



Phylogeny and historical biogeography of Lithospermeae (Boraginaceae): Disentangling the possible causes of Miocene diversifications

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ABSTRACT

Studies about the drivers of angiosperm clade diversifications have revealed how the environment continuously alters the species chances to adapt or to go extinct. This process depends on complex interactions between abiotic and biotic factors, conditioned to the geological and tectonic settings, the genetic variability of species and the rate at which speciation occurs. In this study, we aim to elucidate the timing of diversification of the Lithospermeae, the second largest tribe within Boraginaceae, and to identify the possible morphological and ecological characters associated with shifts in diversification rates of the most species-rich clades. Lithospermeae includes ca. 470 species and 26 genera, among which are some of the largest genera of the family such as *Onosma* (150 spp.), *Echium* (60 spp.), and *Lithospermum* (80 spp.). An exhaustive study of the whole clade is not available to date and its evolutionary history and diversification rates are incompletely known. In the present study, we provide the most comprehensive phylogeny of the group so far, sampling 242 species and all 26 genera. We found that crown-groups and diversification rates of Lithospermeae largely date back to the Mid-Miocene, with high diversification rates in the largest genera, though only significantly high in *Onosma*. Our analysis fails to associate any of the functional or morphological traits considered with significant shifts in diversification rates. The timing of the diversification of the species-rich clades corresponds with Miocene tectonic events and global climate changes increasing aridity across Eurasia and western North America. These results suggest a causal link between known ecological features of Lithospermeae (i.e., pre-adaptation to arid, open habitats, and mineral soils) and their diversification. Future studies should expand the sampling of individual subclades and detailed functional analyses to identify the contribution of adaptations to arid conditions and pollinator shifts.

1. Introduction

Dramatic transformations affecting present day patterns of biodiversity have taken place since the Oligocene and Miocene, ca. 35 million years ago (Ma), when climatic changes affected the diversification of many plant lineages worldwide (e.g., Antonelli et al., 2010; Arakaki et al., 2011; Nagalingum et al., 2011). During the middle Miocene Climatic Optimum and its subsequent cooling phases (ca. 17–10 Ma; Zachos et al., 2001), global large-scale fluctuations in the carbon dioxide levels boosted the diversification of C₄-grasses and led to the expansion of arid and seasonal environments (Latorre et al., 1997; Kürschner et al., 2008; Herbert et al., 2016). Since then, the Earth's landscapes have undergone a series of transformations, driven by a combination of biotic and abiotic processes resulting in the

establishment of new biomes (Donoghue and Edwards, 2014). For instance, the availability of new habitats during the Miocene, rather than a new “key innovation” (Arakaki et al., 2011: page 8382), likely acted as the main driver of plant diversification in succulent lineages. Additionally, extreme fluctuations in temperature and precipitation regimes might have caused widespread extinctions, opening up new suitable habitats and ecological niches for lineage diversification of plant groups pre-adapted to aridity (Jacobs, 2004; Verboom et al., 2009; Byrne et al., 2011, Otero et al., 2019a). Aside from climatic shifts, tectonic, topographic, and geomorphological changes throughout the Miocene are believed to have influenced the diversification of angiosperms and other groups of organisms worldwide (Potter and Szatmari, 2009; Luebert and Weigend, 2014; Wen et al., 2014). Ecological selection via biological interactions and macro ecological shifts in, e.g.,

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pollination syndrome, may have also had a dramatic effect on speciation rates (Renner, 2016; Fernández-Mazuecos et al. 2019).

A recent study on the Cynoglossoideae, the largest subfamily in Boraginaceae (Otero et al., 2019a), investigated the patterns of diversification in this group and found that early and main diversification events took place since the mid-Eocene into the Miocene, correlated to the onset of global cooling, aridisation, and the diversification of large herbivores. Lithospermeae, the second largest Boraginaceae clade (Chacón et al., 2016), represents an interesting parallel case since initial diversification took place between the mid Eocene–Oligocene boundary (ca. 25–40 Ma; Chacón et al., 2017), with most of the extant species diversity arising in a relatively short time period (Mansion et al., 2009; Weigend et al., 2009; Cecchi et al., 2011; Cohen, 2012). More than fifty percent of the current species diversity originated during the Miocene almost in parallel, as suggested by the crown ages of the largest genera: *Onosma* L. (ca. 150 species; ca. 19.8–11.3 Ma), *Lithospermum* L. (ca. 80 species; ca. 14.5–11.3 Ma), and *Echium* L. (ca. 60 species; ca. 10.6 Ma; see Fig. S2 in Chacón et al., 2017). For *Alkanna* Tausch (ca. 40 species), the fourth largest genus in the tribe, no dated phylogeny is available to date. Lithospermeae are thus an interesting plant group for investigating Miocene diversifications in angiosperms. This clade

comprises ca. 470 species of annual and perennial herbs, shrubs, and subshrubs (Fig. 1) distributed in Eurasia, Africa, and the Americas, with centers of species diversity in the Mediterranean and the Irano-Turanian region of Western Asia (Chacón et al., 2016; Weigend et al., 2016; see additional references in Table 1). Lithospermeae in general and the four genera mentioned above (i.e., *Onosma*, *Lithospermum*, *Echium*, and *Alkanna*) are most diverse in semi-arid to arid habitats, with a clear overall center of diversity in western Eurasia and the Mediterranean basin (Weigend et al., 2016). *Alkanna* is widely distributed in the Eastern Mediterranean region and South-Western Asia (Fig. 1A-B; Weigend et al., 2016, Ergen Akçin et al., 2004; Yildirim and Şenol, 2014). *Echium* is very widespread across the Mediterranean and Central European regions, Northern Africa, and Western Asia, but 27 species are endemic to Macaronesia (Fig. 1G-H; Böhle et al., 1996). *Lithospermum* occurs in Africa, Eurasia, and the Americas and is centered in South-Western North America (Fig. 1M-N; Weigend et al., 2009; Cohen, 2018). *Onosma sensu lato* is found from North Western Africa over Europe to Eastern Asia, but has a clear center of diversity in South Eastern Europe and Asia Minor (Fig. 1R-S; Stevanović et al., 2003; Cecchi et al., 2011).

Lithospermeae are also notable for a large suite of specific



Fig. 1. Morphological diversity in Lithospermeae. A. *Alkanna hirsutissima* (Bertol.) A.D.C.; B. *Alkanna pamphylica* Hub.-Mor. & Reese; C. *Arnebia densiflora* (Nordm.) Ledeb.; D. *Moltkia suffruticosa* (L.) Hegi; E. *Buglossoides incrassata* (Guss.) I.M.Johnst.; F. *Cerinthe retorta* Sm.; G. *Echium boissieri* Steud.; H. *Echium creticum* L.; I. *Glandora rosmarinifolia* (Ten.) I.M.Johnst.; J. *Halacsya sendtneri* (Boiss.) Dörf.; K. *Huynhia purpurea* (Erik & Sümbül) L.Cecchi & Selvi; L. *Lithodora hispidula* subsp. *versicolor* Meikle; M. *Lithospermum macbridei* I.M.Johnst.; N. *Lithospermum rodriguezii* Weigend & Nürk; O. *Lobostemon fruticosus* (L.) H.Buek; P. *Pontechium maculatum* (L.) Böhle & Hilger; Q. *Neatostema apulum* (L.) I.M.Johnst.; R. *Onosma rascheyana* Boiss.; S. *Onosma cespitosa* Kotschy; T. *Megacaryon orientale* (L.) Boiss.; U. *Paramoltkia doerfleri* (Wettst.) Greuter & Burdet; V. *Podonosma orientale* (L.) Feinbrun. Photos by F. Selvi and L. Cecchi (A-K, Q, R, T-V), M. Weigend (L-O, S), and A. Coppi (P).

Table 1
Genera of Lithospermeae, species numbers, and geographic distribution.

