

ORIGINAL ARTICLE

Both temperature fluctuations and East Asian monsoons have driven plant diversification in the karst ecosystems from southern China

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Abstract

Karst ecosystems in southern China are species-rich and have high levels of endemism, yet little is known regarding the evolutionary processes responsible for the origin and diversification of karst biodiversity. The genus *Primulina* (Gesneriaceae) comprises ca. 170 species endemic to southern China with high levels of ecological (edaphic) specialization, providing an exceptional model to study the plant diversification in karsts. We used molecular data from nine chloroplast and 11 nuclear regions and macroevolutionary analyses to assess the origin and cause of species diversification due to palaeoenvironmental changes and edaphic specialization in *Primulina*. We found that speciation was positively associated with changes in past temperatures and East Asian monsoons through the evolutionary history of *Primulina*. Climatic change around the mid-Miocene triggered an early burst followed by a slowdown of diversification rate towards the present with the climate cooling. We detected different speciation rates among edaphic types, and transitions among soil types were infrequently and did not impact the overall speciation rate. Our findings suggest that both global temperature changes and East Asian monsoons have played crucial roles in floristic diversification within the karst ecosystems in southern China, such that speciation was higher when climate was warmer and wetter. This is the first study to directly demonstrate that past monsoon activity is positively correlated with speciation rate in East Asia. This case study could motivate further investigations to assess the impacts of past environmental changes on the origin and diversification of biodiversity in global karst ecosystems, most of which are under threat.

KEYWORDS

biodiversity hotspots, climate change, diversification analyses, East Asian monsoons, edaphic specialization, *Primulina*

1 | INTRODUCTION

Karst ecosystems are ecosystems exemplified by a variety of closed surface depressions, underground drainage and surface stream, which are unique landforms with a long geological history formed by

the dissolution of limestones and other soft rocks, from the early Cambrian to the late Mesozoic (Ford & Williams, 2007; Li & Cao, 2015). In total, they represent about 10% of the Earth's land surface and as much as a quarter of the global population depends upon water supplied from karst aquifers (Ford & Williams, 2007;

Hartmann, Goldscheider, Wagener, Lange, & Weiler, 2014). However, many karst environments are under significant threats (Oliver et al., 2017), particularly due to anthropogenic activity and elevated human population density. Karsts are receiving high research priority to understand the evolutionary processes responsible for the origin and diversification of their exceptional biodiversity (Cai, Tian, Xiao, Peng, & Liu, 2015; Chung et al., 2014; Grismer et al., 2016; Oliver et al., 2017).

With more than 800,000 km², the karst landforms of tropical and subtropical Southeast Asia (including southern China) represent an unrivalled biodiversity-rich landscape and have long been regarded as “natural laboratories” for ecological and evolutionary studies (Clements, Sodhi, Schilthuizen, & Ng, 2006; Oliver et al., 2017). In particular, the karst area in southern China and northern Vietnam, situated at the interface between two global biodiversity hotspots, Indo-Burma and the Mountains of Southwest China, is renowned for its remarkable biodiversity and high levels of endemism (Zhu, 2007). Karst areas in southern China are characterized by high edaphic heterogeneity, with contrasting local-scale mosaics of soil types derived from bedrock of differing lithology (Hao, Kuang, & Kang, 2015). This edaphic complexity may also be a strong driver of speciation via habitat specialization (local adaptations) to edaphic microhabitats. Compared with the substantial progress made in other global biodiversity hotspots (Baldwin, 2014; Cook, Hardy, & Crisp, 2015; Lagomarsino, Condamine, Antonelli, Mulch, & Davis, 2016) and southwest China (Wen, Zhang, Nie, Zhong, & Sun, 2014; Xing & Ree, 2017), the evolutionary mechanisms for generating the exceptional species diversity and endemism in the karst areas of southern China have received little attention. One important issue relevant to understanding the high endemism in karst ecosystems is the role of past climate events that occurred between the early and mid-Miocene (23–11.6 Ma) associated with the onset of the East Asian monsoons (20–15 Ma) (Guo et al., 2008; Sun & Wang, 2005).

Climatic change has varying effects on biodiversity patterns and processes (Erwin, 2009; Janssen & Davies, 2008). For instance, warming or cooling events can impact speciation and extinction rates (Condamine, Rolland, & Morlon, 2013), which can in turn affect the dynamic of species richness within biological groups. However, compared to the numerous studies of Pleistocene climatic fluctuations on speciation, the impact of more ancient environmental changes is poorly understood. Yet, ancient climate change may have strongly influenced evolutionary processes (Erwin, 2009), such as during the middle and late Miocene when global temperatures and precipitation regimes underwent remarkable transitions (Zachos, Pagani, Sloan, Thomas, & Billups, 2001). Climate change has been hypothesized as a major driver of early and rapid radiation in numerous plant lineages (Bengtson, Nylinder, Karis, & Anderberg, 2015; Goldblatt & Manning, 2002). These studies proposed that the establishment of winter rainfall/summer arid conditions at the end of the Miocene has resulted in the opening of new niches favourable for species radiation (Goldblatt & Manning, 2002). These studies thus demonstrate the need to test for long-term associations between climate change and speciation rates, as well as other factors to determine the relative

contribution of different factors. However, no study so far has attempted to directly test the correlation between climate variables and speciation rate through time for any plant lineages in southern China and adjacent regions.

