

MORPHOLOGICAL CHARACTER EVOLUTION OF ONOSMA (BORAGINACEAE) IN IRAN, BASED ON NUCLEAR AND PLASTID DNA SEQUENCES

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Onosma is taxonomically a problematic genus and its infrageneric delimitation is not clear. Using nuclear (ITS) and two plastid (*rpl32-trnL*^(UAG) and *trnH-psbA*) markers on 87 species of *Onosma* throughout their distribution range, phylogenetic relationships within *Onosma* was reconstructed. Morphological character evolution was mapped on the previously studied nr DNA ITS tree. In the NeighborNet analysis, the groups formed in the splits graph are correlated to the clades retrieved in the phylogenies. The results suggested that *O. rostellata* is more closely related to Sino-Indian species of *Onosma*. Among 10 morphological characters selected, six characters including basic chromosome number, type of indumentum, corolla length, nectar trichome, sectioned calyx and calyx cleft represent the most important synapomorphies for *Onosma* species.

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تکامل صفات ریخت‌شناسی بر روی سرده **Onosma (Boraginaceae)** در ایران، براساس توالی‌های هسته‌ای و کلروپلاستی فاطمه نصرالهی: دانش آموخته دکتری، گروه زیست‌گیاهی، دانشکده علوم زیستی، دانشگاه تربیت مدرس، تهران، ایران
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از *Onosma* لحاظ تاکسونومی سرده مشکلی است و تعیین حدود فرو سرده‌ای آن به‌طور واضح مشخص نیست. در این مطالعه روابط فیلوژنی با استفاده از نشانگر هسته‌ای (ITS) و دو نشانگر کلروپلاستی (*rpl32-trnL*^(UAG) و *trnH-psbA*) بر روی ۸۷ گونه از سرده *Onosma* از مناطق پراکنش مختلف بویژه ایران بازسازی شد. آنالیز شبکه فیلوژنتیک نشان داد که گروه‌های تشکیل شده بر کلادهای درختان فیلوژنی منطبق هستند. نتایج نشان می‌دهد که *O. rostellata* بیشتر به گونه‌های هندی-چینی *Onosma* مرتبط است. تحلیل صفات ریخت‌شناسی نشان داد که آنها در میان گونه‌های *Onosma* چندین بار تکامل پیدا کرده است، لذا میزان بالایی از همپلازی را نشان دادند. از میان ۱۰ صفت ریخت‌شناسی انتخاب شده، شش تا شامل عدد پایه کروموزومی، نوع کرک، طول جام، کرک در حلقه نوشجای، طویل شدن کاسه بعد از گلدهی و بریدگی کاسه گل در میان گونه‌ها مهمترین صفات پیشرفته مشترک می‌باشند.

INTRODUCTION

Onosma L. with nearly 150 species is the largest genus of the tribe Lithospermeae Dumort. (Cecchi & Selvi 2009; Weigend & al. 2009). The Asia and Mediterranean regions particularly Iran and Turkey are the main centers of diversity of the genus (Johnston 1954; Kazmi 1970; Chacón & al. 2016; Weigend & al. 2016; Cecchi & al. 2016). They are biennial to perennial herbs or shrubs characterized by having scorpioid inflorescence and clavate to tubular corolla. Hair characters have major taxonomic importance in *Onosma* (Riedl 1967). The basic chromosome numbers of *Onosma* species are $X = 6, 7$ and 8 , sometimes $x = 9, 10$ and 11 (Martonfi & al. 2008).

De Candolle (1846) divided *Onosma* into two sections based on the morphology of calyx and the hair cover inside it: (1) *Aponosma* DC. With dense appressed sericeous hairs; (2) *Euonosma* DC. With spreading hairs. Later, Schur (1866), Boissier (1879) and Borbás (1877) recognized the taxonomic value of leaf indumentum, and divided *Onosma* into two or three sections (*Estellatae* Schur = *Haplotricha* Boiss. with glabrous setae, *Stelligera* Schur = *Asterotricha* Boiss. by stellate setae and *Heterotricha* Boiss. covered by both setae types).

Riedl (1967) classified the genus into three sections: *protonosma* Popov., *podonosma* (Boiss.) Gurke, and *Onosma*. Khatamsaz (2002), followed Riedl's treatment and divided section *Onosma* into three subsections *Haplotricha*, *Heterotricha* and *Asterotricha*. A total of 46 species have been reported in Iran (Khatamsaz 2002; Attar & Joharchi 2006; Attar & hamzhee 2007; Mehrabian & al. 2014; Ranjbar & Almasi 2013; 2014; 2015). *Onosma* with vast diversity and intricate evolutionary history is a taxonomically problematic group (Kolarcik & al. 2010).

Molecular studies in the genus *Onosma* limited to amplified fragment length polymorphism (AFLP) study of genetic diversity in European populations of *Onosma echioides* L. (Mengoni & al. 2006). Evolutionary history of the genus *Onosma* in central and southern Europe was performed based on AFLP and nrDNA ITS and plastid *rpl32-trnL*^(UAG) intergenic spacer data by Kolarcik & al. (2010; 2014) and confirmed the hybrid origin of heterotrichous group. Cecchi & al. (2011) investigated evolutionary dynamics of serpentine adaptation in *Onosma* by nrDNA ITS sequence data and found that obligate endemics belonged to six distantly related clades. Mehrabian & al. (2011) applied ISSR and morphological data to show that *Onosma* species in Iran, split to *O. rostellata* Lehm. and the rest of the

genus. Nasrollahi & al. (2018) studied molecular phylogeny and divergence times of *Onosma* (Boraginaceae s.s.) based on nrDNA ITS and plastid *rpl32-trnL*^(UAG) and *trnH-psbA* sequences and revealed that *Onosma* as currently circumscribed is not monophyletic. In the present study, nrDNA ITS and two plastid intergenic spacer (*rpl32-trnL*^(UAG) and *trnH-psbA*) with comprehensive sampling have been used to reveal evolutionary relationships of *Onosma*.

Our objectives in the present study are to use molecular sequence data, to evaluate the evolutionary trends of several diagnostic morphological characters in the context of molecular phylogeny.

MATERIALS AND METHODS

The taxon sampling, DNA isolation, PCR, sequence alignment and phylogenetic inferences were done based on the materials and methods of the previous study (Nasrollahi & al. 2019).

