

**EXPLORING THE TEMPO OF SPECIES DIVERSIFICATION IN *Astragalus* SECTION
Incani DC. BASED ON NUCLEAR AND PLASTID DNA SEQUENCES**

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This study uses phylogenetic relationships of the species-rich section *Astragalus* (*Incani*), to follow up on recent evidence pointing to rapid and recent plant diversification patterns in the west of Iran. Section *Incani* is introduced for its taxonomic complication resulting from overlapping morphological characters, but few studies have been done on this section; hence, we also lack a robust time-calibrated chronogram to address hypotheses (e.g., biogeography and diversification rates) that have implicit time assumptions. Two loci (*rpl32-trnL_(UAG)* and nrDNA ITS) were amplified and sequenced for 87 taxa across *Incani* for phylogenetic reconstruction and a chronogram in BEAST. *Incani* is identified as the sister clade to all remaining sections with high support, and within the clade *Incani*, two strongly supported groups are seen: (1) Clade I includes nine species restricted to eastern Iran and Central Asia, and (2) clade II includes a bulk of the species from west and northwestern Iran, Turkey and southern Europe. Divergence time estimates suggest *Incani* diverged from remaining sections 3 Mya during the late Pliocene. The crown date for *Incani* is estimated at 1.5 Mya (Pleistocene). Biogeography showed significant improvement in the likelihood score when the “jump dispersal” parameter was added. An eastern origin (Central Asia) is implicated as important ancestral area in all deeper nodes. BMM analyses indicate that the best configuration included one significant shift in

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diversification rates within *Incani*: near the crown of *Incani* (1.5-2 Mya) including clade II. Issues with conducting diversification analyses more generally are examined in the context of scale, taxon sampling, and larger sets of phylogenetic trees.

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

INTRODUCTION

Organismal diversification rates (speciation minus extinction) have been variable across lineages and through time, with some lineages showing slowing rates of lineage accumulation and others showing rapid radiations yielding taxon rich clades. Molecular phylogenies of species rich groups supply hypotheses of evolutionary grounds, the tempo and rate and mode of diversification (PYBUS and HARVEY, 2000), and also allow us to test hypotheses about biogeography and migration patterns. The recent expansions of molecular phylogenetics have provided several useful approaches to identify taxonomic groups that have had a history of successful diversification and, more generally, identify areas of the phylogeny in which diversification has been particularly rapid. Rapid or explosive diversifications are generally defined in a way that includes rapid cladogenesis from a common ancestor, within a relatively short time span. Both intrinsic traits (e.g., adaptation to a new habitat) and extrinsic events (e.g., climatic or tectonic changes) may explain variation in diversification rates through time. Consequently, the early speciation burst may be considered adaptive (GRAVILETS and VOSE, 2005), through a key innovation or colonization of a new area, or non-adaptive per se if the diversification is best explained by variance only.

Section *Incani* DC., one of the most species-rich sections in the *Astragalus* with ca. 140 species, most of which are geographically restricted Southern Europe, North Africa, Turkey, Iran, central Asia and Caucasus (PODLECH and ZARRE, 2013). Its main centers of diversity are in Iran and Turkey with about 120 species (PODLECH and ZARRE, 2013), as hot spots of diversity for this section.

The morphological characteristics of the members of the section including a reduced stem, bifurcate hairs, free stipules, sometimes shortly adnate to the petioles and mostly imparipinnate leaves (PODLECH and ZARRE, 2013; MAASSOUMI *et al.*, 2016).