Genus	Species nr.	General distribution	Reference
<i>Aegonychon</i> Gray	3	C Europe, Mediterranean, E Asia	Cecchi et al. (2014)
<i>Alkanna</i> Tausch	ca. 40	Mediterranean and SW Asia (Irano-Turanian)	Johnston, (1953); Yildirim and Şenol (2014); Weigend et al. (2016)
<i>Ancistrocarya</i> Maxim.	1	C and S Japan, Korea	Weigend et al. (2016)
<i>Arnebia</i> Forssk.	ca. 30	NE Africa, SE Mediterranean, Himalaya, SW and C Asia	Coppi et al. (2015)
<i>Buglossoides</i> Moench	6	Macaronesia, Asia, N Europe to S Mediterranean basin	Valdés (2011); Cecchi et al. (2014)
<i>Cerinthe</i> L.	6	Circum-Mediterranean, from the Atlantic region of Morocco to W Irano-Turanian region	Selvi et al. (2009)
<i>Cystostemon</i> Balf.f.	ca. 15	SW Arabia, tropical Africa	Weigend et al. (2016)
<i>Echiostachys</i> Levyns.	3	S Cape Region in South Africa	Retief and van Wyk (1997)
<i>Echium</i> L.	ca. 60	N Africa, Europe, Macaronesia, W Asia	Johnston (1953); Böhle et al. (1996); Romeiras et al. (2011); Weigend et al. (2016)
<i>Glandora</i> D.C.Thomas, Weigend & Hilger	8	N Africa and S Europe	Cecchi et al. (2014)
<i>Halacsya</i> Dörfel.	1	Balkans	Johnston (1953); Thomas et al. (2008), Cecchi and Selvi (2009)
<i>Huynhia</i> Greuter	2	Turkey and Caucasus	Coppi et al. (2015)
<i>Lithodora</i> Griseb.	5	Mediterranean, N Africa, W and SE Europe, SW Asia	Johnston (1953); Cecchi et al. (2014)
<i>Lithospermum</i> L.	ca. 80	Africa, Eurasia, N and S America, with a species diversity center in Mexico and SW USA	Al-Shehbaz (1991); Brand (1930); Johnston (1952); Kartesz (1994); Weigend et al. (2009)
<i>Lobostemon</i> Lehm.	ca. 30	South Africa	Johnston (1953); Retief and van Wyk (1997); Buys (2011)
<i>Maharanga</i> DC.	9	C and E Asia	Zhu et al. (1995)
<i>Mairetis</i> I.M. Johnst.	1	Mediterranean, NW Africa	Thomas et al. (2008)
<i>Megacaryon</i> Boiss.	1	N Turkey (Black Sea region)	Edmondson (1979); Selvi et al. (2017)
<i>Moltkia</i> Lehm.	6	E Mediterranean, S Europe and SW Asia	Johnston (1953); Georgiou et al. (2000)
<i>Molkiopsis</i> I.M. Johnst.	1	Mediterranean, NE Africa and SW Asia	Thomas et al. (2008)
<i>Neatostema</i> I.M. Johnst.	1	Mediterranean and Macaronesia	Thomas et al. (2008)
<i>Onosma</i> L.	ca. 150	C Asia to W Mediterranean, with a major diversity center in SE Europe and W Asia (Irano-Turanian region and S Balkans).	Ball (1972); Riedl (1967), (1979); Meikle (1985); Petrova (1989); Teppner (1991); Cecchi and Selvi (2009); Cecchi et al. (2011); Kolarčík et al. (2014); Weigend et al. (2016)
<i>Paramoltkia</i> Greuter	1	Balkans	Thomas et al. (2008); Cecchi and Selvi (2009)
<i>Podonosma</i> Boiss.	3	NE Africa, E Mediterranean, SW Asia	Weigend et al. (2016)
<i>Pontechium</i> Böhle & Hilger	1	E Europe to W Asia	Hilger and Böhle (2000)
<i>Stenosolenium</i> Turcz.	1	NE Asia	Weigend et al. (2016)

morphological and ecological adaptations characterizing various subgroups of the tribe, which are rare in other subgroups of the family, e.g., in vegetative morphology. Unlike most other groups of Boraginaceae, Lithospermeae comprise a considerable proportion of “woody” growth forms, i.e., shrubs, subshrubs, and sclerophyllous shrublets in genera such as *Lithodora* Griseb., *Glandora* D.C.Thomas, or *Onosma* (Weigend et al., 2016; Kolarčík et al., 2010; Cecchi et al., 2011). A parallel trend can be observed in *Lithospermum*, in which the repeated and independent evolution of woody perennials from herbaceous ancestors appears to have taken place in the Mediterranean climate of California (Thomas et al., 2008). Notably, the genus *Lobostemon* Lehm. is yet another group of sclerophyllous shrubs growing in Mediterranean climates of the Cape Floristic Province (Buys, 2011). The most prominent shrubby representatives are found in *Echium* from Macaronesia (Böhle et al. 1996), which represent the most typical case of “island woodiness” in Boraginaceae (besides from *Selkirkia* and *Trichodesma*; Holstein et al., 2016).

In addition to growth form, edaphic specializations in Lithospermeae are also quite divergent: A tolerance to low calcium levels and high magnesium levels, typical of ultramafic soils, likely promoted the high levels of edaphic endemism of *Onosma* in the Balkans (Stevanović et al., 2003; Cecchi and Selvi, 2009; Cecchi et al., 2011). Facultative serpentinophytes abound in Lithospermeae, as do facultative and obligate edaphic specialists on calcareous rock and siliceous sandstones (see examples in Reehinger, 1965; Cecchi and Selvi, 2009; Yildirim and Şenol, 2014). Previous studies have shown that adaptations to ultramafic soils in *Onosma echioides* (L.) L. include a set of adaptations to aridity such as xeromorphic leaves, reduced plant size, well-developed root systems, and mycorrhizal associations (Brady et al., 2005; Muller and Hilger, 2015), all of which are also common across Lithospermeae irrespective of soil preference (Weigend et al., 2016). Studies on *Onosma* and other elements of the Euro-Mediterranean serpentine flora indicate both a recent and multiple origin of serpentine-

adapted species, strongly arguing for a pre-adaptation to these specific soil conditions permitting multiple colonization events (e.g., Cecchi et al., 2010, 2011). A suite of adaptations to aridity and mineral soils probably also explains the presence of both endemic and non-endemic species of *Lithospermum* growing on gypsum-dominated substrates in arid regions of Mexico and the United States (Hinton and Hinton, 1995; Hoagland and Buthod, 2005; Salinas-Rodríguez et al., 2018).

Lithospermeae are also unusually diverse in what concerns flower morphology and function in contrast to most Boraginaceae, in which radially symmetrical, hypocrateriform flowers with deeply included anthers are the most common condition. In Lithospermeae, anther cones in pendulous flowers, as an adaptation to buzz-pollination (e.g., *Onosma*), zygomorphic flowers (*Echium* and allied genera), and complex forms of herkogamy (the spatial separation of anthers and stigmas as a mechanism to enforce outcrossing; e.g., *Alkanna*, *Lithodora*, and *Lithospermum*) are common. Multiple origins and complex evolutionary patterns of the latter trait in Lithospermeae have been suggested by previous studies (Baker, 1961; Ganders, 1979; Ferrero et al., 2009; Weigend et al., 2009; Cohen, 2011; Ferrero et al., 2011a, b; Cohen and Davis, 2012; Cohen et al., 2012). In addition, Lithospermeae include several bird-pollinated species, a most unusual feature in Boraginaceae. Several studies suggest that adaptation to bird pollination has played a key role in speciation in North America (*Lithospermum*; Boyd, 2002, 2004; Cohen, 2011, 2012, 2016a, b), Macaronesia (*Echium*; Böhle et al., 1996; Dupont et al., 2004), and South Africa (*Lobostemon*; Van Wyk et al., 1997; Buys, 2011).