Phylogenetic networks

NeighborNet (NN) a distance-based network construction method (Bryant & Moulton 2004) was implemented in SplitsTree4 version 4.10 (Huson & Bryant 2006) using a Dice dissimilarity matrix. NeighborNet (NN), allows for graphical representation of conflicting phylogenetic signals and interpretation of evolutionary histories which are not tree-like (Bryant & Moulton 2004). As the plastid markers are inherited as a single unit they were combined prior to analysis. Both the combined cpDNA and ITS matrices were modified prior to analysis by excluding the outgroups. NN splits graphs have been used with varying success to detect reticulate history (Rebernik & al. 2012; Grimm & Denk 2008; Weiss-Schneeweiss & al. 2008).

Analysis of morphological data

Character evolution was explained for 10 characters previously assumed significant diagnostic features in taxonomic treatments of *Onosma* (Candolle 1846; Johnston 1954; Martonfi 2008). The characters (table 1) were collected from Floras (Zhu & al. 1995; Riedl 1967; Popov 1974; Riedl 1978; Fedorov 2001; Khatamsaz 2002) and original observations on field and herbarium material. Likelihood mapping was done using Mesquite v. 2.75 (Maddison & Maddison 2011) on the obtained Bayesian tree based on nuclear dataset. The features were coded in a binary matrix and followed on the molecular tree. Among 10 morphological characters selected, six characters represent the most important synapomorphies for *Onosma* species on the molecular tree.

Table 1. Morphological characters traced on the molecular tree. Characters 1, 2, 3, 5, 6 and 10 are mapped onto the Bayesian tree inferred from nuclear ITS sequence data.

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1. Basic chromosome number: 8 = (0); 6 = (1); 7 = (2); 9 = (3); 10 = (4)
 2. Type of indumentum: simple = (0); stellate = (1); simple - stellate = (2)
 3. Corolla length: 11-20 mm = (0); 1-10 mm = (1); > 20 mm = (2)
 4. Nectar trichome: absence = (0); existence = (1)
 5. Calyx accrescent: existence = (0); absence = (1)
 6. Sectioned calyx: in base = (0); close to base = (1)
 7. Anther exertion: inside = (0); outside = (1)
 8. Corolla shape: tubular = (0); campanulate = (1); clavate = (2)
 9. Corolla to calyx length ratio: 2 = (0); 1/2 = (1); 1 = (2)
 10. Corolla trichome: absence = (0); existence = (1)
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RESULTS

Phylogenetic networks

To visualize phylogenetic structure and possible reticulating relationships within *Onosma*, the NeighborN& algorithm was used to generate phylogenetic networks for the ITS data and combined *rpl32-trnL*^(UAG) and *trnH-psbA* datasets. The splits graph shows vast internal network structure, indicating reticulation. Phylogenetic network for ITS data yielded a high-resolution network, and six major splits (splits A–F) were recovered (fig. 1). Lineage A is composed of haplotrichous and heteritrichous from Europe. Lineage B includes haplotrichous from Iran, while Lineage C comprises asterotrichous species from Iran and Turkey. Lineage D includes asterotrichous species from Europe. Lineage E is composed of haplotrichous and heterotrichous species from mainly Iran, Turkey and, Mediterranean basin. The last one (Lineage F) includes *O. rostellata*, *Maharanga* and Sino-Indian species.

The cpDNA splits graph reveals three main lineages (fig. 2). One of these, lineage A (Ai-Aii) is composed of haplotrichous species from Iran and *O. arenaria*, *O. pseudoarenaria* and *O. viridis* from Europe. Lineage B in the splits graph mainly includes haplotrichous species. Lineage C is composed of asterotrichous and heterotrichous species. Lineage D includes haplotrichous species from North-East of Iran. However, *O. rostellata* is distinct and separate from the remaining samples of NN lineages.

Morphological character evolution

The evolution of morphological characters was investigated by optimizing character-state changes onto the Bayesian tree. In order to reduce terminal polytomies, identical sequences representing more than

one accession of the same species were deleted from the analysis. A total of 75 species have been analyzed. Among the 10 characters studied, the optimization of six taxonomically important characters onto the Bayesian tree is presented in figs. 3–8 and further discussed below. The characters include basic chromosome number (fig. 3), Type of indumentum (fig. 4), corolla length (fig. 5), nectar trichome (fig. 6), calyx accrescent (fig. 7), sectioned calyx (fig. 8). The patterns of homoplasy are shown using various colors.

DISCUSSION

Phylogenetic networks

Phylogenetic network analysis showed a reticulate pattern. The groups formed in the splits graph are (with minor exceptions) readily correlated to the clades retrieved in the phylogenies (Nasrollahi & al. 2019), especially where these received good support. In this study, the term “lineage” is used to refer to groups of specimens in the NN trees, and “clade” to refer to groups in the phylogenies.

Consistent with the groupings generated from the ITS analysis, in phylogenetic network analysis (fig. 1), lineage A is identical to clade V that composed of haplotrichous and heteritrichous species from Europe. Lineage B (= clade IV) includes haplotrichous from Iran, while Lineage C is matching with clade III comprises asterotrichous species from Iran and Turkey. Lineage D (= clade II) includes asterotrichous species from Europe. Lineage E is largely congruent with clades I and IV of the ITS phylogeny that composed of haplotrichous and heterotrichous species from mainly Iran, Turkey and, Mediterranean basin. The last one (Lineage F) includes *O. rostellata*, *Maharanga* and Sino-Indian species.

