

**TEMPORAL DIVERSIFICATION IN THE GENUS *ONOSMA* (BORAGINACEAE)
BASED ON NUCLEAR AND PLASTID DNA SEQUENCES**

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The genus *Onosma* is a homogeneous taxon with high morphological variation and due to the similarities among the *Onosma* taxa, there are many problems in their identification. Hence, systematically and taxonomically, it is considered a difficult genus. In the present study, we included a large number of *Onosma* species throughout their distribution range. Using nuclear (ITS) and two plastids (*rpl32-trnL_(UAG)* and *trnH-psbA*) markers, we analyzed the evolutionary history, divergence time and diversification patterns of *Onosma* across the tropical, subtropical and temperate regions. Divergence time estimates suggest the early radiation of *Onosma* s.l. happened at the Oligocene-Miocene boundary. BMM analyses indicate that the best configuration included one significant shift in diversification rates within *Onosma*: on the branch leading to the clade comprised of species of confined to Iran.

Key words: BMM, BEAST, Biogeography, Diversification, Phylogenetics

INTRODUCTION

Onosma L. (Boraginaceae-Lithospermeae), one of the largest genera in Boraginaceae, is primarily distributed in the temperate zones of the Old-World, with the main center of diversity in the Irano-Turanian regions of Asia, along with the Mediterranean region, especially Iran and

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Turkey region (CHACÓN *et al.*, 2016; WEIGEND *et al.*, 2016; CECCHI *et al.*, 2016; CECCHI and HILGER, 2021).

The genus *Onosma* contained biennale or perennial, hirsute to prickly-strigose herbs (rarely small shrubs), with scorpioid inflorescence, stamens inserted at the middle of the corolla and generally 4 nutlets flat at the base (BINZET *et al.*, 2010; WEIGEND *et al.*, 2016). *Onosma* species have specific indumentums formed by a single large bristle (seta) growing out of the top of a tubercle (BALL, 1972).

By calyx characteristics CANDOLLE (1846) divided *Onosma* into two sections: *Aponosma* DC. and *Euonosma* DC. SCHUR (1866), BOISSIER (1879) and BORBÁS (1877) emphasized trichomes as one of the most important characters for the classification of the genus and divided *Onosma* into two or three sections (ESTELLATAE Schur = Haplotricha Boiss. with glabrous setae, Stelligera Schur = Asterotricha Boiss. by stellately setae and Heterotricha Boiss. covered by both setae types). RIEDL (1967) classified the genus into three sections: *Podonosma* (Boiss.) Gurke, *Protonosma* Popov., and *Onosma* with splitting the latter into two subsections *Onosma* and *Asterotricha* (Boiss.) Gurke, each with several series. KHATAMASAZ (2002), followed RIEDL's treatment and recognized three subsections for section *Onosma*: *Haplotricha*, *Heterotricha* and *Asterotricha*.

Taxonomic treatments within these three subsections are highly controversial (CECCHI and HILGER, 2021). Indistinct morphological characteristics and interspecific hybridization lead to taxonomically problematic group (KOLARCIK *et al.*, 2010).

Molecular studies in the genus *Onosma* are limited. MENGONI *et al.* (2006) studied the genetic diversity in European populations of *Onosma echioides* L. by amplified fragment length polymorphism (AFLP). KOLARCIK *et al.* (2010, 2014) investigated the evolutionary history of European species of *Onosma* based on AFLP and nuclear and plastid DNA sequences and confirmed the monophyly of the Asterotricha group and hybrid origin of heterotrichous group. Evolutionary dynamics of serpentine adaptation in *Onosma* (Boraginaceae) as revealed by ITS sequence data studied by CECCHI *et al.* (2011) and found that obligate endemics belonged to six distantly related clades. MEHRABIAN *et 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 *et al.* (2019; 2020) studied phylogenetic relationships and character evolution of *Onosma* (Boraginaceae) based on nuclear and plastid DNA sequences in Iran and demonstrated that *Onosma* as currently circumscribed is not monophyletic.

The last few decades of plant research have detected an interesting picture of plant evolution and diversification. Simultaneously, the increasing availability of molecular phylogenies and associated divergence times has actuated the development of new methods to estimate rates of speciation and extinction from phylogenetic data of extant species (PARADIS *et al.*, 2004; RABOSKY, 2006) and to find changes in diversification rate through time and across lineages (PYBUS and HARVEY, 2000; HARMON *et al.*, 2003; WEIR 2006; RABOSKY and LOVETTE, 2008; ALFARO *et al.*, 2009).