The range of likely adaptive traits found in Lithospermeae invites a study of their correlation with the diversification, specifically between shifts in diversification rates and the evolution of soil preferences, pollination syndromes, herkogamy, and novel growth habits in the species-rich genera *Alkanna*, *Echium*, *Lithospermum* and *Onosma*. Here, we present a densely sampled molecular phylogeny of Lithospermeae including all currently recognized genera across their entire

geographical range. The molecular phylogeny allowed us to assess the phylogenetic position and monophyly of taxa not yet analyzed, and to estimate divergence times of the major clades using fossil calibrations across Boraginales and related Asterids. The new and expanded dated phylogeny was the basis for estimating absolute diversification rates (Magallón and Sanderson, 2001), and shifts in diversification rates (Rabosky, 2014), as well as to investigate whether geological and/or paleo-climatic events have played a significant role in the diversification of the tribe.

2. Material and methods

2.1. Taxon sampling

Our sampling included 257 taxa of Lithospermeae representing 242 out of ca. 470 species from all genera currently recognized following recent taxonomic revisions of Boraginaceae (Table 1; e.g., Chacón et al., 2016, Luebert et al., 2016). Among these, 139 specimens correspond to taxa that are analyzed for the first time with molecular data. Specimens were obtained from field-collections, herbaria, GenBank accessions with full collection data, and DNA samples provided by the DNA Bank at Kew Royal Botanic Gardens. A DNA sample from *Choriantha popoviana* Riedl, a rare species so far only known from the type locality in Iraq, was also included in our sampling. The taxonomic status of genus *Choriantha* Riedl, however, is still unclear due to its clear affinity to *Onosma*, into which it has been recently included (Weigend et al., 2016). All samples included in this study with available voucher information, geographic origin, and GenBank accession numbers are shown in Table S1.

2.2. Phylogenetic analyses

Four DNA regions that have been used in previous phylogenetic studies of Boraginaceae due to their high levels of phylogenetic information were also sequenced in this study using universal primers and protocols following Chacón et al. (2016). The selected regions are the nuclear ribosomal internal transcribed spacer 1, including the 5.8S ribosomal RNA gene and the internal transcribed spacer 2 (ITS), and the chloroplast (cp) regions tRNA-Leu (*trnL*) gene including the *trnL*-F intron and *trnL*-*trnF* intergenic spacer (overall referred to as *trnL*-F), the *rps16* gene, and the *trnS*-*trnG* intergenic spacer. This marker selection allowed us to make use of the extensive record of Lithospermeae DNA sequences nowadays deposited in GenBank, with full voucher information and reliable taxonomic identification. The resulting DNA sequences were assembled in Geneious 8.1.9 (<http://www.geneious.com>, Kearse et al., 2012), and aligned in MAFFT 7 (Katoh and Standley, 2013), using the FFT-NS-i iterative refinement method (Katoh et al., 2002).

New sequences of the DNA regions selected were generated in this study for 180 Lithospermeae species and 13 subspecies, with duplicate specimens for the monotypic genera. Newly sequenced taxa included species and/or subspecies in *Aegonychon* Gray (2 spp.), *Alkanna* (27 spp., 1 subsp.), *Arnebia* Forssk. (8 spp.), *Buglossoides* Moench (3 spp., 2 subsp.), *Cerinth* L. (4 spp., 2 subsp.), *Cystostemon* Balf.f. D (1 spp.), *Echioschachys* Levyns (3 spp.), *Echium* (23 spp., 2 subsp.), *Glandora* (5 spp.), *Huynhia* Greuter (2 spp.), *Lithodora* (3 spp., 2 subsp.), *Lithospermum* (29 spp.), *Lobostemon* (3 spp.), *Maharanga* DC. (2 spp.), *Moltkia* Lehm. (5 spp.), *Onosma* (50 spp., 4 subsp.), plus the single species of *Ancistrocarya* Maxim, *Halacsya* Dörf., *Mairetia* I.M. Johnst., *Megacaryon* Boiss., *Moltkiopsis* I.M. Johnst., *Neatostema* I.M. Johnst., *Paramoltkia* Greuter, *Podonosma* Boiss., *Pontechium* Böhle & Hilger, and *Stenosolenium* Turcz. Additionally, new sequences of four outgroup species of *Moritzia* DC. ex Meisn. and one of *Chuquiraga* Juss. were obtained (Tables S1 & S2).

The resulting matrices were analyzed using maximum likelihood (ML) approaches to confirm the monophyly of the Lithospermeae

genera, which so far have never been analyzed with molecular data, including multiple accessions for each monotypic genus. The program RAxML (Stamatakis, 2006) was used to infer ML trees using the “partition branch length” option and 1000 ML bootstrap replicates. The nuclear and chloroplast data partitions were analyzed separately and were combined after visual confirmation that no statistically supported topological incongruences (ML bootstrap > 80%) existed between individual data sets (see Wiens, 1998).

2.3. Molecular clock dating

A reduced data set of Lithospermeae with an expanded outgroup sampling was used in the dating analyses (Tables S1 & S2), meaning that individual DNA alignments were pruned to eliminate multiple accessions of the same species and to include only taxa with complete sequence data for the three plastid markers. Only the chloroplast markers were included in the dating and diversification analyses due to the difficulties in aligning ITS sequences from all outgroup and ingroup taxa. The resulting matrices consisted of 172 species of Lithospermeae of all currently recognized genera based on Chacón et al. (2016) except *Ancistrocarya*, for which only a partial ITS sequence was available. We used MrModeltest2 2.4. (Nylander, 2004) to estimate the best-fit model of nucleotide substitution for the selected chloroplast regions based on the Akaike Information Criterion (AIC). The following substitution models were identified as the best fit models of DNA substitution: TVM + G (*trnS*-*trnG*), TIM1 + I + G (*trnL*-F), and TIM1 + G (*rps16*). Among the models available in BEAST, the GTR + G is the most compatible with the models selected in MrModeltest2 and was used in subsequent analyses. Therefore, we ran a lognormal relaxed clock model in BEAST 1.10.4 (Suchard et al., 2018) using the GTR + G site model with four Gamma categories, with a random starting tree, and a speciation Yule Process tree prior. We ran a Markov Chain Monte Carlo with 500 million generations sampling every 50,000 generations. Additionally, 186 outgroup species were included based on Luebert et al. (2017) and Chacón et al. (2017) in order to make use of the extensive fossil record of Boraginales and related Asterid taxa (Fig. S1 & Table S2). The phylogeny was calibrated using four Boraginaceae fossils that were applied to the stem nodes of *Cryptantha* Lehm. ex Fisch. (Cynoglosseae), *Moritzia* (Boragineae), and *Ogastemma* Brummit (Echiochiloideae), and the crown node of *Lappula* Moench (incl. *Rochelia* Rchb.), as well as two fossil calibrations from the Boraginales families Ehretiaceae and Heliotropiaceae (i.e., stem nodes of *Ehretia* P. Browne and *Tournefortia* L. sect. *Tournefortia*, respectively). Additional fossils of other Asterids were used to calibrate the stem node of Asteraceae, the crown node of Ericales, and the crown node of Cornales. A secondary calibration was applied to the root node (crown node of the Asteridae). Details on the exact priors and confidence intervals are shown in Fig. S1.

2.4. Diversification rates

We employed the method of Magallón and Sanderson (2001) to analyse diversification rates. We first calculated net diversification rates at crown nodes of major clades of Lithospermeae using the R-package GEIGER 2.0.3 (Harmon et al., 2008). Species richness of each clade was sourced from Luebert et al. (2016) and Weigend et al. (2016). In order to evaluate whether diversification rates are significantly high across the Lithospermeae clades, as compared to its background diversification rate, we built 95% confidence intervals (CI) for crown- and stem node-diversification rates. We used the rate-estimates of the whole Lithospermeae as background diversification under two extreme values of the extinction/speciation ratio (ϵ): $\epsilon = 0$ (very low) and $\epsilon = 0.9$ (very high; see Magallón and Sanderson, 2001). Upper and lower boundaries of the 95% CI were calculated in GEIGER for both values of ϵ across the whole age-range represented in Lithospermeae. If the whole 95% high posterior density (HPD) interval of a clade was outside the 95% CI, the

clade was considered to have experienced extraordinarily high (above the 95% CI) or low (below the 95% CI) diversification rates.

