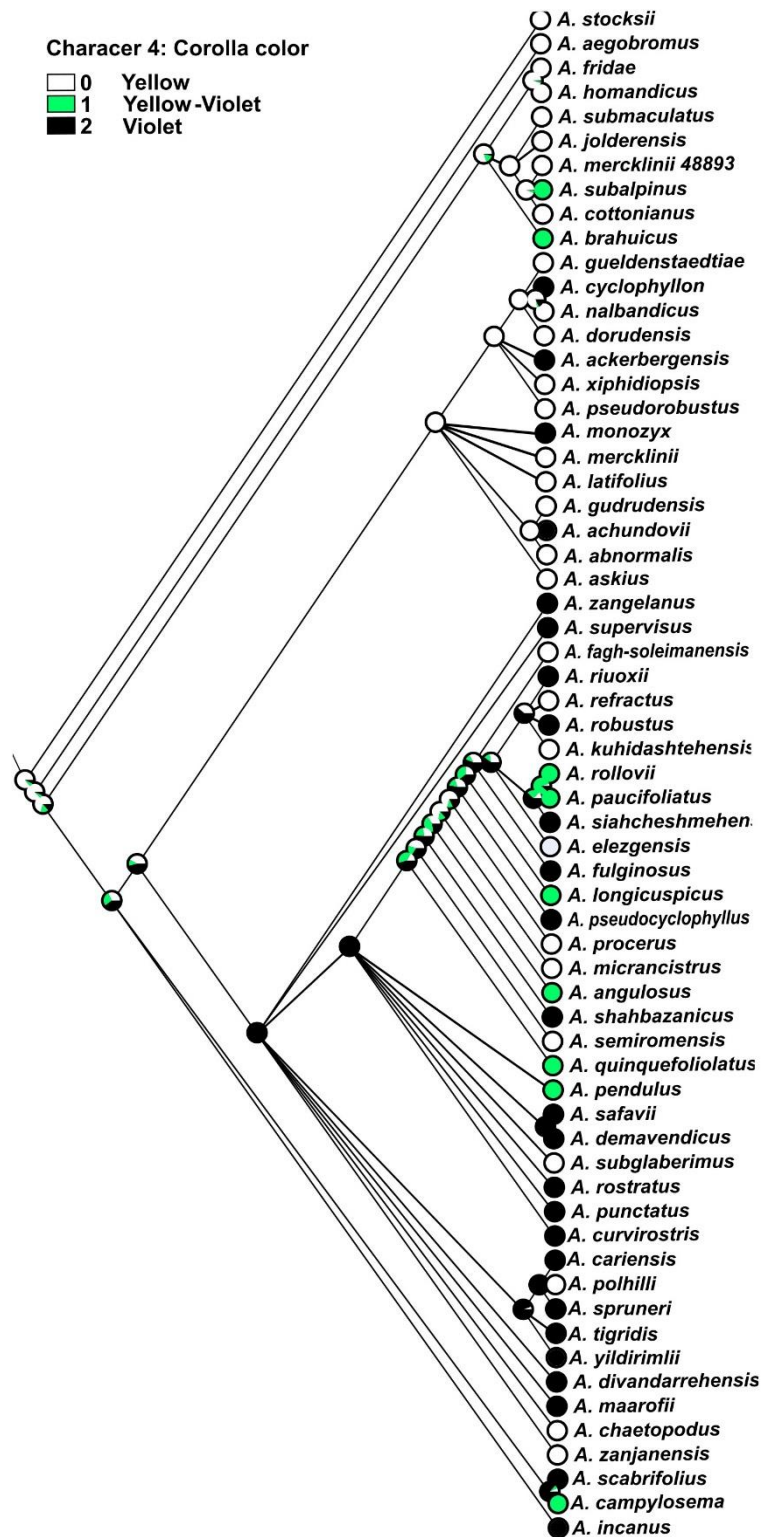


Fig. 6. Evolutionary history of character 4 (corolla color) mapped on the Bayesian tree obtained from the concatenated nr DNA ITS and plastid *rpl32-trnL^(UAG)* sequences.



Fig. 7. Evolutionary history of character 5 (Hair color of calyx) mapped on the Bayesian tree obtained from the concatenated nr DNA ITS and plastid *rpl32-trnL^(UAG)* sequences.