In the present study, we analyze the evolutionary history and diversification patterns of *Onosma* across the tropical, subtropical and temperate regions. We maximized the geographical sampling of all taxa to minimize problems with potential cryptic diversity. Thus, we provide a comprehensive temporal and spatial analysis of the history of this group.

MATERIALS AND METHODS

Data Set

Taxon Sampling, DNA extraction, PCR and sequencing, sequence alignment, phylogenetic inferences, divergence time estimation and ancestral area reconstruction analysis of this work are based on the previous study by authors (NASROLLAHI *et al.*, 2019).

Diversification analyses

We modeled diversification by treating the phylogeny as having been shaped by a mixture of macroevolutionary processes, using a novel Bayesian approach called BAMB (Bayesian Analysis of Macroevolutionary Mixtures, RABOSKY *et al.*, 2014; RABOSKY, 2014). We used the program BAMB to: (1) estimate rates of speciation, extinction, and net diversification, and (2) conduct a rate-through-time analysis of these rates.

Priors for BAMB were generated using the R package BAMBtools v.2.0.2 (RABOSKY, 2014) by providing the MCC tree from BEAST and total species numbers. Two independent MCMC chains of 100,000,000 generations were run in BAMB and convergence was assessed by computing the effective sample sizes of log likelihoods, as well as the number of shift events present in each sample using the R package coda v. 0.16-1 (PLUMMER *et al.*, 2006). After removing 10% of trees as burn-in, we analyzed the BAMB output using BAMBtools and computed the 95% credible rate shift configurations.

RESULTS

Phylogenetic relationships and timing of clade formation in Onosma

In the nrDNA phylogeny, *Podonosma orientalis* and two species of *Alkanna* are resolved as sister taxa and were placed at the base of the tree. A clade including *Onosma rostellata*, *Maharanga emodi* and four Sino-Indian species is, then, sister to the remainder of *Onosma*.

These remaining species fall in a polytomy of six major groups labelled I–VI. Group I and VI comprises species of both haplotrichous and heterotrichous (rarely asterotrichous) leaf indumentum types, mainly distributed in Iran and the East Mediterranean Basin. Group II includes asterotrichous species from Europe, while Group III comprises asterotrichous species from Iran and Turkey, and group IV includes haplotrichous species from Iran. The last group (V) is composed of haplotrichous and heterotrichous species from Europe (NASROLLAHI *et al.*, 2019).

The tree resulting from concatenated plastid dataset reached generally higher resolution and supports than the nrDNA ITS dataset and composed of four groups labelled I–IV. Groups I, II and IV comprise haplotrichous species, while group III includes mainly asterotrichous and heterotrichous species (NASROLLAHI *et al.*, 2019).

The BEAST chronogram based on the nrDNA ITS dataset is consistent with the results from the Bayesian analyses. The molecular dating analyses show that: 1) the early radiation of *Onosma* s.l. happened at the Oligocene-Miocene boundary (95% HPD = 25 mya) after this lineage diverged from *Podonosma orientalis* ca 31.42 mya in Late Oligocene (95% HPD); 2) diversification of *Onosma* s.s. and diversification of the *O. rostellata* + *Maharanga* + Sino-

Indian *Onosma* lineage started in the early and late Miocene, respectively (95% HPD = 19.23, 11.21 mya), and 3) the diversification within *Onosma* s.s. continued during the middle to late Miocene (ca 14–5.33 mya) and continued during Pliocene (95% HPD = 5.33–2.58 mya) and Pleistocene (95% HPD = 2.58–0.11 mya) (Fig. 1).