Shifts in diversification rates across the Lithospermeae phylogeny were assessed using BAMM (Rabosky, 2014) based on the chronogram obtained from the BEAST analysis and accounting for non-random, incomplete sampling using the fraction of species sampled for each clade. Sampling fractions were calculated with respect to the total number of species of each genus following Weigend et al. (2016). We conducted analyses with a Poisson prior of 1.0 in order to test whether clades of Lithospermeae underwent any significant rate shift. This prior generates a distribution with highest probability for zero rate shifts. In each analysis, we varied the prior for the expected number of shifts (set to 1, 2, 5 and 10) in order to account for the potential effects of this prior on the number and location of shifts in diversification rates (Moore et al., 2016). We ran the analyses for 5 million generations, sampling every 5000 steps. The BAMMtools R-package (Rabosky et al., 2014) was used to summarise the output of BAMM. We computed posterior probabilities for the number of rate shifts and selected the most credible rate shift set based on a Bayes factor of 50 or more. Recent critics to BAMM claim that the method estimates dramatically different rates for a given clade depending on whether the analyses are based on a complete tree or on portions of the tree corresponding to that particular clade (Meyer and Wiens, 2018; Meyer et al., 2018). For this reason, the speciation rates estimated in BAMM with the complete Lithospermeae tree were compared with the rates estimated for individual clades.

In order to evaluate the potential relation between selected morphological and ecological characters and shifts in diversification rates, we used the Structured Rate Permutations on Phylogenies (STRAPP) test proposed by Rabosky and Huang (2016) for binary characters, implemented in BAMMtools. This method attempts to detect trait-dependent diversification using the Mann-Whitney U test statistic to compare the value of the diversification rate among clades with different values of a binary trait. A null distribution is generated through permutations of the diversification rate of the individual species. All analyses were carried out with 1000 permutations. We conducted tests for the following traits using the whole phylogeny of Lithospermeae: growth habit (woody/herbaceous), life cycle (annual/perennial), heterostyly (present/absent), stamen cone (present/absent), pollination (vertebrate/insect), and type of substrate where species typically grow (serpentine/other). Character coding for each species with the corresponding references are shown in Table S3.

Using the same morphological matrix, we conducted ancestral character state reconstructions of each of the characters mentioned above, in order to test if any of the characters chosen evolved as a single event that may be associated with a shift in diversification rate. We used a Maximum Likelihood approach for discrete characters as implemented in the R-package APE (Paradis et al., 2004).

2.5. Biogeography analyses

We used the maximum clade credibility (MCC) tree calculated in BEAST to estimate the ancestral geographic ranges in Lithospermeae using the R-package BioGeoBEARS (Matzke, 2013). Analyses were based on the Dispersal Extinction Cladogenesis model (DEC; Ree and Smith, 2008) and its extended version, which includes the jump dispersal parameter (DEC + J) that allows the inference of founder-event speciation (Matzke, 2014). This model is appropriate in the case of Lithospermeae, because several clades in the group appear to have originated after a long-distance dispersal event (Chacón et al., 2017), similarly to other Boraginaceae clades such as Cynoglossoideae and Omphalodeae (Otero et al., 2019a, b). Seven areas were defined, representing the entire distribution range of Lithospermeae, using occurrence data from specimens sequenced in this study, as well as floras and taxonomic revisions by Brand (1930), Johnston (1952, 1953), Ball (1972), Al-Shehbaz (1991), Böhle et al. (1996), Hilger and Böhle

(2000), Boyd (2002, 2004), Cecchi and Selvi (2009), Buys (2011), Cecchi et al. (2011, 2014), Coppi et al. (2015), Weigend et al. (2009, 2016), Luebert et al. (2016), and Cohen (2018). Areas were coded as A: South America, where five species of *Lithospermum* occur. B: North America and Mexico, the main distribution center of *Lithospermum*, with 43 out of 60 species, 32 of them endemic to Mexico. C: South Africa and tropical Africa, with 30 endemic species of *Lobostemon*, five of *Lithospermum*, and three of *Cystostemon*, Macaronesian islands, including the Canary Islands, Madeira and Cape Verde, with 27 endemic species of *Echium*. E: Europe, including the Mediterranean basin and the Balkan region, one of the greatest distribution centers of Lithospermeae with many endemics and widespread species of *Aegonychon*, *Alkanna*, *Arnebia*, *Buglossoides*, *Cerinth*, *Echium*, *Glandora*, *Halacsya*, *Lithodora*, *Lithospermum*, *Mairetis*, *Moltkia*, *Moltkiopsis*, *Neatostema*, *Onosma*, *Paramoltkia*, and *Podonosma*. F: Irano-Turanian region in Western Asia, the second greatest distribution center of Lithospermeae with many endemics of *Alkanna* and *Onosma*. G: Central and Eastern Asia, the center of endemism of *Arnebia* and several species of *Lithospermum* and *Onosma*.

3. Results

3.1. Molecular phylogenies of Lithospermeae based on cpDNA and ITS data

We generated 619 new sequences for this study, mostly from species of *Onosma* (185 new sequences), *Alkanna* (97), *Echium* (89), *Lithospermum* (62), and *Arnebia* (30), analyzed with molecular phylogenetic methods for the first time in this study (see asterisks in Tables S1 & S2). We failed to amplify the aliquot from *Choriantha popoviana* due to the poor quality of the DNA. This sample was originally isolated from the type specimen deposited at K, the only extant herbarium material available to date to the best of our knowledge.

All cpDNA and ITS sequences were clean, meaning that no multiple peaks were observed in any of the chromatograms, thus cloning was not needed. The maximum likelihood trees obtained with the cpDNA and ITS data confirm the monophyly of Lithospermeae with maximal support. Neither data set was able to fully-resolve the relationships within the large genera *Echium*, *Lithospermum* and *Onosma* (Fig. S2-A, B). Seven major clades were retrieved with the plastid dataset (see clade numbering and ML bootstrap values in Fig. 2): Clade I includes *Alkanna* and *Podonosma* forming a monophyletic group with maximum ML bootstrap support (MLB). This clade is sister to the remaining Lithospermeae, in which the six currently recognized species of *Moltkia* form a clade (clade II, 100% MLB) that is sister to the rest of the species. Clade III consists of species belonging to *Cerinth*, *Halacsya*, *Lithodora*, *Mairetis*, *Moltkiopsis*, *Neatostema*, and *Paramoltkia*, which form a well-supported but poorly resolved clade (93% MLB). In clade IV, the genera *Aegonychon*, *Buglossoides*, *Glandora* [non-monophyletic because *G. gastonii* (Benth.) L.Cecchi & Selvi is retrieved as sister to *Aegonychon*], and *Lithospermum* form a well-supported clade (99% MLB), with the latter genus forming a largely unresolved clade (71% MLB). Clade V comprises *Arnebia* [excluding *A. hispidissima* (Sieber ex Lehm.) DC.] with *Stenosolenium* nested in it (94% MLB). *Echiostachys*, *Echium*, *Lobostemon*, *Megacaryon*, and *Pontechium* are grouped in clade VI (100% MLB, here after referred to as “*Echium* clade”), with *Echium* paraphyletic with respect to *Lobostemon* and *Megacaryon*. The remaining species belong to *Cystostemon*, *Maharanga*, and *Onosma*, and form a well-supported clade (clade VII, 96% MLB). Apart from the successively sister relation of clades (I)–(III) to the rest of Lithospermeae, the backbone of the tree receives only moderate to low support (Fig. 2). The ITS phylogeny (Fig. S2-B) shows almost the same clades, but the backbone has weaker support than the cpDNA phylogeny. The position of *Huynhia* remains unresolved in both cpDNA and ITS trees (Fig. S2-A, B). In contrast to the tree reconstructed using cpDNA, the monophyly of *Echium* and *Glandora* is highly supported in the ITS phylogeny. In view of these incongruences, the chloroplast and ITS data sets were not combined.