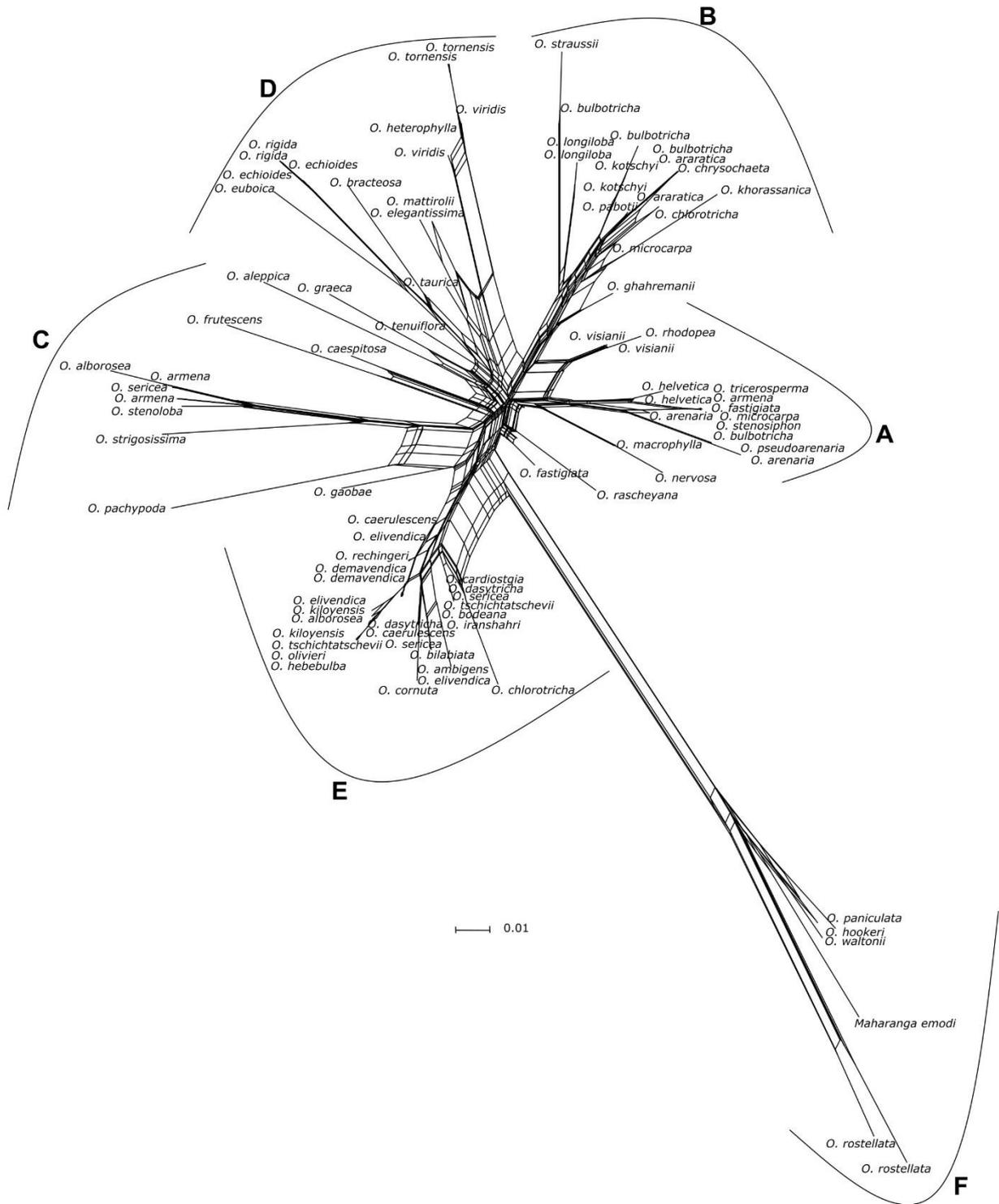


Fig. 1. Splits graph for ITS sequences of *Onosma*. Six major branches were recovered.

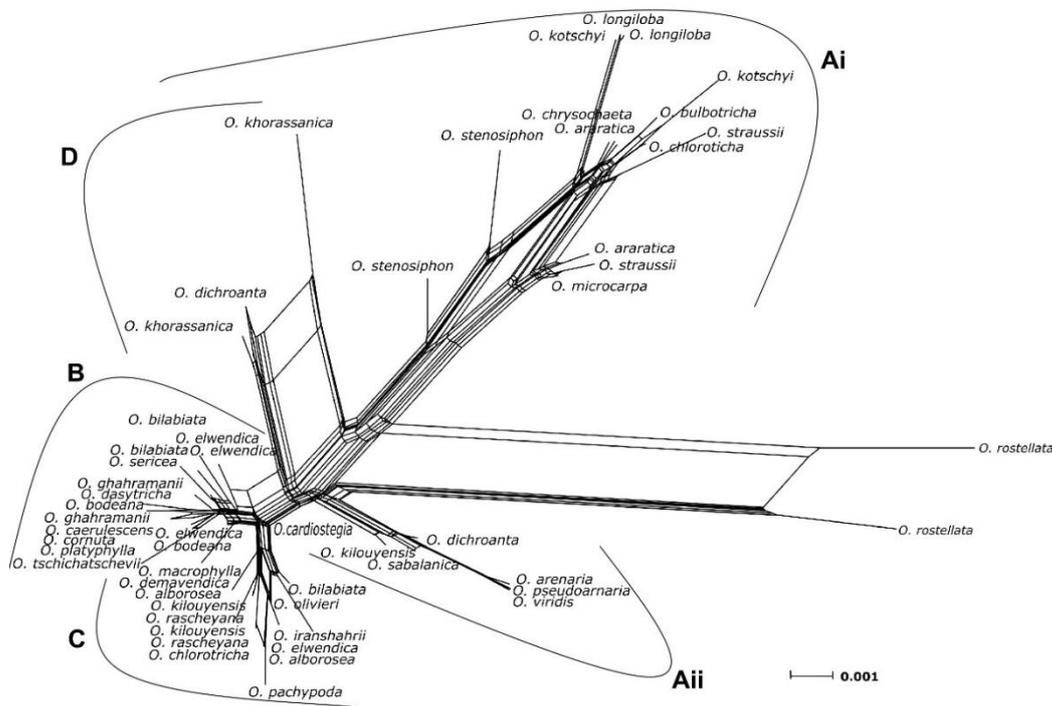


Fig. 2. Splits graph for chloroplast DNA (cpDNA) sequences of *Onosma*. Tree major branches were recovered.

The cpDNA splits graph reveals four main lineages (fig. 2). One of these, lineage A in the splits graph is largely congruent with clade I of the cpDNA phylogeny (Nasrollahi & al. 2019, fig. 2) that mainly includes haplotrichous species. Lineage B (= clade II) is composed of asterotrichous and heterotrichous species. Lineage C (Ci-Cii), correlates to clade III in cpDNA phylogeny and is composed of haplotrichous species from Iran and *O. arenaria*, *O. pseudoarenaria* and *O. viridis* from Europe. Lineage D (= clade IV) includes haplotrichous species from North-East of Iran. However, *O. rostellata* is distinct and separate from the remaining samples of NN lineages.