Several morphological studies comprising leaf hair micromorphology (GHAHREMANINEJAD, 2004 a,b), petiolar anatomy (MEHRABIAN *et al.*, 2007; AL-JOBOURY, 2016), embryology (RIAHI *et al.*, 2003), and cytology (RANJBAR *et al.*, 2010; 2011) have been done on some members of *Incani*. Lately, some new species of section *Incani* have been recognized from Iran (PODLECH and MAASSOUMI, 2003; RANJBAR and KARAMIAN, 2003; MAASSOUMI and KAZEMPOUR-OSALOO, 2006; RANJBAR, 2007; BAGHERI *et al.*, 2011; RANJBAR *et al.*, 2011; GHAHREMANINEJAD and BAGHERI, 2009; 2012; BIDARLORD and GHAHREMANINEJAD, 2021). KAZEMPOUR-OSALOO *et al.* (2003, 2005) examined a few species of section *Incani* using nuclear ribosomal internal transcribed spacer (nrDNA ITS) sequences, and demonstrated that *Incani* along with *A. subsecundus* Boiss. & Hohen. (Section *Laguropsis* Bunge) is monophyletic. DIZKIRICI *et al.* (2014) introduced the first molecular phylogenetic analysis of *Incani* based on 30 species from Turkey, individually based on ribosomal DNA (nrDNA ITS) and showed this section is monophyletic and evolutionarily separated from sections *Hypoglottidei* DC. and

Dissitiflora DC. More recently, AMINI *et al.* (2018) examined *Incani* in greater detail using a cpDNA (*rpl32-trnL*_(UAG)) and a nuclear (ITS) genomic regions and determined that all studied species, excluding of *A. platyphyllus*, formed *Incani* s.s. clade. This section is phylogenetically split into two main groups with largely separate biogeographical ranges. BEAST analysis placed the separation of the section from other *Astragalus* groups in the late Pliocene and a bulk of the speciation events within the section in the last 1–1.5 Myr (middle Pleistocene). Newly, AMINI *et al.* (2020) studied morphological character evolution of section *Incani* DC. using mesquite software based on nuclear and plastid DNA sequences.

Even though *Incani* is introduced for its taxonomic complication resulting from overlapping morphological characters, few studies have been done on this section. In this study we test the hypothesis that there is significant among lineage diversification rate variation across section *Incani*. Analyses of diversification rates for a sparsely sampled higher-level phylogeny of other sections as a whole, and *Incani* densely sampled species-level phylogenies of individual *Astragalus* clades are presented, to gain preliminary insights into the extent of among lineage variation in diversification rates across the section. Models that allow different diversification rates in different parts of the tree are used to identify putative phylogenetic locations of diversification rate shifts. Despite the early stage of these analyses, initial ideas about the dynamics of *Incani* diversification as well as the potential of such studies to shed light on the underlying factors that may have driven the evolutionary success of *Astragalus* section *Incani* are highlighted.

In the present study, we analyzed the evolutionary history and diversification patterns of *Incani*. Thus, we provide a comprehensive temporal and spatial analysis of the history of this section.

MATERIAL AND METHODS

Taxon sampling and Data analysis

Taxon sampling, DNA extraction, PCR and sequencing, sequence alignment, phylogenetic inferences, divergence time estimation and ancestral area reconstruction analysis of this work is based on a previous study (AMINI *et al.*, 2018).

In previous research (AMINI *et al.*, 2018), phylogenetic and divergence time analyses were performed using BEAST v1.8.3 (DRUMMOND *et al.*, 2012) on the CIPRES Science Gateway (Cyber infrastructure for Phylogenetic Research cluster) (MILLER *et al.*, 2010, < www.phylo.org >) on the concatenated datasets. AMINI *et al.* (2018) used the recently developed statistical dispersal–vicariance analysis (S–DIVA) implemented in RASP (YU *et al.*, 2015) to reconstruct the possible ancestral ranges of the section on Bayesian trees obtained from BEAST analysis of the data set of 73 accessions of *Incani* (nrDNA ITS dataset) and identify factors responsible for the current disjunctive distribution pattern.

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 BAMM (Bayesian Analysis of Macroevolutionary Mixtures, RABOSKY *et al.*, 2013; RABOSKY and MATUTE, 2013; RABOSKY, 2014). We used the program BAMM to: (1) estimate rates of speciation, extinction,

and net diversification, and (2) conduct rate-through-time analysis of these rates. The chronogram obtained from the BEAST program related to nrDNA ITS analysis was used to perform macroevolution analyzes due to the presence of sequences related to the Turkish region in newic format. Due to the fact that the relevant analyzes are performed by considering factors such as the percentage of sampled species and compensate for incomplete sampling percentages through simulation, it is better to remove outgroups. To remove outgroup taxa from the chronogram, the tip function coded in the ape package available in the R language was used. Priors for BAMM were generated using the R package BAMM tools 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 BAMM 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 BAMM output using BAMM tools and computed the 95% credible rate shift configurations.