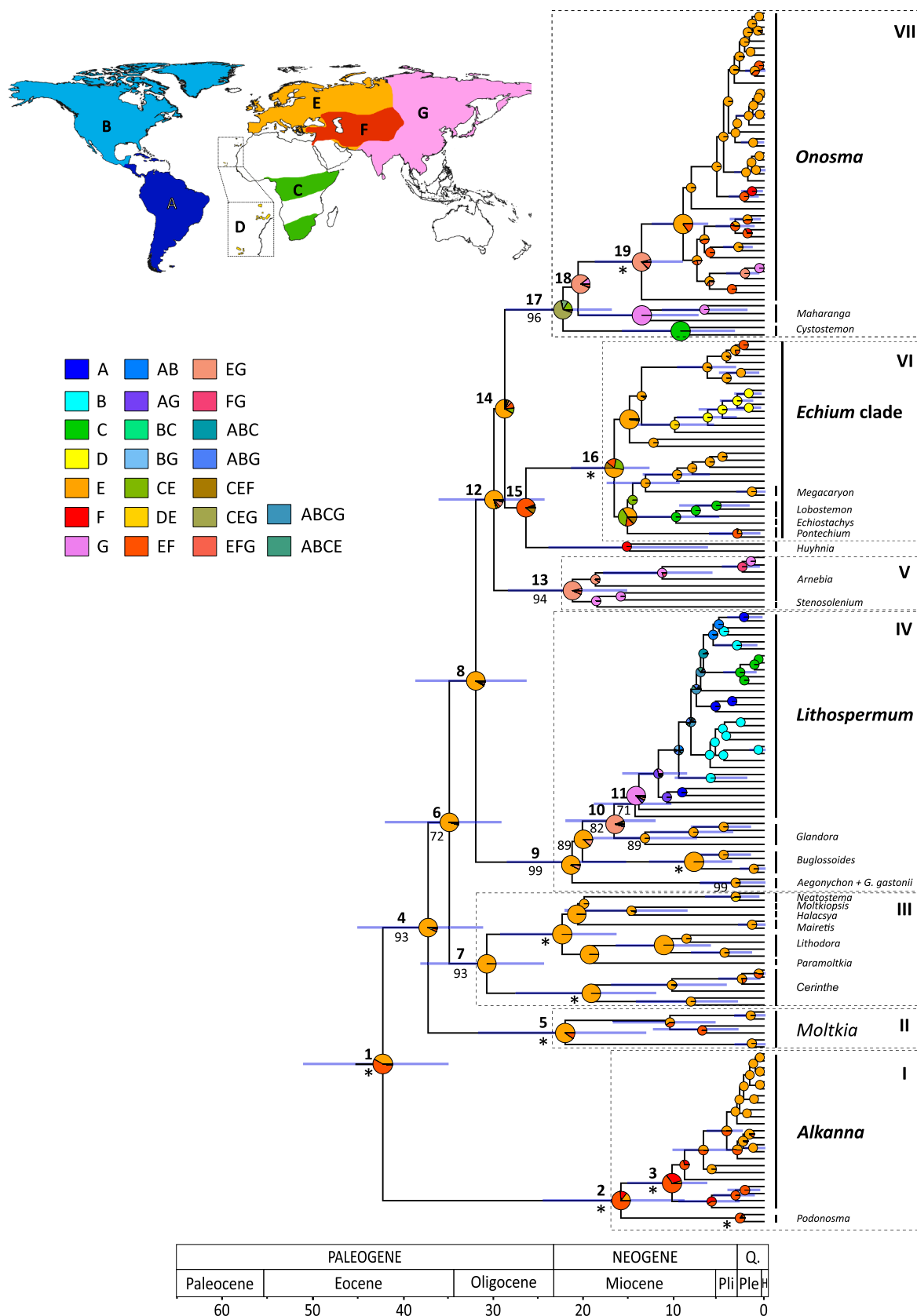


Fig. 2. Dated phylogeny of Lithospermeae based on concatenated chloroplast DNA sequences (*trnL-trnF*, *rps16* and *trnS-trnG*), and ancestral ranges inferred in BioGeoBEARS for the clades of interest (dotted boxes with roman numbers) based on the area coding shown in the map. Only areas reconstructed with $p > 0.5$ are shown in the inset. Pie charts indicate the probability of each reconstruction as in Table 2. Bars at nodes with $> 95\%$ posterior probability indicate the 95% confidence intervals on the estimated times (see complete trees with species names and dates in Figs. S1 & S3, and dates in Table 2). Numbers above branches (bold) are the node numbers as in Table 2. Numbers below branches are Maximum Likelihood bootstrap support (MLB) values of clades. Only values $> 70\%$ are shown. Asterisks indicate maximally supported nodes (100% MLB). The *Echium* clade refers to the monophylum formed by *Echiostachys*, *Echium*, *Lobostemon*, *Megacaryon* and *Pontechium*.

Table 2

Node ages (million years ago, Ma) and ancestral ranges inferred with $p > 0.5$ for the clades of interest, with 95% highest posterior density intervals (HPD) indicated in parenthesis. Nodes numbers are indicated in Fig. 2. Area codes are as follows: A: South America, B: North America, C: South Africa and Tropical Africa, D: Macaronesia, E: Western Europe and Mediterranean basin, F: Irano-Turanian region, G: Central and Eastern Asia.

Node	Clade	Age (95% HPD)	Ancestral range
1	Crown Lithospermeae	42.5 (35.3–51.5)	EF (0.55), E (0.42)
2	Crown clade I	16.0 (9.0–24.8)	EF (0.73), E (0.15) F (0.12)
3	Crown <i>Alkanna</i>	10.3 (6.5–15.4)	EF (0.65), F (0.31)
4	Stem clade II	37.5 (31.4–45.5)	E (0.92), EF (0.04)
5	Crown clade II	22.2 (13.3–32.0)	E (0.90), EF (0.10)
6	Stem clade III	35.1 (29.4–42.4)	E (0.96)
7	Crown clade III	30.9 (24.7–38.4)	E (1.0)
8	Stem clade IV	32.1 (26.6–39.0)	E (0.91)
9	Crown clade IV	21.4 (15.5–28.8)	E (0.92)
10	Stem <i>Lithospermum</i>	16.8 (12.2–22.3)	EG (0.89)
11	Crown <i>Lithospermum</i>	14.4 (10.5–19.1)	G (0.86)
12	Stem clade V	30.1 (24.6–36.4)	E (0.79)
13	Crown clade V	21.4 (15.4–28.7)	EG (0.89)
14	Stem clade VII	28.9*	E (0.67)
15	Stem clade VI (stem <i>Echium</i> clade [†])	26.6*	EF (0.84), CEF (0.1)
16	Crown clade VI (crown <i>Echium</i> clade)	16.7 (12.9–21.7)	E (0.47), CE (0.24)
17	Crown clade VII	22.4 (17.1–29.0)	CEG (0.66), CE (0.2)
18	Stem <i>Onosma</i>	20.8*	EG (0.82), G (0.11)
19	Crown <i>Onosma</i> (= crown clade VII excl. <i>Maharanha</i> + <i>Cystostemon</i>)	13.7 (9.2–19.0)	EG (0.82), EFG (0.12)

* Node ages below the 95% HPD.

[†] The *Echium* clade includes *Echiostachys*, *Echium*, *Lobostemon*, *Megacaryon* and *Pontechium*.

Ancistrocarya, absent in the plastid dataset, was recovered within a maximally supported clade together with *Lithospermum*, *Buglossoides*, *Aegonychon*, and *Glandora* (Fig. S2-B). *Onosma* is not monophyletic with respect to *Maharanga* (both in the ITS and cp trees; Fig. S2-A, B), due to the position of the Chinese species *O. waltoni* Duthie, *O. hookeri* C.B. Clarke, *O. sinicum* Diels, and *O. pyramidalis* Hook.f., which are retrieved on a sister clade to the remainder of *Onosma* together with *Maharanga emodi* (Wall.) A.DC. and *M. bicolor* (Wall. ex G. Don) A. DC.