Geographical isolation

Taxonomic issues in *Onosma* range from typification to additional fieldwork needed to understand species variation over geographic distribution ranges. Our results confirm a gross geographical differentiation of the whole lineages into six well supported regional branches in Iran, Turkey and Mediterranean basin, Europe and China-India. Data from evolutionary dynamics of serpentine adaptation (Cecchi & al. 2011) have suggested that *Onosma* should be classified as two major groups, Euro-Mediterranean and Western Asiatic (excluding the eastern Asiatic accessions) group. Those species in the Western Asiatic group are restrictedly distributed in

Iran, Turkey and Syria, and those species in the Euro-Mediterranean group are widely distributed in Italia, Greece and Balkan regions. Phylogenetic network analysis suggested that lineages from Euro-Mediterranean and Asiatic regions were evolutionarily distinct, which is in good agreement with the suggestion of Cecchi & al. (2011).

Phylogenetic position of *O. rostellata*

Previous published studied inferred from morphology (Arabameri & al. 2014), Palynological diversity (Mehrabian & al. 2012), ISSR analysis (Mehrabian & al. 2011), ITS sequence and cpDNA (Nasrollahi & al. 2019) displayed that *O. rostellata* (sect. *Protonosma*) is different from the remainder of *Onosma*. Phylogenetic network graph showed that the branching length of node including *O. rostellata*, *O. hookeri*, *O. waltoni*, *O. paniculata* and *Maharanga emodi* is more than that of other species, indicating that *O. rostellata* may be the oldest diverged taxon. These results suggested that *O. rostellata* is more closely related to Sino-Indian species of *Onosma*. Relatedness of *O. rostellata* and Sino-Indian species may reflect the same morphological characters such as the tubular-campanulate corolla, mainly latterly coherent anthers to form a tube, villous nectar, (Popov 1951; Johnston 1954; Riedl 1967, 1987; Zhu & al. 1995; Liu & al. 2010).

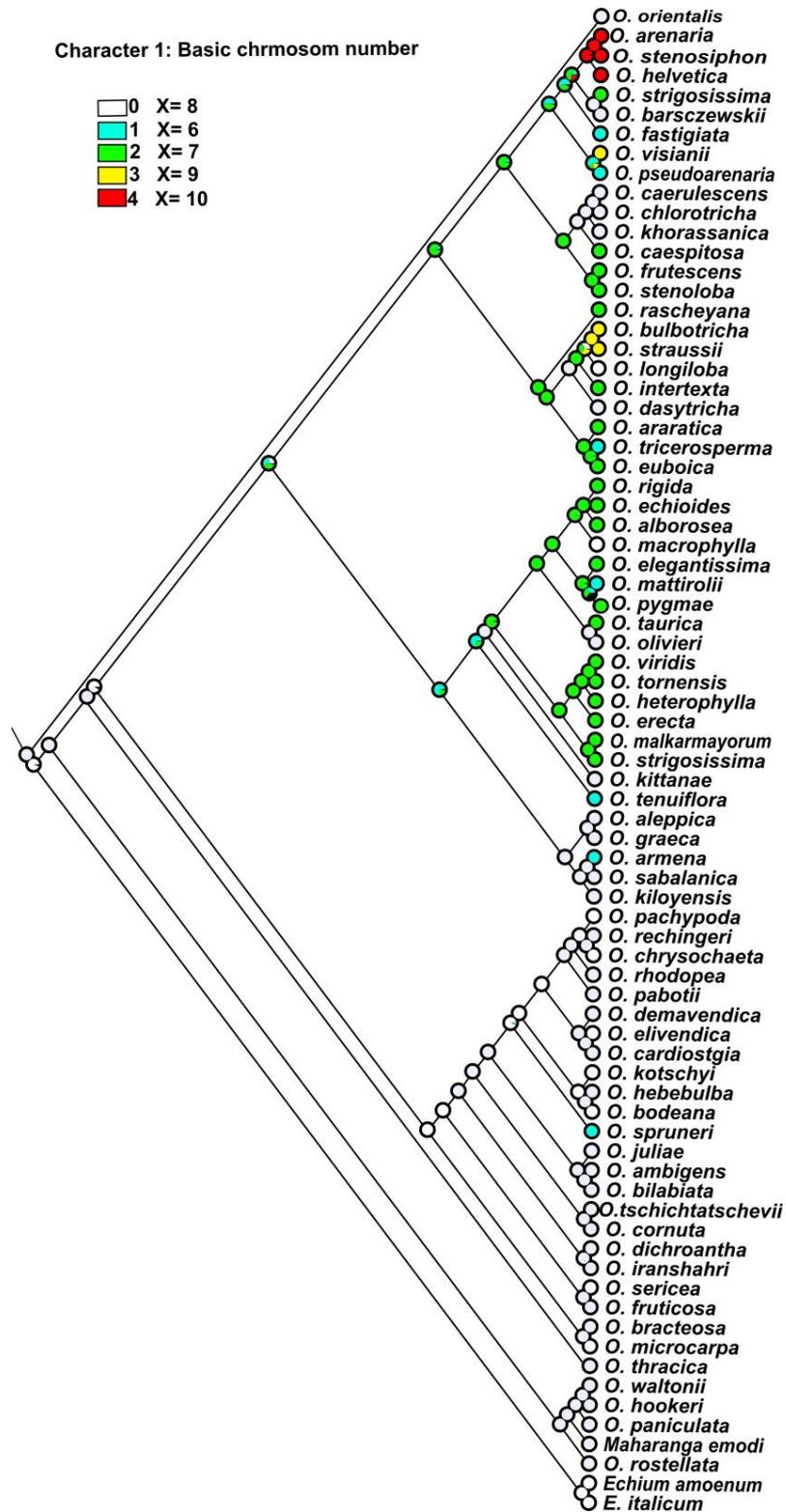


Fig. 3. Evolutionary history of character 1 (basic chromosome number) mapped on the Bayesian tree obtained from the nr DNA ITS sequences.