RESULTS

Phylogenetic relationships and timing of clade formation in Incani

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 *Laguropsis*), except for *A. platyphyllus* Kar. & Kir. formed a well-supported clade (PP = 1, ML/BS = 100) (AMINI *et al.*, 2018). The *Incani* s.s. clade is composed of two groups. One group (I) which is restricted to East Iran, Pakistan and Central Asia. The other group (II) comprises the rest of the species which are confined to northwest, west of Iran, Turkey, southern Europe and North Africa (AMINI *et al.*, 2018). The chronogram based on the BEAST analysis of the combined (nrDNA ITS and *rpl32-trnL_(UAG)*) dataset is compatible with those resulting from Bayesian analyses (AMINI *et al.*, 2018). Divergence time estimates suggest *Incani* s.s. diverged from relatives sections 3 Myr (Figure 1) during the late Pliocene. The BEAST analyses show that: 1) the primary radiation of section *Incani* s.s. is concluded to have occurred in the late Pliocene, about 3 Myr (95% HPD:1.9-4.2), and 2) diversification of main lineages of *Incani* s.s. started in the early Pleistocene (2.5 Myr) and with the bulk of speciation events being younger than 2 Myr.

Biogeographical patterns of diversification in Incani

S-DIVA performed by AMINI *et al.* (2018), proposed three probable ancestral ranges, (ab) (Central Asia+west and northwest Iran), (ac) (Central Asia+Turkey) and (ae) (Central Asia+Europe) for *Incani* s.s. (node 143, Figure 2) offering that the clade II might have originated in west and northwest Iran (AMINI *et al.*, 2018). This node proposes an primary dispersal to Turkey and Europe. The ancestral area reconstruction at these nodes suggests west and northwest Iran+Europeas ancestral areas, which likely represented a dispersal phenomenon.