3.2. Age estimates and ancestral ranges of the main subclades of *Lithospermeae*

Divergence times and ancestral range estimates for the major clades are summarized in Table 2 and Figs. 2 & S3. Effective Sample Sizes (ESS) were verified in Tracer 1.7.1 (Rambaut et al., 2018) and were all above 200. Our data suggest that *Lithospermeae* originated during the early Eocene (ca. 56 Ma) and started to diversify during the middle Eocene (ca. 42.5 Ma). The ancestral range inferred at the crown node of *Lithospermeae* comprises Europe, the Mediterranean and the Irano-Turanian regions (node 1 in Table 2). Initial diversification of clade I took place in the same area about 16.0 Ma (node 2). Crown node ages of clades II, III and IV were dated to ca. 22.2 Ma, 30.9 Ma, and 32.1 Ma (nodes 5, 7 & 9), respectively, with ancestral geographic ranges inferred as Europe and the Mediterranean basin for these three nodes (i.e., ancestral range E). Initial diversification of clade V was dated to 21.4 Ma within a large ancestral range comprising Europe, the Mediterranean, C and E Asia (node 13, EG). The crown node age of clade VII was dated to ca. 22.4 Ma with an estimated ancestral range extending from Eurasia and the Mediterranean to tropical Africa and South Africa (node 17, CEG). All four most diverse genera of *Lithospermeae* diversified in the Miocene: *Alkanna* (ca. 10.3 Ma), *Echium* and allies (ca. 16.7 Ma; node 16), *Lithospermum* (ca. 14.4 Ma; node 11), and *Onosma* (ca. 13.7 Ma; node 19). Especially the Mediterranean groups *Alkanna* and *Onosma* show considerable diversification during the Pleistocene (Fig. 2). Further dispersals of *Lithospermum* into North America, South America and Africa, and *Echium* into Macaronesia, took place within the last ca. 11 Ma, although these age estimates are poorly supported and fall below the 95% high posterior density interval (Fig. S3).

3.3. Diversification rates and inferred shifts in diversification rate in *Lithospermeae*

Diversification rates estimated in GEIGER were highest in the four highly diverse genera *Alkanna*, the *Echium* clade, *Lithospermum*, and *Onosma*. However, only diversification rates in *Onosma* were significantly higher than expected (Fig. 3). All BMM runs were convergent after 5 million generations with a 10% burnin, with the ESS > 200 on both the number of shifts and the log-likelihood. Results of the BMM analyses indicated four shifts in diversification rates, one in each of these highly diverse clades (Fig. 4): at the stem branch of *Alkanna*; at the stem branch of *Lithospermum* excluding *L. bolivariensis* Weigend & Nürk, *L. rodriguezii* Weigend & Nürk, *L. erythrorhizon* Siebold & Zucc., and *L. tschimganicum* B.Fedtsch.; at the stem branch of the *Echium* clade (i.e., *Echiostachys* + *Echium* + *Lobostemon* + *Megacaryon* + *Pontechium*); at the stem branch of *Onosma* excl. *O. barszewskii* Lipsky. These results did not change with different priors on the expected number of shifts (Fig. S4). We found no significant association between any individual ecological and morphological trait and the shifts in diversification rates according to the STRAPP analysis (Table 3).

Ancestral character state reconstructions did not suggest any associations with the identified shifts in diversification rates in spite of the fact that all characters evolved several times on the phylogeny of *Lithospermeae* (Fig S5). Herbaceous habit is the ancestral condition in the tribe with switches to woody habit only occurring in a few species of *Buglossoides*, *Cystostemon*, *Echium*, *Glandora*, *Onosma*, *Lithodora*, *Lithospermum*, *Lobostemon*, *Moltkia*, as well as in *Halacsya sendtneri* Dörf. and *Moltkiopsis ciliata* (Forssk.) I.M. Johnst. Concerning the life cycle, the ancestral state in *Lithospermeae* is the perennial habit and only in few species of *Alkanna*, *Arnebia*, *Buglossoides*, *Cerinth*, and *Echium* did the annual life cycle evolve. Heterostylous flowers evolved independently in the most recent common ancestor of *Arnebia*, in some species of *Glandora* and *Lithodora*, and in *Huynhia pulchra* (Willd. ex Roem. & Schult.) Greuter & Burdet. The absence of a stamen cone is the ancestral condition of the tribe and this character arose independently in the most recent common ancestor of *Cerinth* and the *Onosma* + *Cystostemon* + *Maharanga* clade, as well as in *Lithospermum macromeria* J.I. Cohen and *Podonosma orientalis* (L.) Feinbrun. Insect

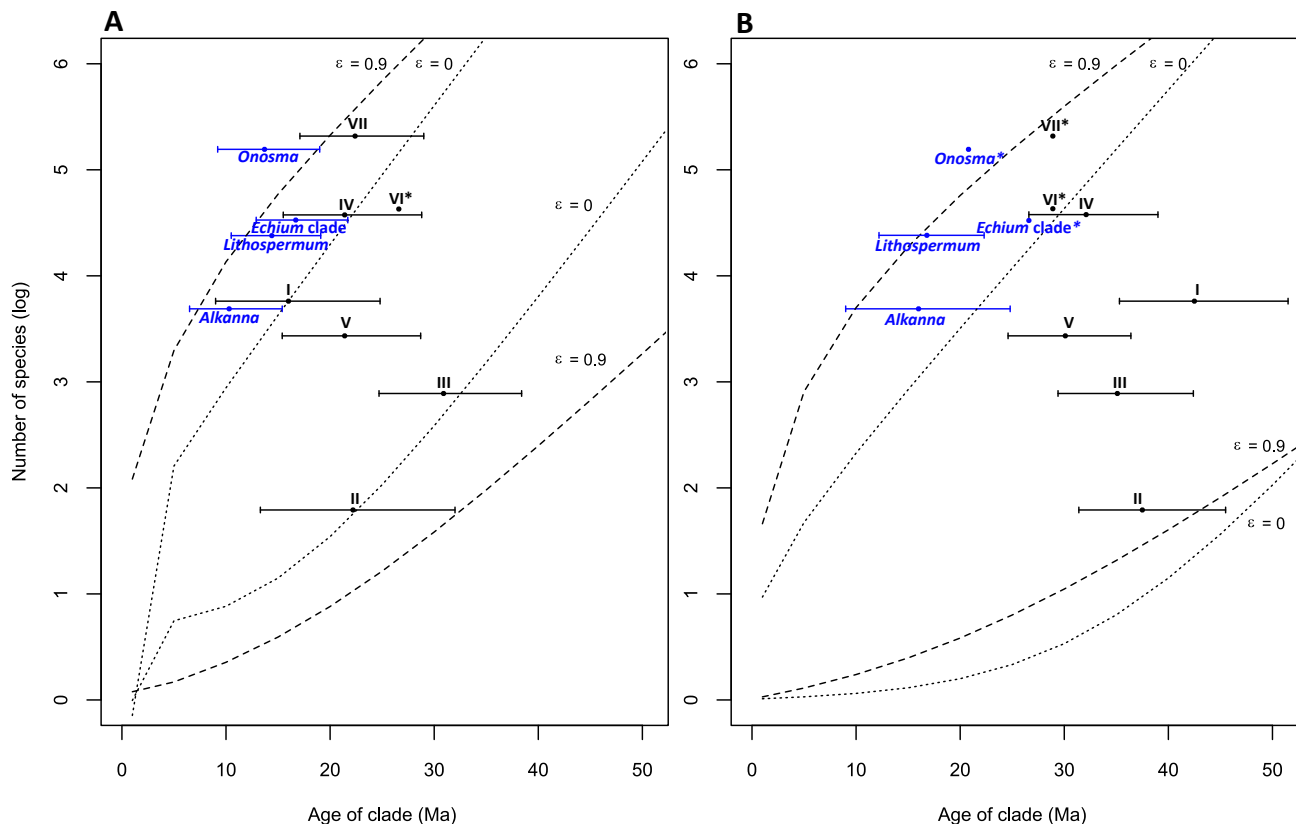


Fig. 3. Lithospermeae species diversity based on crown (A) and stem node ages (B) based on the chloroplast dated phylogeny. Confidence intervals indicate 95% high posterior density limits of age estimates. Dotted lines are the background diversification of Lithospermeae rate under two extreme values of the extinction/speciation ratio (ϵ); $\epsilon = 0$ and $\epsilon = 0.9$ (see Magallón and Sanderson, 2001). Age-estimates below the 95% HPD are indicated with an asterisk. Clades are number as in Fig. 2. Species-rich genera of interest are shown in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pollination is the ancestral and most widespread condition in Lithospermeae, with vertebrate pollination only evolving in *Lobostemon belliformis* Buys and in a few species of *Lithospermum*. Finally, the ancestral state for the substrate preference in the tribe was inferred as other than serpentine, with serpentinophytes only evolving in some *Alkanna*, *Aegonychon*, *Onosma*, and *Echium* species, as well as in the monotypic genera *Halacsya*, *Neatostema*, *Paramoltkia*, and *Pontechium*. According to the ancestral range estimations obtained with BioGeoBEARS (Table 2 & Fig. 2), these shifts in diversification rates took place in the Mediterranean basin and the Irano-Turanian region (*Alkanna*), Europe, and the Mediterranean basin (*Onosma* and *Echium* clade), as well as during the colonization of North America from Central-Eastern Asia (*Lithospermum*), respectively.