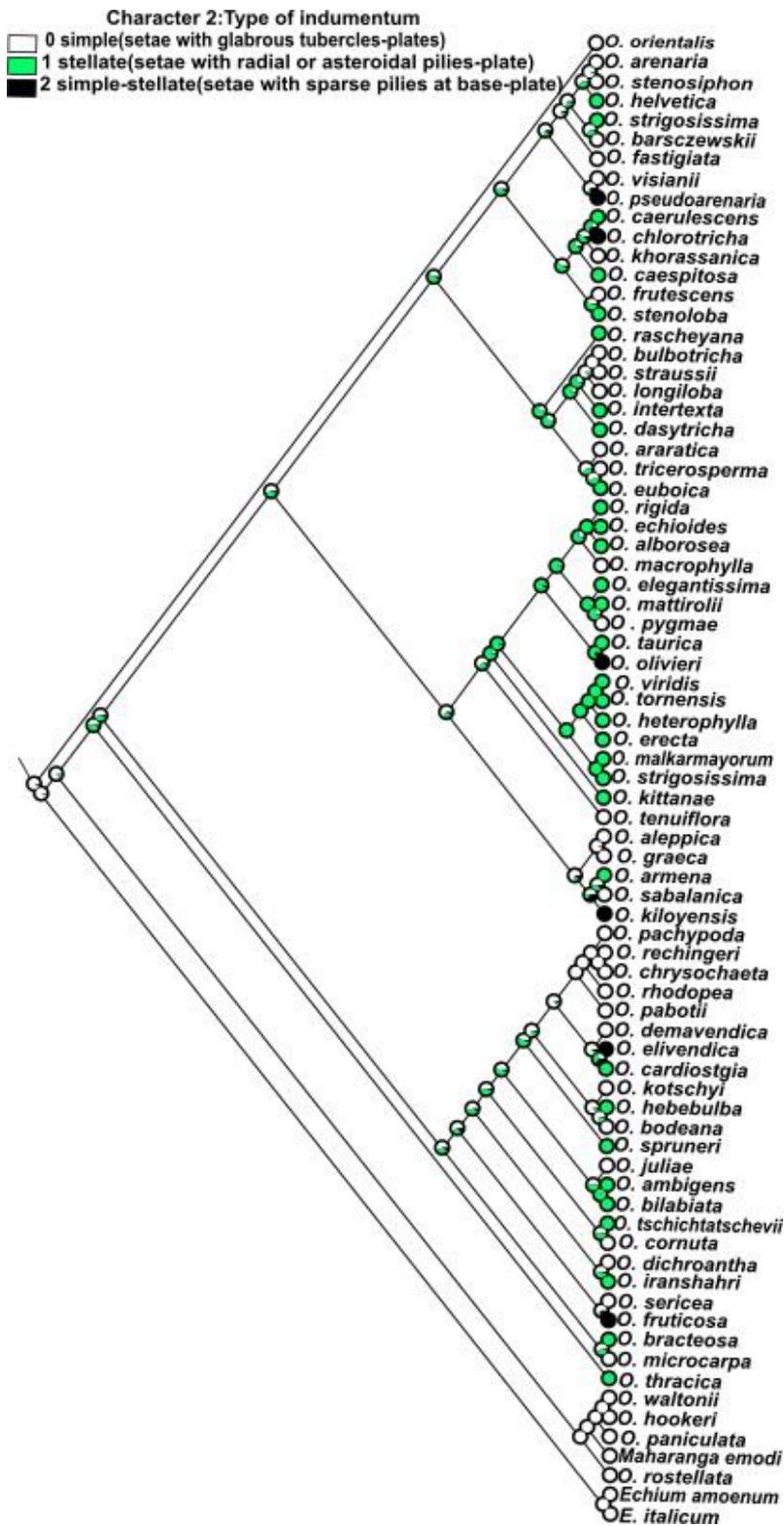


Fig. 4. Evolutionary history of character 2 (type of indumentum) mapped on the Bayesian tree obtained from the nr DNA ITS sequences.