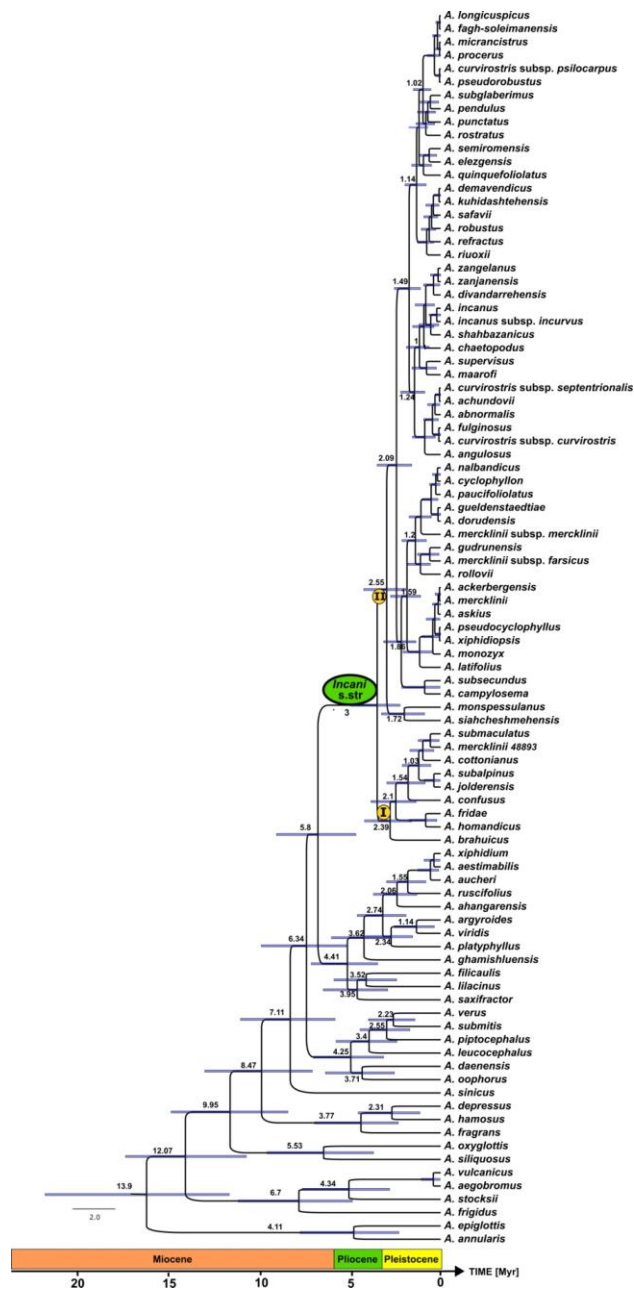


Figure 1. Chronogram using BEAST Bayesian inference with ages given in million years ago (Myr). Mean divergence dates are shown for key nodes.

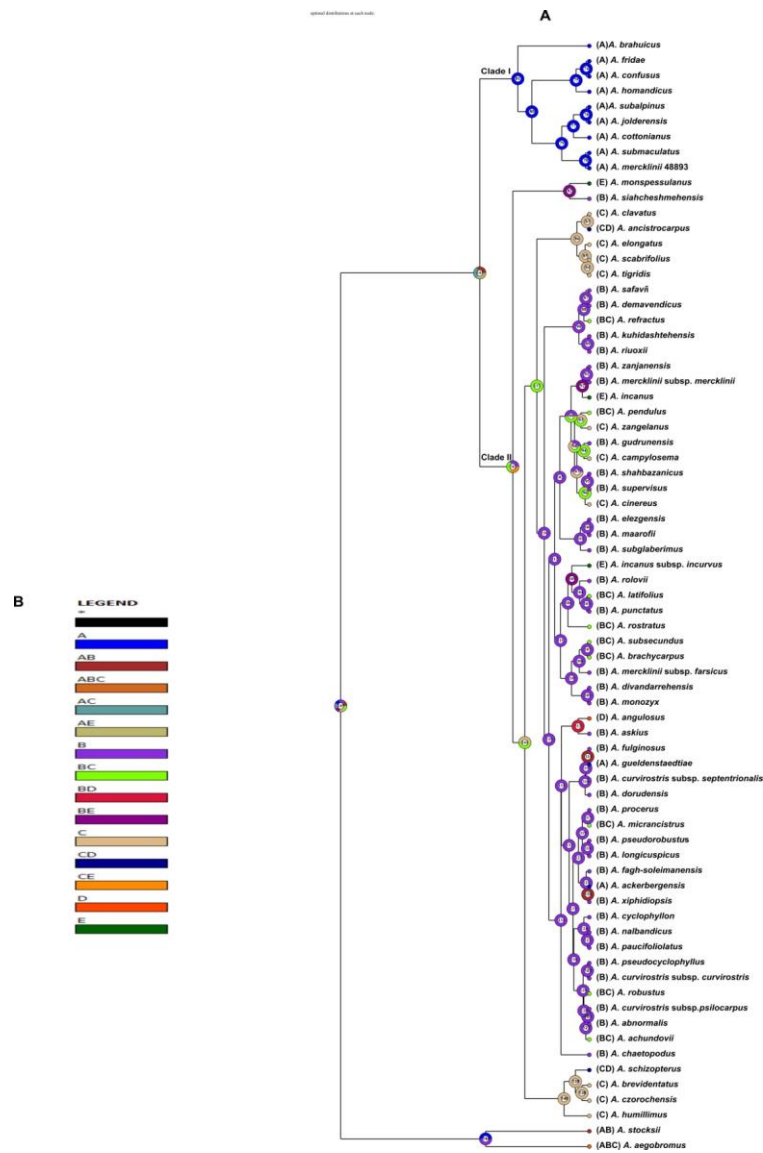


Figure 2. Graphical output from S-DIVA (RASP)

(A) Graphical results of ancestral distribution areas at each node of the phylogeny of *Astragalus* sect. *Incani* s.s obtained by S-DIVA. (B) Colour legend to possible ancestral ranges; black with an asterisk represents other ancestral ranges.

Speciation and extinction rates and identification of rate shift in species diversification in section Incani DC.