Speciation rates estimated in BAMM for *Onosma*, *Lithospermum*, *Echium*, and *Alkanna* using the whole tree of Lithospermeae differed from the rates estimated for each clade separately, in 6.5%, 14.2%, 15.3%, and 12.7%, respectively. In both cases, we obtained the highest rates for *Onosma* followed by *Lithospermum*, *Echium* and *Alkanna*, and the 95% confidence intervals for the estimated rates were fully overlapping in each clade for both types of analyses (Table S4).

4. Discussion

4.1. Molecular phylogeny, incongruences between cpDNA and ITS trees, and novel phylogenetic placements in Lithospermeae

In this study we provide the most comprehensive cpDNA and ITS phylogenies of Lithospermeae to date (Fig. S2-A, B), including all currently recognized genera and about half of the species from across their range. The only genus not included in the phylogeny is *Choriantha*

Riedl., which we failed to amplify and sequence. According to Riedl (1961) this taxon may be closely related to the Chinese members of *Onosma* based on its peculiar growth habit. Most genera were shown to be monophyletic and their phylogenetic relationships are in agreement with previous studies that are not discussed here. We found conflicting results between the cpDNA and the ITS data sets concerning *Arnebia*, *Echium*, and *Glandora*. Incongruence, however, was significantly supported only in the case of *Echium*, which is retrieved as a grade together with *Lobostemon*, *Echiostachys*, and *Megacaryon* in the cpDNA tree (Figs. 2 & S2-A). A similar result was obtained by Selvi et al. (2017), in which *Echium* and *Lobostemon* were also retrieved as non-monophyletic (i.e., *Echiostachys* embedded within *Lobostemon* in the chloroplast tree, and *Pontechium* and *Lobostemon* embedded in *Echium* in the ITS tree). Johnston (1924, 1953) originally proposed a wider circumscription of *Echium* (i.e., including *Echiostachys*, *Lobostemon*, and *Megacaryon*) and suggested that *Lobostemon* descended from “ancestors of modern *Echium* which became isolated in South Africa” (Johnston, 1953: page 285), perhaps different from those that became isolated in Macaronesia and gave rise to the island radiation. This author argued that Mediterranean *Echium* share very similar floral morphologies and woody habit with *Lobostemon*. Later on, Hilger and Böhle (2000) proposed a broader circumscription of *Echium*, including *Lobostemon* and *Echiostachys*, based on a combined analysis of the *trnL-F*_{UAA} region and ITS1. Conversely, Selvi et al. (2017) re-instated the genus *Megacaryon* based on morphological, karyological, and molecular data (i.e., *trnL-F* and ITS). Here, we found that only the nuclear ribosomal ITS supports the segregation of the genera above (Fig. S2-A, B), suggesting that this incongruence between cpDNA and ITS trees may have resulted from introgression/ancient hybridization or undetected paralogous copies in the most recent common ancestor of the *Echium* clade.

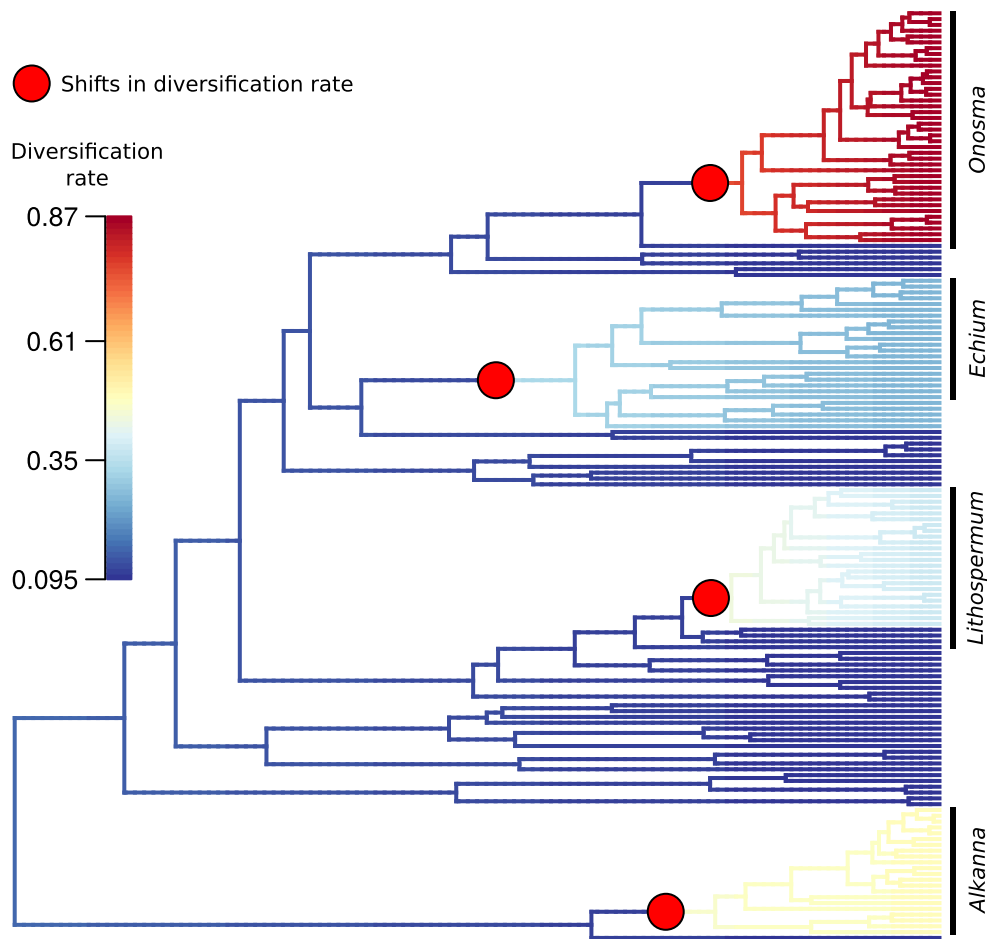


Fig. 4. Maximum credible rate shift set of Lithospermeae (red circles) according to the BAMM analysis (Rabosky, 2014) with a prior on the expected number of shifts equals to one ($f = 0.089$). Clades in which a shift in diversification rate occurred are indicated next to the corresponding stem branch. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3

Results of the Structured Rate Permutations on Phylogenies (STRAPP; Rabosky and Huang, 2016) analyses for each of the six traits analyzed (first column, see methods for details). *P*-values of the Mann-Whitney *U* test are indicated for net diversification, speciation and extinction rates with respect to each of the traits studied. Any of the STRAPP tests resulted in a statistically significant association between traits and rates.