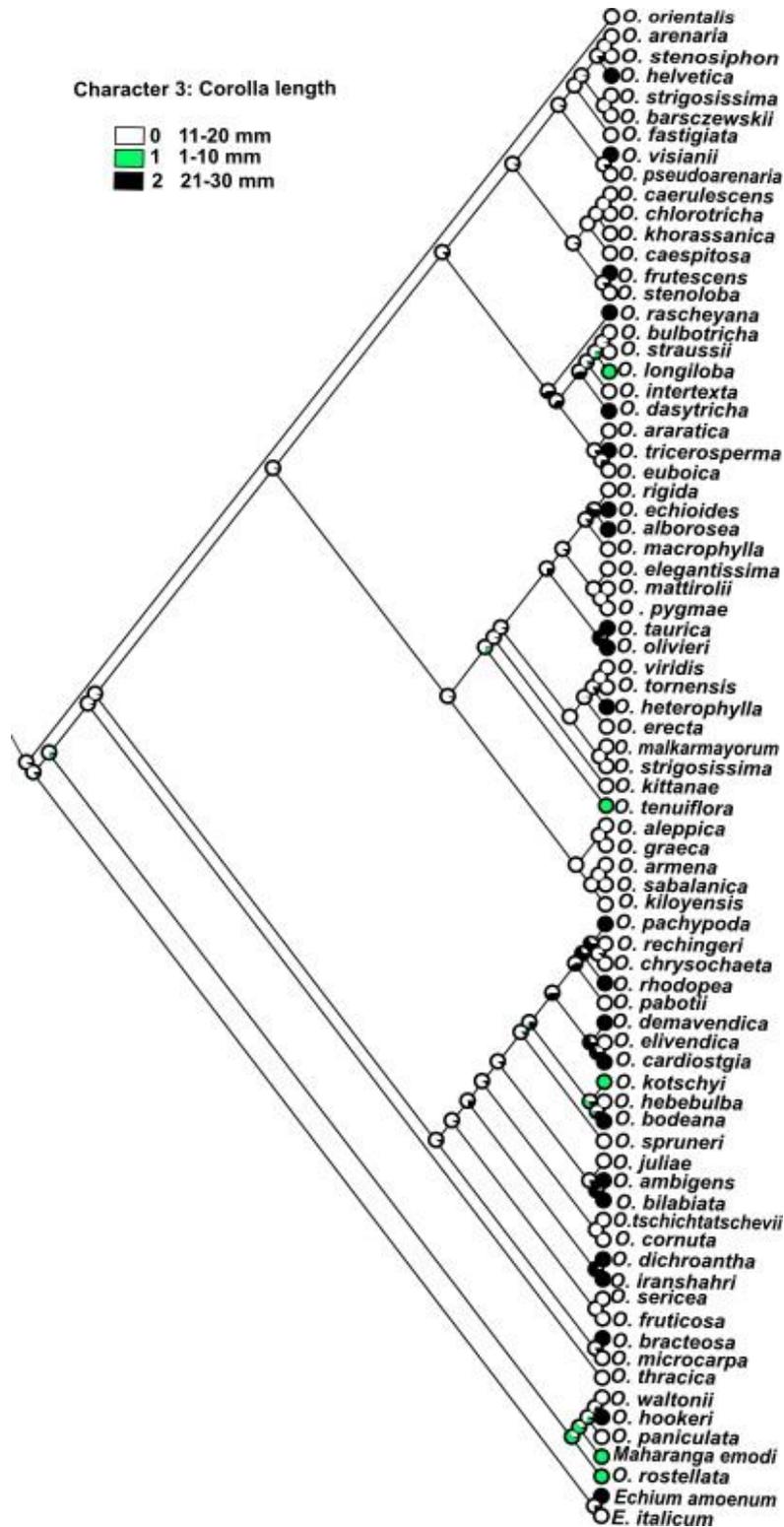


Fig. 5. Evolutionary history of character 3 (corolla length) mapped on the Bayesian tree obtained from the nr DNA ITS sequences.

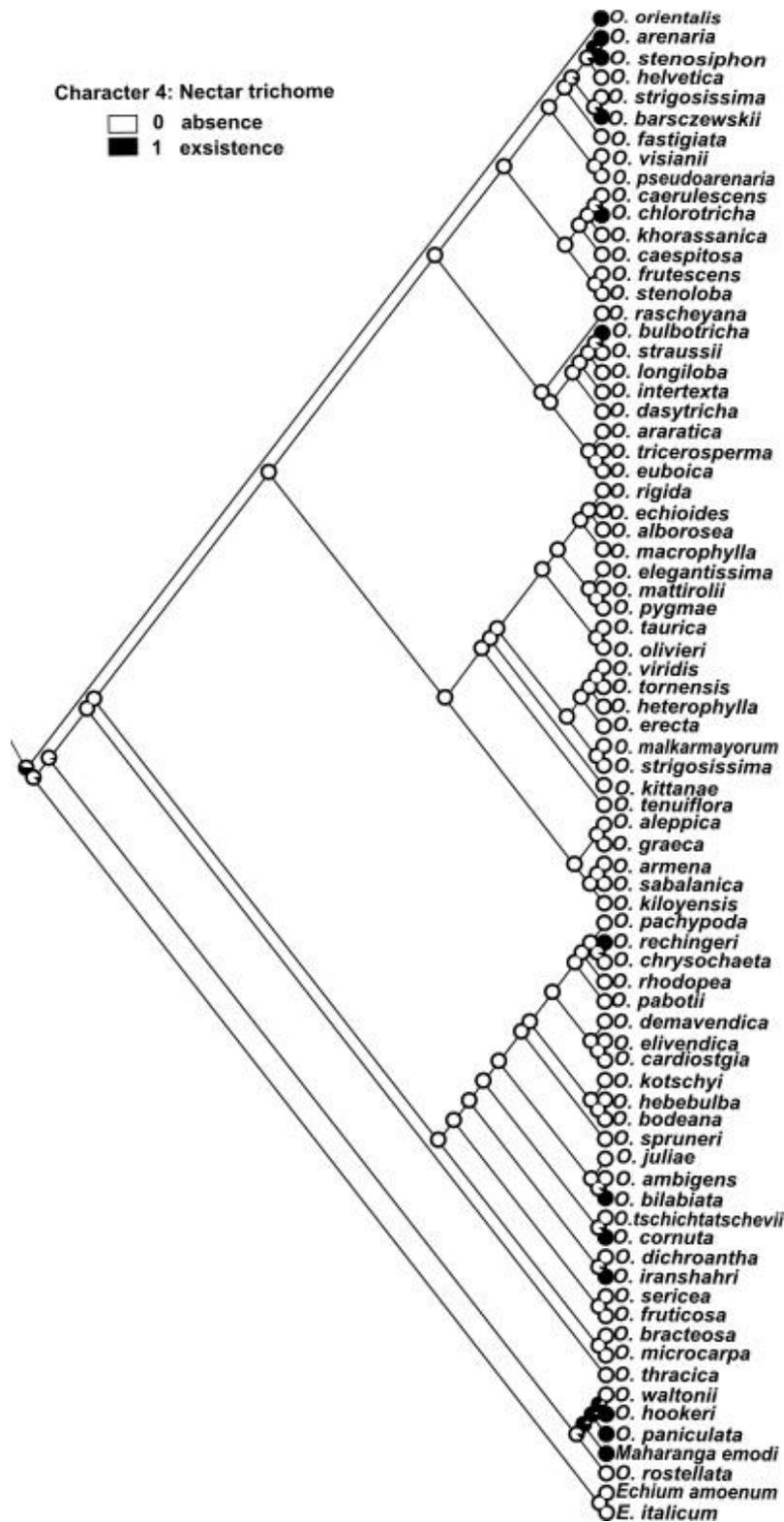


Fig. 6. Evolutionary history of character 4 (nectar trichome) mapped on the Bayesian tree obtained from the nr DNA ITS sequences.