After discarding the burn-in, we confirmed convergence of the MCMC chains in the BAMM analyses, as well as effective sample sizes >700 for both the number of shifts and log likelihoods. BAMM analyses strongly supported a diversity-dependent speciation process across *Incani* with a net diversification rate (speciation minus extinction) of 0.22 species/Myr (Figure 3). The highest speciation rates are seen at nodes within clade II (1.9 species/Myr). This clade also show the fixed extinction rates (0.9 species/Myr). Clade I exhibit the lowest speciation and extinction rates within the section. Rate-through-time plots for *Incani* revealed a increased speciation rate until 3 Mya, at which point speciation began to increase, with the highest average rate (0.32 species/Myr) seen at present (Figure 3). Extinction rates were low and fixed (0.9 species/Myr) from 4 Mya until at present (Figure 3).

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. Figure 4 represents the single best configuration and includes one shift detected within *Incani* including clade II, on the branch leading to the clade comprised of species of confined to northwest, west of Iran and Turkey (Figure 4).

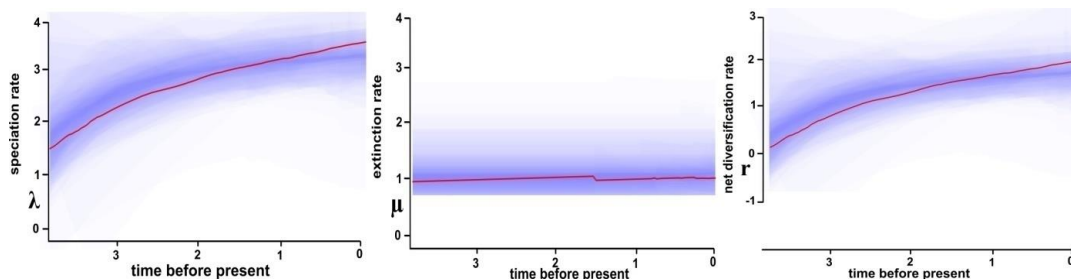


Figure 3. Rates-through-time plots of speciation, extinction, and net diversification in *Incani* utilizing BAMM. Curved red lines represent the median values with the 95% confidence intervals.

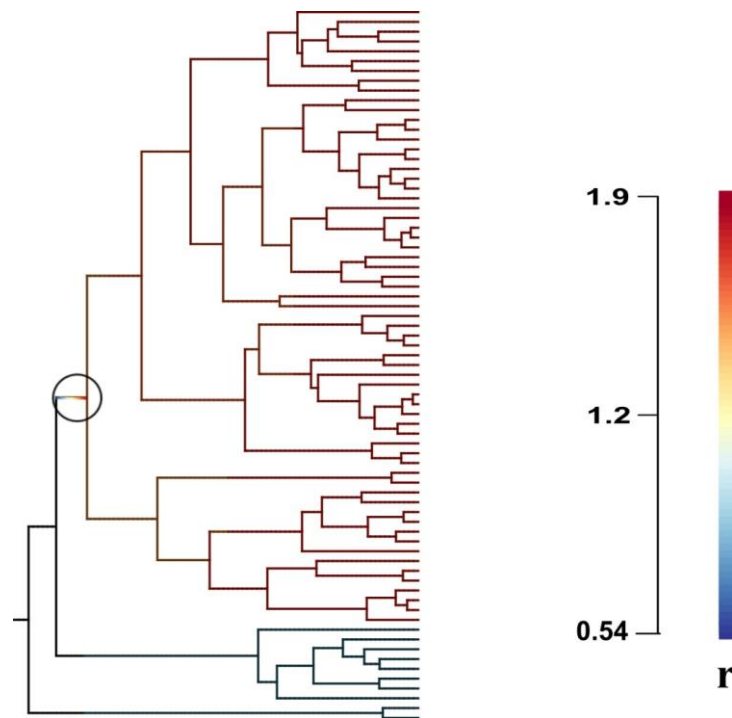


Figure 4. Speciation rates in *Incani* utilizing BAMM. Chronogram of *Incani* with branch lengths colored according to speciation rate. Red clade indicate significant increases in speciation rates present in the best shift configuration.