Another putative factor of speciation in the karst ecosystems from southern China is the onset and evolution of the East Asian monsoons since the mid-Miocene in South and East Asia (Li et al., 2007). The East Asian monsoon is considered to be one of the principal causes of environmental and biological changes in East Asia (An, 2000). The formation of the East Asian monsoon system is traditionally ascribed to the uplift of the Himalayas and the Tibetan Plateau (An, Kutzbach, Prell, & Porter, 2001; Guo et al., 2008; Li et al., 2007); however, recent studies argued that global cooling played a more important role in the evolution of the East Asian monsoons (Lu, Wang, & Li, 2010; Miao, Herrmann, Wu, Yan, & Yang, 2012; Miao et al., 2016; Passey et al., 2009). Although the Asian monsoons have been suggested as a possible driver for species diversification in East Asia (Chung et al., 2014; Guo et al., 2016; Wang, Schneider, Zhang, & Xiang, 2012), the association between Asian monsoon activity and diversification dynamics has never been explored. Recently, Clift, Wan, and Blusztajn (2014) used the sediments recovered at Ocean Drilling Program (ODP) Sites 1146 and 1148 in the northern South China Sea to reconstruct the monsoon activity in South and East Asia since 25 Ma in the Oligocene, and this allows direct testing of the correlation between the diversification rate and the East Asian monsoons.

Among the plants distributed within the karsts of southern China and northern Vietnam, the genus *Primulina* (Gesneriaceae) represents an exceptional model system for addressing the roles of environmental change on diversification processes. *Primulina* contains about 170 described species that comprise a monophyletic group of perennials with a stem age of ca. 16 Ma (Gao, Ai, Kong, Kang, & Huang, 2015), and it is one of the most diverse plant genera associated with karst landscapes. Most species are micro-endemics with narrow, island distributions, often limited to a single cave or karst limestone hill system. This is because karst landforms in this region are generally scattered and isolated as small limestone hills and *Primulina* has poor dispersal capacities (Hao et al., 2015; Kang et al., 2014). Moreover, the genus displays a high degree of edaphic habitat specialization, with the majority of species occurring in calcareous soils originated from limestone bedrock (i.e., calciphiles, Hao et al., 2015). However, the mechanism of ancient environmental changes, including the palaeotemperature and the strength of East Asian monsoons, generating the geographical distribution pattern as well as the great species diversity and endemism of *Primulina* in the karst ecosystems from southern China remains unclear.

In this study, we investigate the evolutionary history of a species-rich genus occurring on the karst landforms in southern China and address the causes of species diversification. We generated a dated molecular phylogeny with nearly complete taxon sampling using 20 plastid and nuclear regions. We applied phylogenetic and macroevolutionary analyses as well as palaeoenvironmental-dependent models to test the contributions of temperature changes,

fluctuations in the strength of East Asian monsoons, and edaphic specialization in shaping the evolutionary radiation of the genus *Primulina*. Relying on current knowledge of the species geographical distributions, palaeoenvironmental records, and edaphic specialization of species, we tested several diversification scenarios and evolutionary hypotheses related to the drivers of diversity dynamics in the karst ecosystems.

2 | MATERIALS AND METHODS

2.1 | Taxon sampling

The samples used in this study were collected throughout the entire geographical range of *Primulina* in China and adjacent regions during our field investigations from 2011 to 2015. These samples represented 199 populations of 159 described *Primulina* species, as well as 31 undescribed species based on preliminary morphological characters (coded as *Primulina* sp. nov. 1–31) (Table S1). Geographical coordinates were recorded using a Garmin-eTrex GPS instrument. We associated species of *Primulina* with the soil types in which they occur using our previously published data from field studies (Hao et al., 2015), the monograph of Gesneriaceae in South China (Wei et al., 2010) and herbarium records (Table S1). In each population, leaf samples were taken from randomly selected plants and rapidly dried with silica gel in sealed plastic bags prior to DNA extraction. We used two species of *Petrocodon*: *P. hancei* (Hemsl.) A.Weber & Mich. Möller and *P. dealbatus* Hance, the sister genus of *Primulina* (Möller, Forrest, Wei, & Weber, 2011; Weber et al., 2011) as outgroups in all analyses.

2.2 | DNA extraction, PCR amplification and sequencing

We extracted total genomic DNA from up to 60 mg of dried leaves using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle, 1987). We used the DNA to amplify nine noncoding regions of chloroplast DNA (*atpB-rbcL*, *rpl32-trnL*, *trnL-trnF*, *rpoB-trnC*, *trnC-petN*, *ndhA_intron*, *ndhH-rps15-ycf1*, *ycf1_1* and *ycf1_2*) and the nuclear ribosomal internal transcribed spacer (ITS) as described in Kang et al. (2014) and Gao et al. (2015). We amplified additional single-copy nuclear markers with primers designed in PRIMER 3.0 (Rozen & Skaletsky, 2000) using transcriptome resources of *Primulina* (Ai et al., 2015). After the preliminary screen for PCR amplification and polymorphism, we selected 10 single-copy nuclear genes (7FR, 13FR, 97FR, 117FR, 155FR, 165FR, 166FR, 248FR, 302FR and 383FR) for the phylogenetic analysis in this study. In total, we sequenced 20 regions (nine chloroplast and 11 nuclear). We have significantly increased both the sampling of molecular markers and accessions compared to previous studies (Gao et al., 2015; Kang et al., 2014), which showed that the phylogeny of *Primulina* was unresolved with few molecular markers and the level of molecular diversity within species was low. The amplified products from the cpDNA regions and most of the nuclear regions were sequenced directly. However, direct sequencing revealed some accessions with

polymorphic reads for the single-copy nuclear genes, so we obtained sequences for these by cloning with JM109 cells using the pGEM-T Vector System (Promega, Madison, WI, USA) following the manufacturer's protocol. We amplified six to eight colonies using PCR and sequenced the products using M13 forward and reverse primers. The DNA sequences generated for this study were deposited in GenBank (accession numbers in Table S2).

2.3 | Gene alignment, molecular partitioning and phylogenetic analyses

We used a set of 20 molecular sequence regions, including almost all described species in *Primulina* and aligned gene matrices of the DNA sequences in CLUSTAL X (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997). We created a concatenated molecular matrix with the individual alignments of the 20 markers for 199 samples representing 159 species (including varieties and 31 undescribed species) and manually defined 10 alternative partitioning schemes based on the genomic origin and function of the markers (Table S3). We compared the 10 partitioning strategies and selected corresponding nucleotide substitution models under the Bayesian information criterion implemented in PARTITIONFINDER 1.1.1 (Lanfear, Calcott, Ho, & Guindon, 2012).