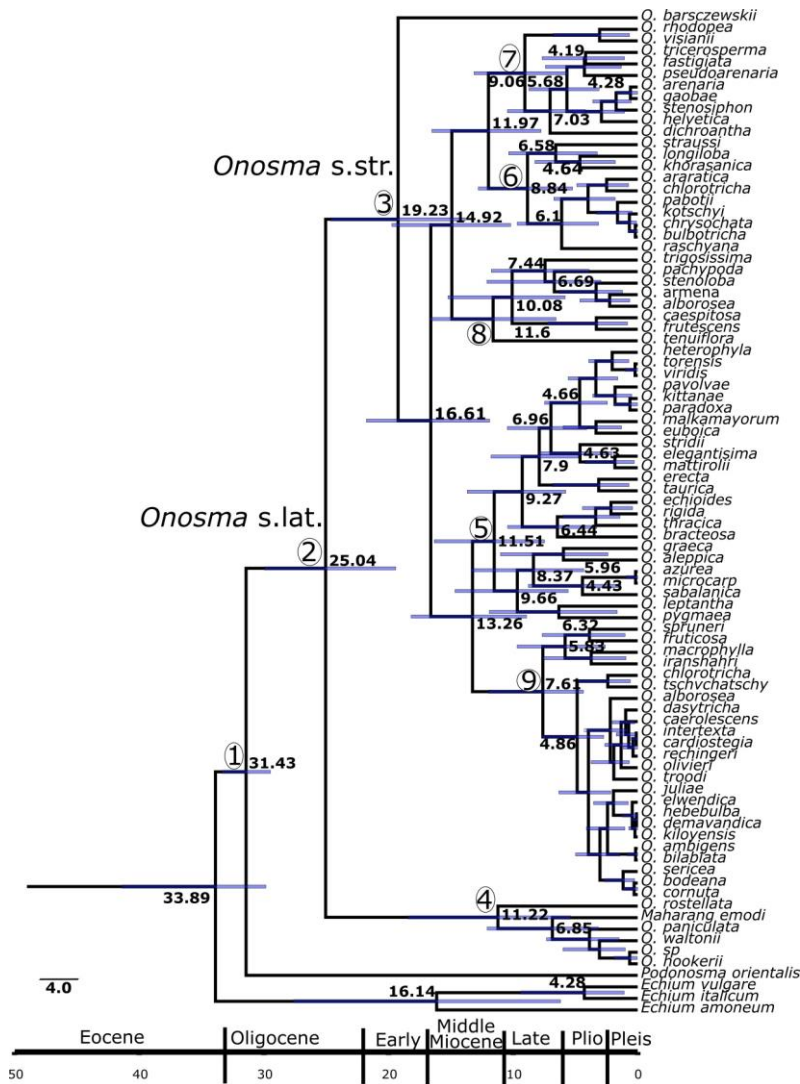


Figure 1. Chronogram inferred from BEAST. Each node represents the mean divergence time estimate and grey bars represent the 95% highest posterior density intervals around mean nodal ages.

Speciation and extinction rates in species diversification in Onosma

After discarding the burn-in, we confirmed convergence of the MCMC chains in the BAMM analyses, as well as effective samples sizes >700 for both the number of shifts and log likelihoods. BAMM analyses strongly supported a diversity-dependent speciation process across *Onosma* with a net diversification rate (speciation minus extinction) of 1.96 species/Myr (Fig. 2).

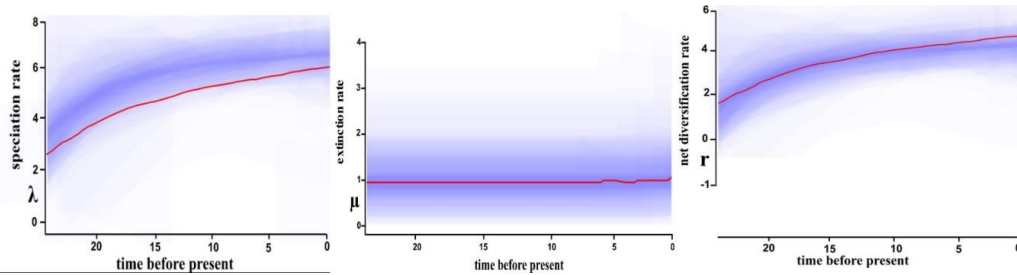


Figure 2. Rates-through-time plots of speciation, extinction and net diversification in *Onosma* utilizing BAMM. Curved black lines represent the median values with the 95% confidence intervals shown in gray.