DISCUSSION

Our results, which are based on the two clades of section *Incani*, provide strong evidence for phylogenetic relationships among *Incani*, for the ancestral areas, timing and biogeographical mode of this remarkable radiation, and for the specific subclades that have undergone significant shifts in speciation. We show for the first time, stronger support for the placement of *Incani* as sister to all remaining sections. This relationship and other early cladogenetic events within *Incani* signal the importance of northeast and eastern Iran, Pakistan and Central Asia as ancestral areas in the early biogeographic diversification of the section during the early Pleistocene. This biogeographic diversification involves both vicariant and dispersal events among biogeographically regions. Lastly, we find one significant shift in speciation rates within section (clade II).

Phylogenetic perspective of evolution within Incani

The lack of adequate taxon sampling of *Incani* contributed to the uncertainty in its phylogenetic placement in previous studies (KAZEMPOUR-OSALOO *et al.*, 2003, 2005; DIZKIRICI *et*

al., 2014; AZANI *et al.*, 2017). In the study conducted by AMINI *et al.* (2018), based on the MP, ML and BI analyses, all the studied species of the section *Incani* plus *A. subsecundus* (section *Laguropsis*), but excluding *A. platyphyllus*, belong to a well supported monophyletic group (PP = 1.00, ML/BS = 100/100), referred to as the *Incani* s.s. clade (AMINI *et al.*, 2018).

A large assemblage 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.

Origin and biogeography of Incani

Previous phylogenetic studies of *Incani* resolved most relationships among extant species and supported an eastern origin (Central Asia) of the section (AMINI *et al.*, 2018). The S-DIVA analysis revealed an east–west pattern of dispersal between the eastern (node 81: clade I) and western (node 142: clade II) lineages (Figure 2). Clade I contains nine species, from *A. brahuicus* through *A. subamculatus* which are restricted to northeast and eastern Iran, Pakistan and Central Asia. However, the most number of species (Clade II) occurs in the western part of the distribution area of the section, ranging from western and northwestern Iran, through Turkey and the Caucasus, to southern Europe/North Africa (Figure 2; MAASSOUMI, 1998; PODLECH and ZARRE, 2013). Fewer species in the eastern part of the distribution range may be clarified by lack of proper habitat towards the eastern parts of the distribution, where the climate maybe gets too dry for *Incani* species. It is very likely that topographic heterogeneity and geologic and climatic history have been significant for determining the distribution of the species (DJAMALI *et al.*, 2012; MANAFZADEH *et al.*, 2014). The main centers of biodiversity of the section are Iran and Turkey with about 120 species, making the area a hotspot of *Incani* s.s. diversity (PODLECH and ZARRE, 2013).

Incani and species diversification rates

An important question now being addressed more critically in evolutionary biology is the nature of the processes that lead to significant 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.*, 2015). The program BAMM, as now implemented, can address a number of these issues. The baseline or average rate of net diversification for *Incani* is not striking (0.06–0.07 species/Myr). In general, and demonstrated in rates-through-time analysis (Figure 3), *Incani* exhibited long-term speciation rate increase 2 Myr and extinction rate fixed throughout its entire 4 Myr diversification. However, at 2 Myr speciation (and net diversification) increases rapidly (Figure 3). Thus, our dates (late Pliocene/early Pleistocene) suggest a rapid radiation of section all now largely adapted and confined to a wetland biome that would appear in Western and northwestern Iran, through Turkey for at least another 2 Myr.

What climatic factors may be correlated with this shift in species diversification? It is very likely that topographic heterogeneity and geologic and climatic history have been

significant for distinguishing the distribution of the species (DJAMALI *et al.*, 2014; MANAFZADEH *et al.*, 2014). In addition, high levels of ultraviolet light allow rapid mutations to be induced (DAVIES *et al.*, 2004; WILLIS *et al.*, 2009). We do find evidence of rapid diversification in clade II. The rapid and extensive radiation of *Incani* within western and northwestern Iran, through Turkey and the Caucasus, to southern Europe/North Africa (often unresolved in phylogenetic analyses) occurs from the late Pliocene into the early Pleistocene as higher altitude and humidification intensified. Biodiversity of the section drops sharply in the westernmost part of its distribution (southern Europe/North Africa). The uplift of the Elburz and Zagros Mountains from the Iranian plateau during the middle Miocene promoted high levels of endemism (DJAMALI *et al.*, 2012; AMINI *et al.*, 2018). The Zagros mountain chain expands from northwest to southeast of Iran and the Zagros chain has been separated into three primary domains (MOZAFFARIAN, 2013). The 'Humid Zagros' in the northwest, which includes the higher mountains, is affected by Mediterranean winds and the 'Dry Zagros' in the southeast, with a drier climate, and the Karkas mountains of central Iran.