We performed Bayesian inference (BI) of phylogenies with the best partition strategy using MRBAYES 3.2.6 (Ronquist et al., 2012) on the high-performance computer cluster available in the CIPRES Science Gateway 3.3 (www.phylo.org; Miller et al., 2015). We unlinked models across the data partitions so each partition had independently estimated parameters, and we ran four Markov chain Monte Carlo (MCMC) for 80 million generations using default settings for priors and saved one tree every 1,000 generations. We excluded the first 25% of posterior trees as burn-in. We also conducted maximum-likelihood (ML) analyses using RAXML 7.2.8 (Stamatakis, 2006). We evaluated the ML tree using nonparametric bootstrapping (1,000 replications) with the thorough bootstrap option of RAXML under the optimal partition schemes by PARTITIONFINDER and the general time-reversible (GTR) model with a gamma model (Γ) of the rate of heterogeneity.

Prior to the species-tree analysis, we used MRMODELTEST 3.7 (Posada & Crandall, 1998), which is incorporated in PAUP* 4.0b10 (Swofford, 2002), to select the best-fit nucleotide substitution model for each sequenced region according to the corrected Akaike information criterion (AICc). We input the 1,000 bootstrap-resampled data sets to ASTRAL-II (Mirarab & Warnow, 2015), which is a summary-based species-tree inference program and reasonably robust to the effects of incorrectly estimated gene trees.

2.4 | Test for incongruence between chloroplast and nuclear DNA

We first visualized the amount of incongruence among phylogenetic trees resolved according to each of the 20 molecular markers. Thus, we compared trees according to their shared clades. To accomplish this, we generated a tree by clade matrix, $m \times n$, where m contains

each tree and n contains all clades present across all trees. This is similar to the matrix representation by parsimony (MRP) matrices used for building super trees and originally described by Baum (1992) and Ragan (1992), and also similar to other node-based methods of measuring congruence (Larget, Kotha, Dewey, & Ané, 2010; Puigbò, Garcia-Vallvé, & McInerney, 2007). We defined a clade as a monophyletic group possessing at least two terminals and excluded stand-alone terminals. We generated the tree by clade matrix using Python and Biopython and provide our script in Dryad (<http://www.datadryad.org/>) with Accession no. dryad.fq4h3. We analysed the matrix using the uncorrected p -distance in PAUP* 4.0b10 (Swofford, 2002). After a preliminary analysis, we concatenated the cpDNA regions and used the resulting tree as a single in the tree by clade matrix. We compared the distances among gene trees visually using a heat map of distances generated in MEV (Saeed et al., 2003, 2006).

Additionally, we used a distance-based method, namely Procrustean Approach to Cophylogeny (PACo) (Balbuena, Míguez-Lozano, & Blasco-Costa, 2013; Hutchinson, Cagua, Balbuena, Stouffer, & Poisot, 2017) to detect those outliers, which independently derived from chloroplast and nuclear sequence data, contributing to conflicting phylogenies. For this analysis, we converted the nuclear and chloroplast phylogenies to patristic distance matrices using the method proposed by de Vienne, Aguileta, and Ollier (2011) and sorted each distance matrix according to the nuclear–chloroplast association matrix. We ran PACo for 100,000 permutations with the R-packages APE 3.5 (Paradis, Claude, & Strimmer, 2004) and VEGAN 2.3 (Pérez-Escobar, Balbuena, & Gottschling, 2016) using the association matrix (<http://www.ues/cophylpaco/>; accessed on 11 July 2016), and used the jackknife method to estimate the importance of each individual link to the overall sum of squares score. To consider the effect of phylogenetic uncertainty and statistical support, we discarded the first 10% (10,000) of trees as burn-in for PACo analyses.

2.5 | Molecular dating

We performed molecular dating analyses under a Bayesian relaxed molecular clock using BEAST 1.8.3 (Drummond, Suchard, Xie, & Rambaut, 2012). Due to the absence of any fossil pollen or macrofossils in Gesneriaceae (Roalson, Skog, & Zimmer, 2008; Perret, Chautems, de Araujo, & Salamin, 2013; no occurrence in Paleobiology Database, last accessed on 29 June 2017), secondary calibrations were used to calibrate the clock. The stem group age of *Primulina* has previously been estimated as 16.04 Ma with a 95% highest posterior density (HPD) between 19.87 Ma and 10.79 Ma (Gao et al., 2015; Roalson et al., 2008). These ages were employed as secondary calibration points with a uniform distribution, because under uniform priors, the node ages have an equal probability of taking any age between the minimum and maximum bounds, and typically age estimates tend to have larger 95% HPD than with other prior calibrations (Foster et al., 2017; Schenk, 2016).

We compared the outcomes of analyses using an uncorrelated log-normal relaxed clock (UCLN) and a strict clock, both with the Yule model and the birth–death model as the branching process prior (as tree priors may impact estimates of molecular dating Condamine,

Nagalingum, Marshall, & Morlon, 2015)). We ran two independent analyses (with different random seeds) in BEAST for 80 million generations of MCMC using the partitions and nucleotide substitution model as selected by PARTITIONFINDER. We used TRACER 1.6 to assess convergence and to check that the effective sample size (ESS) was greater than 200 for each parameter. We discarded the first 10% of trees as burn-in and then generated the maximum clade credibility (MCC) chronogram from the remaining trees with nodal mean heights and 95% confidence time intervals with TREEANNOTATOR in the BEAST 1.8.3 package.