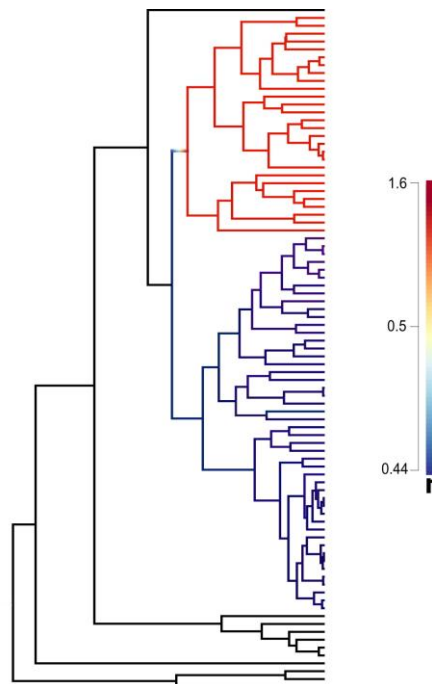


Figure 3. Speciation rates in *Onosma* utilizing BAMM. Chronogram of *Onosma* with branch lengths colored according to speciation rate. Red cladogram indicates significant increases in speciation rates present in the best shift configuration.

The highest speciation rates are seen at nodes within clade of *Onosma* s.lat. (3.41 species/Myr) (Fig. 2). This clade also shows the fixed extinction rates (1.45 species/Myr) (Fig. 2). Rate-through-time plots for *Onosma* revealed a fixed speciation rate until 25 Mya, at which point speciation began to increase, with the highest average rate (3.41 species/Myr) seen at present (Fig. 3).

Extinction rates were low and fixed (1.45 species/Myr) from 25 Mya until at present. The 95% credible set of rate shift configurations sampled with BAMM included several distinct configurations of which the configurations with the highest probability included one shift represents the single best configuration and includes one shift detected within *Onosma* including clade of *Onosma* s.lat., on the branch leading to the clade comprised of species of confined to Iran (Fig. 3).

DISCUSSION

Our results revealed strong evidence for phylogenetic relationships among species, for the ancestral areas and timing of this remarkable radiation, and for the specific subclades that have undergone significant shifts in speciation.

However, none of the three traditionally recognized subsections of sect. *Onosma* are monophyletic. The nuclear and plastid trees favors a hybrid (allopolyploid) origin of the heterotrichous group. Several factors could account for the lack of monophyly, including recombination of divergent alleles, unrecognized amplification of a paralogous locus, inadequate phylogenetic signal, introgressive hybridization and incomplete lineage sorting (DRAPER *et al.*, 2007; SYRING *et al.*, 2007; FLAGEL *et al.*, 2008; DRAGON and BARRINGTON, 2009; RAMDHANI *et al.*, 2009).

Polyploidization and hybridization of heterotrichous group

The last few decades of plant research have apparented an interesting image of plant evolution and diversification. Now, it is accepted that polyploidization and hybridization are two significant drivers in angiosperm evolution (WOOD *et al.*, 2009; SOLTIS and SOLTIS, 2016; KOVAŘÍK *et al.*, 2017). Both polyploidization and hybridization are united, and most of the polyploids are allopolyploids (PADILLA-GARCÍA *et al.*, 2018; JULCA *et al.*, 2018), which are derived from the hybridization between two different taxa. The study of the effects of a specific driver on diversification is complex. In addition, polyploids are often correlated with morphological, anatomical, reproductive, cytological and niche variations (GRANT, 1971). The effects of polyploids on physiology and ecology are complicated (KOLARCIK *et al.*, 2019).

Onosma is a genus with interspecific hybridization, so, there are many challenges, such as problems in identifying species as well as species delimitation. Our result show that the discordant patterns on nuclear and plastid trees are conflicting regarding the position of the heterotrichous taxa. This already been suggested based on bimodal karyotype features, intermediate morphology, Pollen morphology and molecular data (VOUILLAMOZ, 2001, KOLARCIK *et al.*, 2014; KOLARCIK *et al.*, 2019). Polyploidization and hybridization have also been observed to increase the diversification of the genus *Onosma* in Europe (KOLARČIK *et al.*, 2014).

Phylogenetic perspective of evolution within Onosma

The lack of sufficient taxon sampling of the *Onosma* contributed to the uncertainty in its phylogenetic placement in old studies. In our study, we explicitly meliorated sampling in *Onosma* by including representatives of Central Asia and Turkey, China and southern Europe. All species of *Onosma* except five falls in a single clade that is well-supported in the nrDNA ITS tree.