The low nucleotide variability and poor resolution in clade II of the section might represent a rapid and recent radiation. A rapid and recent diversification pattern is not a event limited to this section but has been showed in other groups of *Astragalus*, including the New World aneuploids, the so-called Neo-Astragalus, and the sections *Ammodendron* Bunge and *Hymenostegis* Bunge (WOJCIECHOWSKI *et al.*, 1999; SCHERSON *et al.*, 2008; DASTPAK *et al.*, 2013; BAGHERI *et al.*, 2017; *et al.*, 2018).

Analysis of the evolution of traits show that the violet corolla in clade II has a high level of support, while this trait is much less observed in clade I and the majority of species of clade I have yellow corolla (AMINI *et al.*, 2020). This trait has been a key innovation in clade II species, and may have attracted more pollinators and accelerated speciation so that they could increase their numbers in less than two million years. Although the amount of drought or soil moisture in the region has been the most important factor in stimulating of the speciation, factors such as high altitudes, soil types, pollinators, different mechanisms of seed distribution and polyploidy can also be considered as important factors in stimulating of speciation (JABBOUR and RENNER, 2011; SALVO *et al.*, 2011; DJAMALI *et al.*, 2012; MANAFZADEH *et al.*, 2014).

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**ISTRAŽIVANJE TEMPA DIVERZIFIKACIJE VRSTA U SEKCIJI *Astragalus incani* DC.
ZASNOVANO NA NUKLEARNIM I PLASTIDNIM DNK SEKVENCIJAMA**

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Izvod

Ova studija koristi filogenetske odnose odeljka bogatog vrstama *Astragalus* (*Inceni*), da prati nedavne dokaze koji ukazuju na brze obrasce diverzifikacije biljaka na zapadu Irana. Odeljak *Inceni* dobro poznat po svojoj taksonomskoj složenosti koja je rezultat preklapanja morfoloških karaktera i visoke fenotipske plastičnosti, malo je studija urađeno na ovom odeljku; stoga nam takođe nedostaje robustan vremenski kalibriran hronogram da bismo odgovorili na hipoteze (npr. biogeografija i stope diversifikacije) koje imaju implicitne vremenske pretpostavke. Dva lokusa (rpl32-trnL_(UAG) i nrDNA ITS) su amplifikovana i sekvencionirana za 87 taksona širom *Inceni* za filogenetičku rekonstrukciju i hronogram u BEAST-u. *Inceni* su identifikovani kao sestrinska klada za sve preostale delove sa visokom podrškom, a unutar klade *Inceni* se vide dve grupe koje su snažno podržane: (1) Klada I uključuje devet ispitivanih vrsta ograničenih na istočni Iran i centralnu Aziju, i (2) klada II obuhvata većinu vrsta iz zapadnog i severozapadnog Irana, Turske i južne Evrope. Procene vremena divergencije sugerišu da su se *Inceni* odvojili od preostalih delova pre 3 miliona godina, tokom kasnoj pliocena. Datum vrhunca za *Inceni* se procenjuje na 1,5 milion godina (pleistocen). Biogeografija je pokazala značajno poboljšanje u rezultatu verovatnoće kada je dodat parametar „raspršivanje skoka“. Istočno poreklo (centralna Azija) je uključeno kao važno područje predaka u svim dubljim čvorovima. BAMM analize pokazuju da je najbolja konfiguracija uključivala jedan značajan pomak u stopama diverzifikacije unutar *Inceni*-ja: blizu vrhunca (1,5-2 miliona godina) uključujući kladu II. Pitanja sa sprovođenjem diverzifikacionih analiza uopštenije se ispituju u kontekstu razmera, uzorkovanja taksona i većih skupova filogenetskih stabala.

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