2.6 | Diversification analyses

We used either the Bayesian MCC tree or 100 randomly sampled post burn-in chronograms to estimate diversification rates with different approaches. To visualize the tempo and mode of diversification of *Primulina*, we used a suite of ML models and a Bayesian model of diversification. For each type of diversification model relying on a ML framework, we computed the AICc for model comparison. The model with the lowest AICc was considered the best fit for ML analyses, and a Bayes factor difference 10 units ($BF > 10$) between the first and the second completing models suggests decisive support for the first model (Kass & Raftery, 1995). Unless otherwise specified, diversification analyses were performed using R 3.3.2 (R Development Core Team, 2016).

2.6.1 | Time-dependent diversification

We assessed whether diversification rates remained constant during the evolutionary history of *Primulina*. To accomplish this, we used BAMM 2.5 to estimate speciation and extinction rates through time along the *Primulina* phylogeny (Rabosky et al., 2013). BAMM allows studying complex evolutionary processes on phylogenetic trees, and detecting the diversification rate shifts and comparing how many and where these shifts occurred. We ran four MCMC for 50 million generations and sampled every 1,000 generations. Other parameters were set to default values except the Poisson process prior, which we set to 1.0 following recommendations from the authors of the package (Rabosky, Mitchell, & Chang, 2017; Rabosky et al., 2014). To test the effect of the prior setting on the model, we also ran the analyses with different values (0.1, 5.0 and 10.0) of the Poisson process. We used the R-package BAMMTOOLS 2.2.0 (Rabosky et al., 2014) to compute the posterior distribution and estimate the best global diversification rates through time and the configuration of the diversification rate shifts by evaluating alternative diversification models as compared by Bayes factors.

We also used the R-package RPANDA 1.2 (Morlon et al., 2016) to independently assess speciation and extinction rates through time, because BAMM has received criticisms on the independence of priors with respect to the posterior and likelihood (Moore, Höhna, May, Rannala, & Huelsenbeck, 2016; but see Rabosky et al., 2017). We specifically used RPANDA to detect potential rapid and global changes in diversification rates that might be due to environmental factors, such as climatic shifts following major geological events. We

TABLE 1 Sequence characteristics and models of DNA evolution selected for the 20 sequence regions analysed

	Aligned length (bp)	Invariable characters (bp)	Variable characters (bp)	Parsimony-informative characters (bp)	Best-fit model of nucleotide by AIC
<i>trnL-trnF</i>	876	660	121	95	GTR+G
<i>rpl32-trnL</i>	1,208	808	193	207	GTR+G
<i>atpB-rbcL</i>	1,232	926	158	148	GTR+I+G
<i>rpoB-trnC</i>	924	675	122	127	GTR+G
<i>trnC-petN</i>	797	555	111	131	GTR+G
<i>ndhA-intron</i>	873	617	136	120	GTR+G
<i>ndhH-rps15-ycf1</i>	878	570	155	153	GTR+I+G
<i>ycf1_1</i>	542	343	92	107	GTR+G
<i>ycf1_2</i>	790	477	126	187	GTR+G
<i>ITS</i>	910	388	110	412	GTR+I+G
<i>7FR</i>	1,038	705	115	218	GTR+I+G
<i>13FR</i>	900	529	156	215	GTR+I+G
<i>97FR</i>	759	484	94	181	GTR+I+G
<i>117FR</i>	693	497	86	110	GTR+G
<i>155FR</i>	948	515	179	254	GTR+I+G
<i>165FR</i>	702	428	117	157	SYM+G
<i>166FR</i>	1,478	745	284	449	GTR+G
<i>248FR</i>	1,002	687	134	181	GTR+I+G
<i>302FR</i>	534	356	70	108	GTR+I+G
<i>383FR</i>	681	471	99	111	GTR+I+G

employed four time-dependent models as follows: (i) BVAR, speciation rate varies exponentially; (ii) BVARDCST, speciation rate varies exponentially and extinction rate is constant; (iii) BCSTDVAR, speciation rate is constant and extinction rate varies exponentially; and (iv) BVARDDVAR, speciation and extinction rates are both varying exponentially. We also fit two null models (i.e., rates do not vary through time): a time constant birth model, and a time constant birth–death model, which were used for comparison purpose.

2.6.2 | Palaeoenvironment-dependent diversification

Regional environments have changed drastically in eastern Asia during the Cenozoic (Zhang, Smith, Yang, & Li, 2016). Therefore, we hypothesized that past environmental changes (e.g., palaeotemperature and East Asian monsoons) might have had consequences on the diversification of *Primulina*. To test this hypothesis, we used a model that allows speciation and extinction rates to vary according to an environmental variable, such as temperature, which changes through time (Condamine et al., 2013). Following this approach, we designed four palaeoenvironmental-dependent models as follows: (i) BVAR, speciation rate is exponentially varying with temperature and East Asian monsoons and extinction rate is missing; (ii) BVARDCST, speciation rate is exponentially varying with temperature and East Asian monsoons and extinction rate is constant; (iii) BCSTDVAR, speciation rate is constant and extinction rate is exponentially varying with temperature and East Asian monsoons; and (iv) BVARDDVAR,