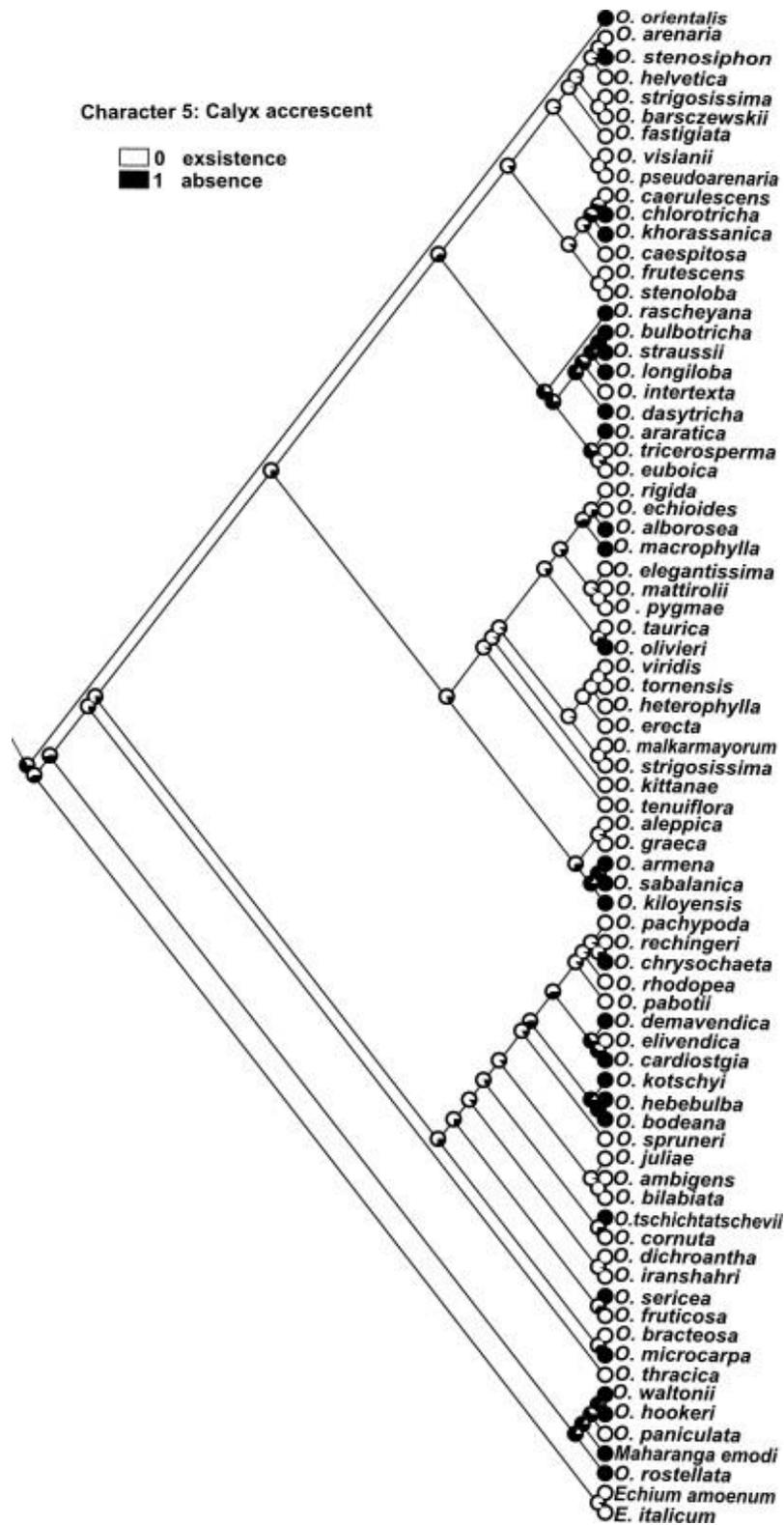


Fig. 7. Evolutionary history of character 5 (calyx accrescent) mapped on the Bayesian tree obtained from the nr DNA ITS sequences.