Trait	Net diversification	Speciation	Extinction
Habit	0.56	0.40	0.44
Life cycle	0.18	0.14	0.29
Heterostyly	0.25	0.18	0.39
Stamen cone	0.38	0.40	0.49
Pollination	0.83	0.88	0.65
Soil type	0.45	0.35	0.36

Onosma (Fig. S2-A, B) is clearly paraphyletic since some Eastern Asian taxa (e.g., *O. waltoni*, *O. hookeri*, *O. sinicum*, and *O. pyramidalis*) are more closely allied to *Maharanga* than to the remainder of *Onosma*. The non-monophyly of *Onosma* and the position of *Maharanga* embedded within this genus corroborates earlier findings by Nasrollahi et al. (2019). In that study, however, a direct comparison of the topology and the ages inferred was precluded by the lack of species of the closely related genus *Cystostemon*. We were also able to provide a first indication about the relationships of the rare Japanese endemic *Ancistrocarya japonica* Maxim., retrieving it within a clade with *Lithospermum*, *Buglossoides*, *Aegonychon*, and *Glandora* (Fig. S2-B).

4.2. Shifts in diversification rates inferred for the largest clades of Lithospermeae cannot be shown to correlate with specific morphological and ecological traits

Rapid diversifications or shifts in diversification rates have been associated with the appearance of specific morphological traits, or adaptive types, purportedly triggering adaptive radiation (Simpson, 1953). These traits have been termed “key innovations” (Schluter, 2000). Overall, Lithospermeae show a range of particular traits that could be interpreted as potential “key innovations”, such as fruticose habit, bird pollination, and heterostyly (increasing outcrossing) in *Lithospermum* (e.g., Thomas et al., 2008; Cohen, 2011, 2012, 2016a), and edaphic adaptations in *Onosma* (e.g., Cecchi et al., 2011). Our results, however, are unable to confirm a direct connection between the appearances of any trait considered and increased diversification rates. In other words, the phylogeny does not show replicated associations between single traits and shifts in diversification rates. Three possibilities may still be considered following this result: (1) the association between these traits and shifts in diversification rates are single historical events not captured by the STRAPP analyses; (2) the shifts in diversification rates have been triggered either by unknown interactions among the traits studied, or (3) by other factors not considered in this study. The results of the ancestral character state reconstructions rule out the first possibility, pointing to unknown trait interactions as a more plausible cause for the rate shifts in *Alkanna*, the *Echium* clade, *Lithospermum*, and *Onosma*. Such interactions may have increased divergence and reproductive isolation, especially in floral traits (e.g., buzz-pollination in *Onosma*, zygomorphy in *Echium*, heterostyly in

Lithospermum) very similar to what has been inferred for Cactaceae (Hernández-Hernández et al., 2014) or Boraginaceae subfam. Cynoglossoidae (Otero et al., 2019a).

4.3. Relevant Eocene-Miocene climatic and geologic events parallel the diversification of *Lithospermeae*

The main diversification events in *Lithospermeae* were dated to the Oligocene and the early Miocene, ca. 30–16 Ma in Western Eurasia (i.e., Europe incl. the Mediterranean and Irano-Turanian regions; Figs. 2 & S3; Table 2). Paleomagnetic and magneto-stratigraphic data indicate that the central Tibetan Plateau was uplifted by ca. 40 Ma, changing the atmospheric circulation above the Tethys Ocean, with profound effects on the paleoclimate of Eurasia and its vegetation (Wang et al., 2008; Lippert et al., 2014). This period coincided with shifts in global vegetation cover due to global cooling (Zachos et al., 2001). All extant lineages of *Lithospermeae* are mostly found in open, semi-arid habitats, differentiating them strongly from their predominantly mesic sister clade Boragineae. The origin and present-day diversity of *Alkanna*, *Echium*, and *Onosma* in the Mediterranean basin were likely affected by tectonic events that took place there during the Middle Miocene, which modified local climates and atmospheric circulation. These Miocene tectonic events were related to increased volcanism due to plate subduction and extension in the Northern African coast and the formation of the Alboran Sea between the Iberian Peninsula and the north of Africa (Rosenbaum et al., 2002). Although the shift in diversification rates in the *Echium* clade (ca. 26–17 Ma; Fig. 2 and Table 2) predate these geological events, the middle Miocene colonization of South Africa (*Lobostemon*) and Macaronesia (*Echium*) likely also contributed to the overall diversification of that clade (Figs. 3, 4 & S4; Böhle et al., 1996; Mansion et al., 2009). The shift in diversification rates in *Lithospermum* appears to coincide with the colonization of North America during the middle Miocene (Chacón et al., 2017). *Lithospermum* is most diverse in the arid southwestern part of North America (Cohen, 2018), a region that has been subject to considerable aridisation since the middle Miocene (Axelrod, 1979). The inferred Miocene dispersals from Europe into North America paralleled those inferred in other northern hemisphere Lamiid clades (Drew and Sytsma, 2012; Chacón et al., 2017; Otero et al., 2019a), which also took place during periods of global cooling and aridisation. On a more recent timescale, the Mediterranean groups of *Onosma* and *Alkanna* showed considerable Pleistocene diversification (Fig. 2), similar to other Mediterranean plant groups (e.g., Mansion et al., 2009; Chen et al., 2014; Salvo et al., 2010), in which range expansions and contractions, and subsequent isolation during the glacial-interglacial cycles likely played a significant role.

Absolute diversification rates estimated for the main clades shown in Fig. 2 underscore that *Onosma* is the most diverse genus in the tribe and the only one with significantly increased diversification rates (Fig. 3 & Table S4). The bulk of the diversification in this genus dates back to the end of the Miocene, ca. 13.7–9.1 Ma (Table 2; Figs. 4 & S4), and is thus older than previously suggested (i.e., 6–5 Ma, see Cecchi et al., 2011). Our age estimates indicate that diversification in *Onosma* predates the latest Miocene aridisation of the Mediterranean, also known as the Messinian salinity crisis (ca. 5.96 Ma, Manzi et al., 2013), triggered by glacial conditions in the northern hemisphere and aridity in Northern Africa (Fauquette et al., 2006). Most *Onosma* species grow in semi-arid regions in montane habitats, often on serpentine soils (Cecchi et al., 2011; Wójcik et al., 2017). Hughes et al. (2001) showed that drought tolerance has been an important adaptation during the evolution of serpentine tolerance in some plants, helping to explain the multiple colonization events of serpentine soils by *Onosma*. Aridity also promotes habitat openness, which has been identified as a precursor to soil specialization in other groups such as the *Streptanthus* clade of Brassicaceae (Cacho and Strauss, 2014). *Lithospermeae*, across all clades, are most diverse and most abundant on mineral – mostly calcareous, sandy or ultramafic – soils in open, at least seasonally arid

habitats. They are adapted to these habitats by a range of vegetative traits, such as a dense, heavily mineralized indument, microphyllly, fruticulose habit, and the possession of deep tap-roots. These adaptations to arid habitats likely played a key role in permitting the diversification of *Lithospermeae*, with the increasing availability of semi-arid, open habitats in the Mid-Miocene across much of their range.

5. Conclusions

The molecular phylogeny of *Lithospermeae* is now well resolved and the overall evolution of the group is well understood. Neither *Echium* nor *Onosma* are retrieved as monophyletic in their current circumscription. The circumscription of *Onosma* needs to be adjusted, either with the transfer of several Eastern Asian and Himalayan taxa into *Maharanga*, or an expansion of *Onosma* to include *Maharanga*. The situation in *Echium* is more complex and the phylogenetic relationships among *Echium*, *Echiostachys*, *Lobostemon*, and *Megacaryon* remain unresolved.

The available evidence does not allow for an association of any particular morphological or ecological trait in *Lithospermeae* with shifts in diversification rates. This is probably partly explained by the fact that characters such as edaphic preferences and heterostyly have been shown to be evolutionarily labile in previous studies, especially in *Onosma* and *Lithospermum*. Due to lack of data, we have been unable to investigate the possible role of morphological and physiological adaptations to open, semi-arid habitats, and mineral soils in the diversification of *Lithospermeae*, but their present day patterns of diversity suggest that these adaptations must have played a crucial role in the Miocene diversification of the tribe. A denser sampling of individual subclades, coupled with detailed functional analyses are necessary to identify the contribution of adaptations to aridity conditions and pollinator shifts in the diversification of *Lithospermeae*.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ympcv.2019.106626>.

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