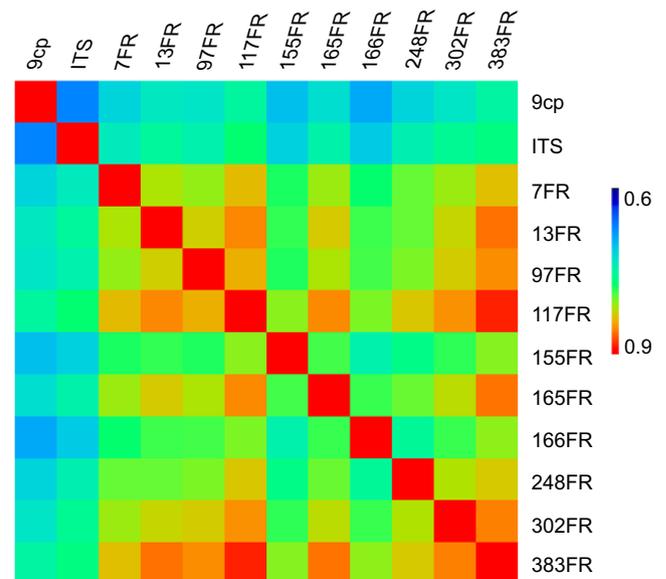


FIGURE 1 A heatmap showing levels of congruence among genes based on analysis of a binary tree by cladogram. Warmer colours (towards red) indicate greater congruence among the gene pairs. The cpDNA and ITS gene trees had the most divergent cladogram compositions and differed from one another by 32.98% of their clades. The cpDNA gene tree differed from the single-copy nuclear gene trees in cladogram composition by 24.80%–31.00%, and the ITS tree differed from the single-copy nuclear gene trees by 22.69%–28.89%. Differences among single-copy nuclear gene trees ranged from 11.48% to 25.33%

speciation and extinction rates are both exponentially varying with temperature and East Asian monsoons. For temperature, we relied on the well-known Cenozoic temperature curves published and updated by Zachos, Dickens, and Zeebe (2008). For the East Asian monsoons, we used the haematite/goethite proxy calculated from colour spectral data from the Ocean Drilling Program (ODP) Site 1148 in the South China Sea, which is measured by the relative strength of 565 and 435 nm wavelengths in the colour spectra of the freshly cut core since the Oligocene (about 25 Ma) (Clift et al., 2014). We used the R-package `PSPLINE` 1.0-18 to reconstruct smooth lines of the palaeo-data for the environmental variable. Given the dated phylogeny, the model then estimates speciation and extinction rates as a function of this value (Condamine et al., 2013).

2.6.3 | Soil habitat-dependent diversification

To evaluate the effect of different soil habitats on the diversification of *Primulina*, we used the multiple state speciation and extinction (MuSSE; FitzJohn, Maddison, & Otto, 2009) model implemented in the R-package `DIVERSITREE` 0.9-2 (FitzJohn, 2012). We built the likelihood function using '`make.musse`', then optimized by maximum-likelihood using '`find.mle`'. We ran eight different models to test whether speciation, extinction or transition rates among soil types were dependent on trait evolution. We estimated posterior density distributions in Bayesian MCMC analyses (10,000 steps) performed with the best-fitting models and resulting speciation, extinction and transition rates.

2.7 | Estimation of ancestral area of origin and ancestral soil type

We defined six geographic regions representing the current distribution of *Primulina* according to the floristic regions of China proposed by Wu and Wu (1998): A, East China; B, Central China; C, South China mountain region; D, Yunnan, Guizhou and Guangxi limestone mountain and hill regions; E, South China Sea region; F, Tonkin Bay region. These floristic regions are generally based on the floral composition and vegetation types (Wu & Wu, 1998). We performed ancestral range estimation using the dispersal–extinction–cladogenesis (DEC, Ree & Smith 2008) model as implemented in `RASP` 20160518 (Yu, Harris, Blair, & He, 2015). The maximum areas at each node were set to two, which corresponds to the largest range of any extant species (Table S1).

We also reconstructed the most likely ancestral soil types with `RASP`. We used the ancestral soil types of each node according to the

current distribution from monographs and fieldwork data (Wei et al., 2010). We carried out the analyses using the same time-calibrated phylogeny as used for the diversification analyses.

3 | RESULTS

3.1 | Phylogenetic relationships and divergence time estimates

The detailed statistics for the 20 DNA regions sequenced in this study are summarized in Table 1. The nuclear data set was more informative than the chloroplast data set, with 39.81% variable characters and 24.84% parsimony-informative characters of 9,645 bp, and 30.65% and 15.70% of 8,120 bp, respectively. The complete concatenated alignment including outgroups was 17,765 bp long, of which 11,436 characters were constant, 2,658 were parsimony-uninformative, and 3,671 were parsimony informative. For the complete data set, `PARTITIONFINDER` favoured the six-partition scheme as the best-fitting partitioning strategy. We present additional details on models and partitioning schemes in Table S4.

The Bayesian tree based on the concatenated data set of chloroplast and nuclear regions revealed four major well-supported clades, denoted A, B, C and D (Fig. S1). The analyses of incongruence using the tree by clade matrix and `PACo` showed only moderate incongruence between the chloroplast and nuclear DNA data sets (Figure 1 and Fig. S2). The tree topology from the concatenated data was similar to the species tree resolved in our coalescent analysis in `ASTRAL-II` (Fig. S3).

The `BEAST` analyses revealed the age estimates are very similar between the UCLN and the strict clock for the crown age of *Primulina*: both recovered an origin of the genus in the mid-Miocene, ca. 14 Ma (Table S5). However, the strict clock model outperformed the UCLN model based on the marginal likelihood estimate as calculated with stepping-stone sampling ($MLE_{SC-BD} = -150,900.69$, $MLE_{SC-Yule} = -150,913.37$, $MLE_{UCLN-Yule} = -152,566.49$, $MLE_{UCLN-BD} = -152,567.75$), and Bayes factors (25.36, 3,331.60 and 3,334.12, respectively) (Table S6). Hence, all subsequent analyses were conducted using the chronogram resulting from the strict clock model and the birth–death branching process prior.