The vast majority of *Onosma*, therefore, appear to form a monophyletic group, to which we will henceforth refer as *Onosma* s.str. This clade is characterized by a mixture of morphological, anatomical and pollen traits (RIEDL, 1978; AZIZIAN *et al.*, 2000; LIU *et al.*, 2010; MEHRABIAN *et al.*, 2012; BINZET *et al.*, 2011). It also has various basic chromosome numbers, $x = 6, 7, 8, 10, 11$ (ALMASI and RANJBAR, 2013).

Significant shift and species diversification rates

A significant question now being addressed more critically in evolutionary biology is the nature of the processes that lead to important shifts in speciation and/or extinction rates within clades (e.g., ALFARO *et al.*, 2009; ANTONELLI *et al.*, 2015; BIFFIN *et al.*, 2010; GIVNISH *et al.*, 2014; LINDER *et al.*, 2014; MCGUIRE *et al.*, 2014; RABOSKY *et al.*, 2014; SMITH *et al.*, 2011). Relevant issues in detecting significant rate shifts include incorporating extinction, phylogenetic uncertainty, phylogenetic scale, sampling density, correlation and/or causality of biotic or niche attributes driving the rate shifts (BERGER *et al.*, 2016). The program BAMM, as now performance, can address a number of these issues. The baseline or average rate of net diversification for *Onosma* is not significant (1.96 species/Myr).

In general, and demonstrated in rates-through-time analysis, *Onosma* exhibited long-term speciation rate increase and extinction rate fixed throughout its entire 25.04 Myr diversification. Thus, our dates suggest that the early radiation of *Onosma* s.l. took place at the Oligocene-Miocene boundary and the diversification within *Onosma* s.s. occurred during middle to late Miocene and Pliocene.

What biotic factors may be correlated with this shift in species diversification? Presumably, topographic heterogeneity and geologic and climatic history have been important for determining the distribution of the species (DJAMALI *et al.*, 2012; MANAFZADEH *et al.*, 2014). We do find evidence of rapid diversification in clade of Iranian species. The diversification of *Onosma* s.s. may have started at Early Miocene (Burdigalian) characterized by warm subtropical climatic conditions where thermophilous plants were favored. But the continued cooling process was already evident by the late-middle Miocene (KAYSERI-ÖZER, 2014; KAYSERI-ÖZER *et al.*, 2014). The rapid and extensive radiation of Irano-Anatolian species also started in the late Miocene (95% HPD =11.88–7.61 mya) and extended into the Pleistocene. This period roughly corresponds to the Messinian salinity crisis of the Mediterranean region (FAUQUETTE *et al.*, 2006; JOLIVET *et al.*, 2006; ROUCHY and CARUSO, 2006), which led to an extremely dry climate, which could have favored such a rapid radiation in *Onosma* as in many other Mediterranean taxa (FAUQUETTE *et al.*, 2006; SALMAKI *et al.*, 2016).

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**VREMENSKA DIVERZIFIKACIJA U RODU *ONOSMA* (BORAGINACEAE)
ZASNOVAN NA NUKLEARNIM I PLASTIDNIM SEKVENCIMA DNK**

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Izvod

Rod *Onosma* je homogen takson sa velikim morfološkim varijacijama. Zbog sličnosti među taksonima *Onosma* postoji mnogo problema u njihovoj identifikaciji. Otuda se sistematski i taksonomski smatra kompleksnim rodом. U ovoj studiji proučavali smo veliki broj vrsta *Onosma* u čitavom njihovom rasponu distribucije. Koristeći nuklearne (ITS) i dva plastidna (rpl32-trnL(UAG) i trnH-psbA) markera, analizirali smo evolucionu istoriju, vreme divergencije i obrasce diversifikacije *Onosme* u tropskim, subtropskim i umerenim regionima. Procene vremena divergencije sugerišu da se rano razdvajanje *Onosma* s.l. dogodilo na granici oligocena i miocena. BMM analize pokazuju da je najbolja konfiguracija uključivala jedan značajan pomak u stopama diversifikacije unutar *Onosme*- na granu koja vodi do klade koja se sastoji od vrsta ograničenih na Iran.

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