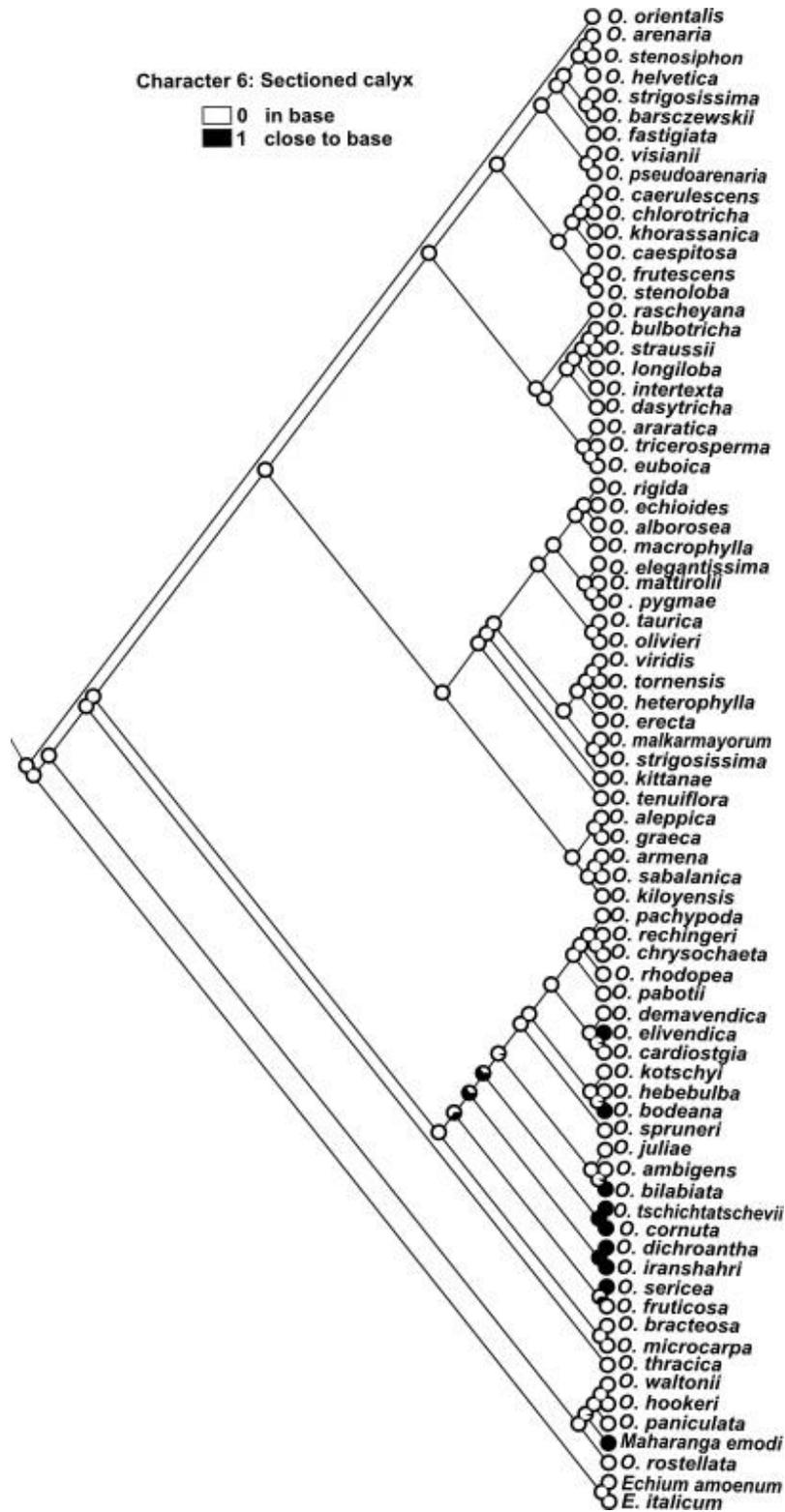


Fig. 8. Evolutionary history of character 6 (calyx cleft) mapped on the Bayesian tree obtained from the nr DNA ITS sequences.

Morphological character evolution

Most of the morphological traits that have been used in delimitation of species of *Onosma* present high levels of homoplasy. Our analysis displays among 10 morphological characters selected (table 1) six characters including basic chromosome number, type of indumentum, corolla length, nectar trichome, calyx accrescent and sectioned calyx represent some synapomorphy for the groups on the molecular trees. Most of the characters traced diverged several times in the *Onosma*. The remaining four characters provided some support for grouping of taxa. These are: anther exertion, corolla trichome, corolla shape, corolla to calyx length ratio. Our results proposed that morphology cannot clarify relationships in *Onosma* exactly. Evolutionary trends of six diagnostic morphological characters are discussed below (figs. 3-8).

Basic chromosome number

The analyzed species show $x = 8$ is ancestral in the *onosma* species as in the outgroup, then evolved to $x = 9$ in *O. bulbotricha*, *O. straussii*, *O. visianii* and to $x = 10$ in *O. arenaria*, *O. stenosphon*, *O. helvetica*. The Basic chromosome number evolved to $x = 7$ in asterotrichous species two times. In haplotrichous group $x = 6$ (is dominant in Europe) and $x = 8$ (is dominant in W and SW Asia) are the prevailing basic chromosome number and $x = 9$ and $x = 10$ are less frequent (Almasi & Ranjbar 2013). Members of asterotrichous group shows $x = 7$ as prevailing basic chromosome number, while $x = 11$ is less frequent (Teppner 1971). In the heterotrichous group, two cytotypes with bimodal chromosome sets are known (Kolarcik & al. 2014) (fig. 3).

Type of indumentum

The simple indumentum is ancestral. This character evolved multiple times to stellate and six times to simple - stellate within the *Onosma* species. Some authors classify the genus into sections or subsections based on type of leaf indumentum (Schur 1866; Boissier 1879; Borbás 1877). Most species of the *Onosma* have simple or stellate setae. Six members including *O. pseudoarenaria*, *O. chlorotricha*, *O. olivieri*, *O. kiloyensis*, *O. elivendica* and *O. fruticosa* show both setae types (fig. 4).

Corolla length

intermediate corolla is ancestral and evolved multiple times to major and five times to minor. Measuring the size of plant organs and parts is important in description and identification. Within each taxon, the size of the flower parts is very variable. Although all of the species of *Onosma* have a medium corolla (11-20 mm), this trait has evolved several times

in the tree. This character is a valuable character according to Riedl (1967). Moreover, Peruzzi & Passalacqua (2008) and Mehrabian & al. (2012) used corolla length for interpretation of population variability in *Onosma*. Arabameri & al. (2014) revealed taxonomic significance as it in some *Onosma* taxa, for example *O. kotschyi* (corolla length ca. 9 mm) and *O. asperrima* (corolla length ca. 25 mm) as closely related species, can be distinguished based on corolla length (fig. 5).

Nectar trichome

Most species of the *Onosma* lack the nectar trichome, as in the outgroup, and the existence of it is apomorphic. Due to the similarities among the *Onosma* taxa, there are many problems in their identification (Binzet & Akcin 2009). Our result displayed that this trait as a weak limiting character can be used along with other characters for description of restricted taxa. Arabameri & al. (2014) suggested that the nectary trichomes appeared to be diagnostic in limited taxa: *O. straussii*, *O. cornuta*, *O. stenosphon* and *O. orientalis* (fig. 6).

Calyx accrescent

Calyx accrescent is ancestral and was observed in most species of the *Onosma*, as in the outgroup and lacking of calyx accrescent is scarce in this genus. As shown in fig. 7 species without calyx accrescent evolved from ancestors with calyx accrescent. Arabameri & al. (2014) suggested that this character can be used as a diagnostic character for delimitation of some closely distinguishable taxa. For instance, *O. elivendica* can easily be distinguished from its close species *O. olivieri* based on calyx accrescent (fig. 7).

Calyx cleft

Sectioned calyx has a taxonomic importance. De Candolle (1846) based on this valuable character classified the genus into two sections. Calyx cleft to the base have been shown in most species of *Onosma*, as well as in the outgroup species and cleft to near the base of the calyx is an apomorphic state. In accordance with our results, Arabameri & al. (2014) indicated that only *O. cornuta*, *O. dichroantha* belonging to subsect. *Haplotricha* and *O. elivendica* from subsect. *Heterotricha* represent calyx division near the base (fig. 8).

Conclusions

The present study inferred phylogenetic hypothesis of *Onosma* with broad taxon sampling using three DNA regions. Phylogenetic network analysis is a reticulate pattern, as the groups formed in the splits graph are readily correlated to the clades retrieved in the phylogenies. Our results proposed that morphology

cannot clarify relationships in *Onosma* exactly. Most of the morphological characters traced diverged several times in *Onosma*. Further studies using fast evolving nuclear genes or whole plastid genome data are certainly necessary for resolving phylogenetic relationships within this critical species group.

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