The divergence time estimates (Figure 2) yielded a crown age of *Primulina* at about 14.14 Ma, with a 95% HPD of 10.79 to 19.09 Ma, which is consistent with our uniform prior calibration (Table S5). The four major clades of *Primulina* originated during the middle and late Miocene, between 13.55 and 7.58 Ma (Figure 2,

FIGURE 2 Bayesian molecular chronogram and historical biogeography of *Primulina*. The upper-left corner map represents southern China and adjacent region delimited in six floristic divisions (A–F) according to Wu and Wu (1998). Codes: A, East China; B, Central China; C, South China mountain region; D, Yunnan, Guizhou and Guangxi limestone mountain and hill regions; E, South China Sea region; F, Tonkin Bay region. The large pie chart indicates the relative species richness of *Primulina* within each region. At the tips of the chronogram, the present-day distributions of the species (Table S1) are given. Coloured circles at each node correspond to the most likely ancestral area recovered by the dispersal–extinction–cladogenesis (DEC) model, and coloured arrows on branches indicate dispersal events. Three vertical bars represent the following major climatic events: Onset of the East Asian Monsoons (OEAM), Mid-Miocene Climatic Optimum (MMCO) and Pleistocene Climatic Change (PCC)

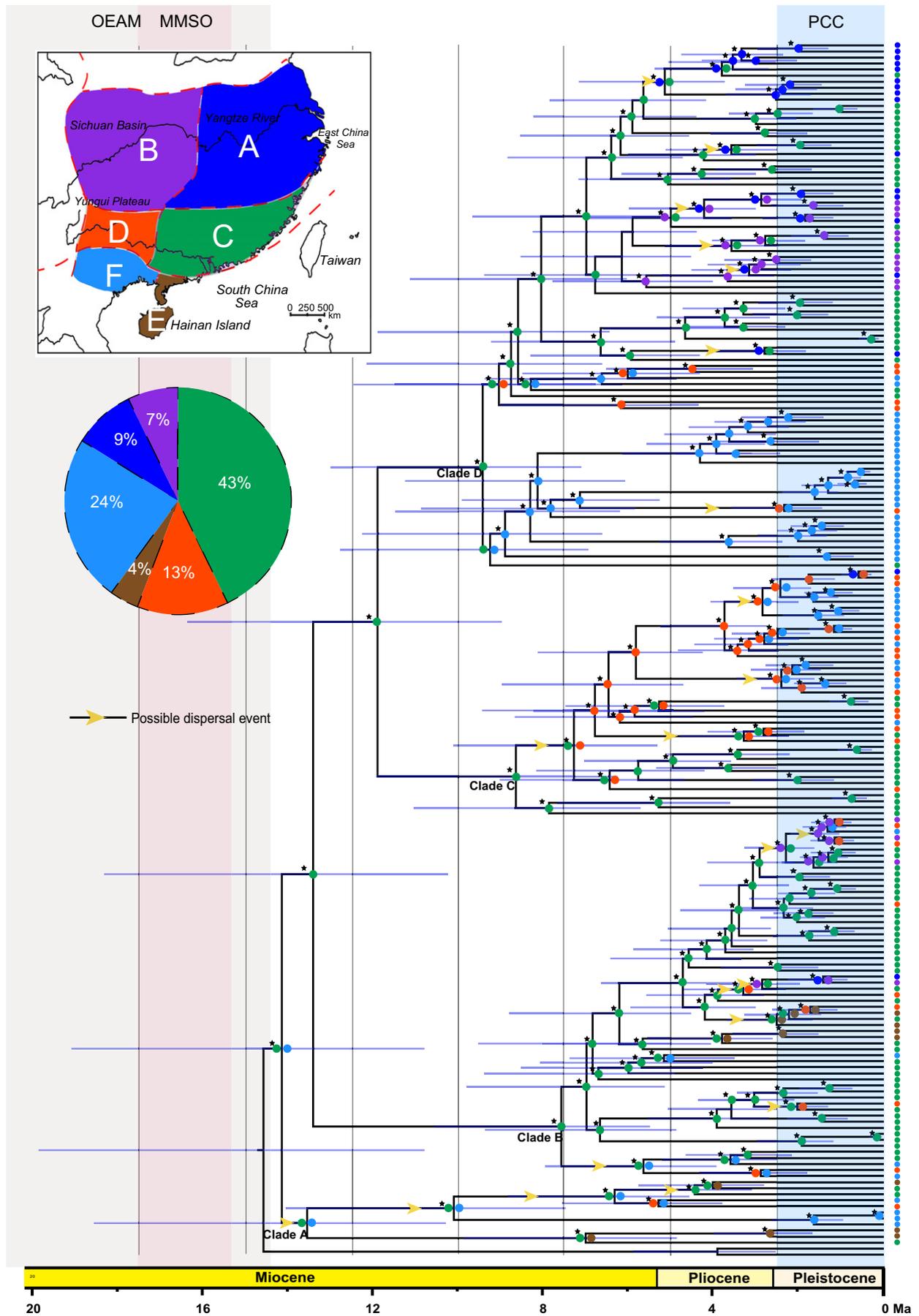


Table S5). Our age estimates were younger than the age estimates found in two previous studies of *Primulina* (Perret et al., 2013; Woo, Funke, Smith, Lockhart, & Garnock-Jones, 2011). This could be due to the secondary calibrations that often yield younger age estimates (Sauquet et al., 2012; Schenk, 2016) or due to the choice of outgroups (both studies used *Streptocarpus*, but not *Petrocodon* as sister group of *Primulina*). However, the age estimates largely overlap with each other when considering the 95% HPD that we inferred in a prior study (Gao et al., 2015). The difference for divergence time of *Primulina* between the current and previous studies is negligible and does not affect the remaining analyses.

3.2 | Diversification analyses

The B_{AMM} analyses showed that a single macroevolutionary rate better explains the diversification of *Primulina* over time (Table 2, Fig. S4). The analyses indicate an elevated net diversification rate in the early stages of the evolution of the group and a steady decline through time (Figure 3a). These results were very similar with different Poisson prior values (Table S7), that is, a single diversification regime is the most likely scenario for all different priors. Additional time-dependent analyses with R_{PANDA} also revealed the *Primulina* radiation as an early-burst pattern with high initial speciation rates followed by a steady decline of speciation towards the present and a constant extinction rate since the middle Miocene (Figure 3a, Table 2). The R_{PANDA} analyses supported a diversification model with varying-rates that outperformed other rate-variable models and the constant-rate models (Table 3). The B_{AMM} and R_{PANDA} analyses are thus congruent on inferring no detectable diversification rate shift, decreasing speciation rate through time and constant extinction.