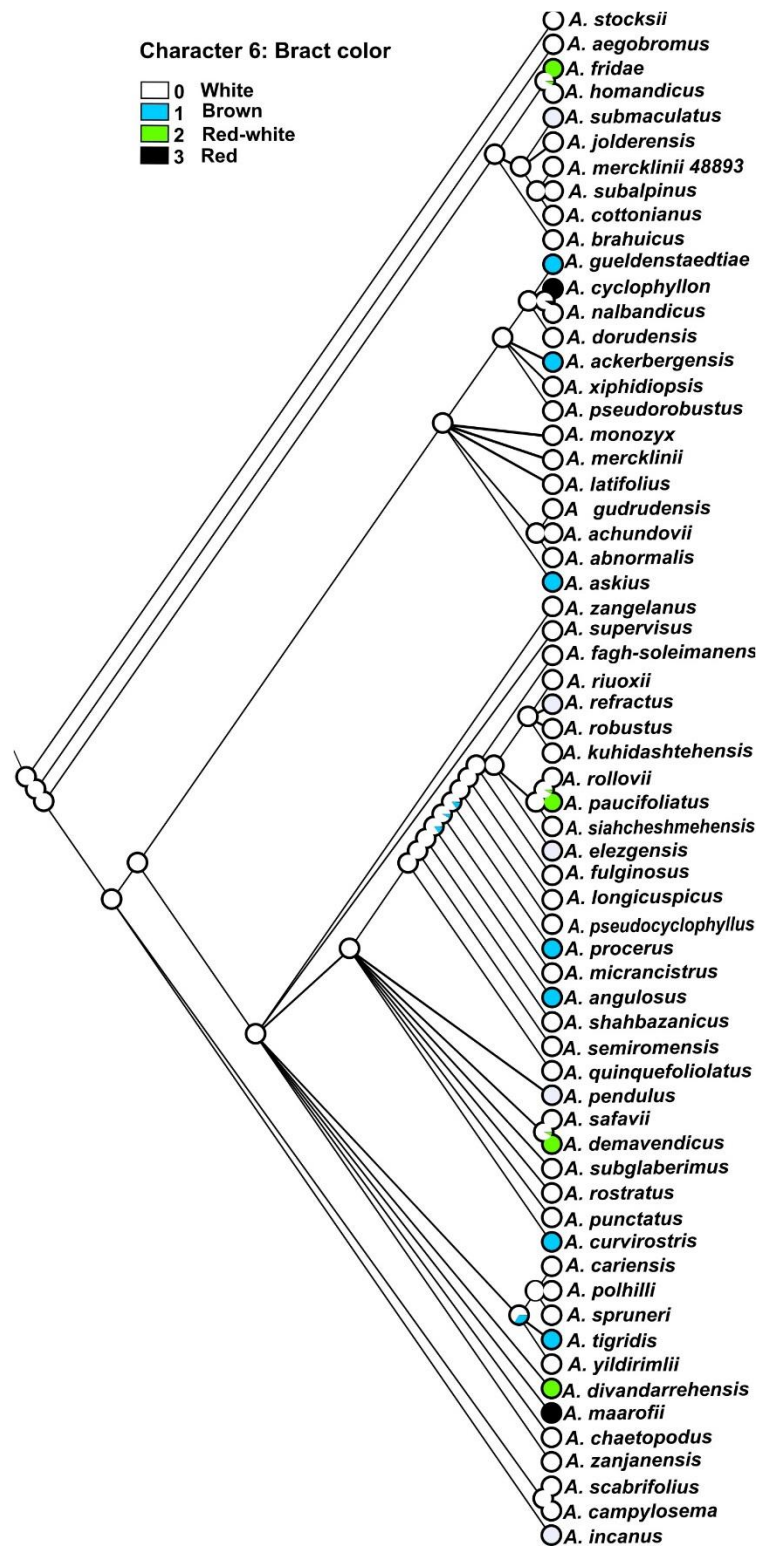


Fig. 8. Evolutionary history of character 6 (Bract color) mapped on the Bayesian tree obtained from the concatenated nr DNA ITS and plastid *rpl32-trnL^{UAG}* sequences.

Bracteole: Most species of the *Incani* have bracteole, as in outgroup and the lack of bracteole is infrequent in this section (*A. brahuicus*, *A. monozyx*, *A. refractus*, *A. fulginosus* and *A. kuhidashtehensis*). As shown in fig. 5, species without bracteole apparently evolved repeatedly from ancestors with bracteole.

Corolla color: Yellow corolla is ancestral and yellow-violet and violet corolla show parallel evolution several times. Most species of *Incani* have yellow or violet corolla but nine members including *A. subalpinus*, *A. brahuicus*, *A. rollovi*, *A. paucifolius*, *A. longicuspicus*, *A. pendulus*, *A. campylosema*, *A. quinquefolialatus* and *A. angulosus* show both corolla color. Yellow corolla occurred in some members of *Incani*, especially clade I. However, this trait changed to yellow-violet and violet corolla in clades II several times (fig. 6).

Hair color of calyx: In this trait, black and white hair is ancestral but white hair shows parallel evolution two times in *A. incanus* and *A. cottonianus* (fig. 7).

Bract color: White bract is ancestral and other states evolve several times from ancestors with this trait. Red bract evolved in *A. cyclophyllon* and *A. maarofii* two times independently (fig. 8).

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