The palaeoenvironment-dependent analyses showed that the speciation rate of *Primulina* positively correlates with both past temperatures (Figure 4a,c) and the East Asian monsoons (Figure 4b,d), respectively. The most likely models are the temperature- and East Asian monsoon-dependent ones with two parameters (AIC_c = 873.46 and 903.71, respectively). Comparing all models using the AIC_c, we found that the best palaeoenvironmental model is the one including the past temperature variation (Δ AIC = 30.25; Table 3). Constant-rate models, time-dependent models and the monsoon-dependent models were outperformed. Thus, speciation rates have decreased over time as the climate cooled towards the present, and this was corroborated by the B_{AMM} and R_{PANDA} models.

The MuSSE analyses suggested that there was a significant difference between the null model and the soil-dependent models (Table 4). The best-fitting model was a model in which speciation and transition rates were variable, and extinction remained the same between the three traits (AIC_c = 1029.39; the second best model with two additional parameters had an AIC_c = 1,033.97; Δ AIC = 4.58; Table 4). Under the best MuSSE model, we found that speciation rates were twice higher on Danxia soil (0.64 lineage/Ma) than on acid soil (0.29), and the speciation rates on acid soil were two times higher than on karst soil (0.11). The best MuSSE model

TABLE 2 Summary of results of the various diversification analyses performed

Type of birth–death	Method used	Reference	Data used	Settings	Result
Palaeoenvironmental and East Asian monsoons dependence (rates vary “continuously” as a function of time)	RPANDA (fit_env)	Condamine et al. (2013)	100 posterior chronograms	8 ML models testing whether rates vary or not (exponential and linear variation)	Speciation is “positively” linked to past temperatures and the East Asian monsoons
Among clade and time variation of rate	B _{AMM} & B _{AMM} tools	Rabosky et al. (2014)	Bayesian (MCC) chronogram from BEAST	Bayesian model testing rate shift(s) among clade and through time	No significant rate shift detected: Speciation decreased through time
Time dependence (rates vary “discretely” as a function of time)	RPANDA (fit_bd)	Morlon et al. (2016)	100 posterior chronograms	8 ML models testing whether rates vary or not (exponential and linear variation)	Speciation rate is cliffy decreased, and extinction rate is constant
Trait dependence (rates vary as a function of a character state for a trait)	diversitree (MuSSE)	FitzJohn (2012)	100 posterior chronograms	8 ML models testing whether rates depend on the trait	The rates depend on the different soil types

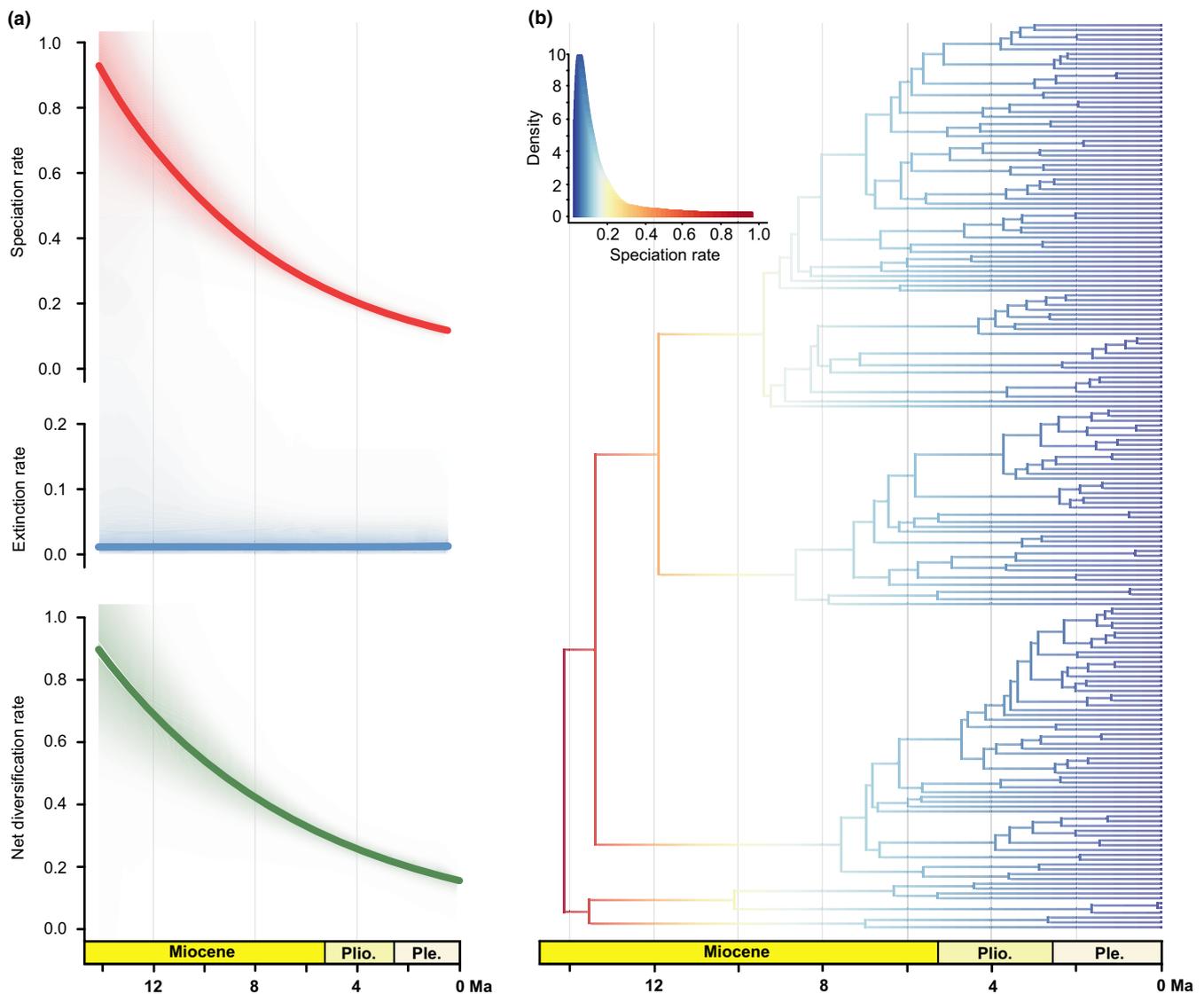


FIGURE 3 Bayesian analysis of macroevolutionary mixtures in *Primulina*. (a) Bayesian reconstruction of rate variations in speciation, extinction and net diversification through time. (b) Maximum a posteriori probability shift configuration represented as a phylorate plot showing variations in speciation rates (cool colours = slow, warm = fast) along each branch of the *Primulina* phylogeny. Each unique colour section of a branch represents the mean of the marginal posterior density of speciation rates on a localized segment of a phylogenetic tree. The rate variation pattern for lineages involves a uniform temporal deceleration in speciation rates. Plio. = Pliocene; Ple. = Pleistocene

also indicated that transition rates were different between all traits, with the transition between Danxia towards karst soil being at least fourfold higher than other transition rates. The second most elevated transition rate was between acid and karst soil.

3.3 | Ancestral area of origin and evolution of soil habitat

The biogeographic analysis identified the South China mountain region and the Tonkin Bay region as the ancestral area of origin of *Primulina* and its centre of diversification for most of the middle and late Miocene (Figure 2). The dispersal events for the common

ancestors of *Primulina* towards other regions mostly came from the South China mountain region in the late Miocene (Figure 2).

Primulina exhibits a high degree of soil specialization, with only five of the 120 sampled species being found in more than one soil type (Fig. S5). Species from an early-diverging lineage (e.g., clade A; Figure 2) displayed wider habitat occupancy for all three soil types with two species growing on karst and acid soils. Only four sister species pairs out of the 60 deepest sister species (6.67%) showed a pattern consistent with allopatric speciation with fully distinct soil type preferences. Additionally, partial shifts in soil type were observed in five sister species pairs (Fig. S6). These results indicated that soil type shifts have not frequently coincided with divergence events.

TABLE 3 Results of diversification analyses for *Primulina*. In bold are displayed the best-fit models for each birth–death series that explain the phylogeny of the group. Values represent the mean of each parameter as estimated from 100 randomly selected trees of the posterior distribution of the dating analyses. B, birth or speciation; D, death or extinction; NP, number of parameters in the model; logL, the log-likelihood of the model; AICc, the corrected Akaike information criterion; AIC wt, Akaike weight; λ , speciation rate at present; α , shape of speciation rate; μ , extinction rate at present; and β , shape of extinction rate

Model type	Model descriptions	Rate variation	NP	logL	AICc	AIC wt	λ	α	μ	β
Constant rate	Yule model	Constant	1	−452.315	906.651	0	0.2423	—	—	—
	Constant BD	Constant	2	−452.315	908.694	0	0.2423	—	0	—
Time dependence	B variable no D	Exponential	2	−439.035	882.134	0	0.1567	0.1277	—	—
	B variable D constant	Exponential	3	−439.036	884.202	0	0.1567	0.1277	0	—
	B constant D variable	Exponential	3	−452.315	910.760	0	0.2424	—	0	0.0344
	B variable D variable	Exponential	4	−439.035	886.288	0	0.1567	0.1276	0	0.0145
Monsoon dependence	B variable no D	Exponential	2	−449.824	903.713	0	0.2023	0.4477	—	—
	B variable D constant	Exponential	3	−449.837	905.804	0	0.2009	0.4650	0	—
	B constant D variable	Exponential	3	−452.401	910.932	0	0.2420	—	0	0.0270
	B variable D variable	Exponential	4	−449.838	907.894	0	0.2008	0.4669	0	0.0365
Temperature dependence	B variable no D	Exponential	2	−434.699	873.463	0.675	0.0943	0.2632	—	—
	B variable D constant	Exponential	3	−434.699	875.529	0.240	0.0943	0.2632	0	—
	B constant D variable	Exponential	3	−452.400	910.931	0	0.2420	—	0	0.0235
	B variable D variable	Exponential	4	−434.699	877.617	0.085	0.0943	0.2632	0	0.0236

4 | DISCUSSION

It is commonly accepted that climate change has played a significant role in the history of life (Erwin, 2009). Yet, confirmation with empirical data and identification of the underlying mechanism behind the relationship has long been a challenge. In the current study, using an explicit approach linking past environment to species diversification (Condamine et al., 2013), we demonstrated that species diversification in *Primulina* was temporally associated with palaeoenvironmental changes. In particular, the past climate change around the mid-Miocene apparently triggered an early burst of speciation which was followed by a slowdown in diversification rate towards the present that correlates temporally with and is potentially caused by global cooling. The onset of global cooling and increased seasonality after the mid-Miocene (Zachos et al., 2001) was a pivotal time for explosive diversification of a large number of modern plant lineages (Baldwin, 2014; Schnitzler et al., 2011; Xiang et al., 2016).

The temperature dependency of speciation for the karst-adapted plants could be explained by a number of direct and indirect factors. According to the evolutionary speed hypothesis (Rohde, 1992), higher temperatures are associated with high rates of mutation and speciation. For instance, a palaeontological study of biodiversity patterns of over the last 540 Ma using fossil marine invertebrates found a strong temporal association between temperature and diversification rate (Mayhew, Bell, Benton, & McGowan, 2012), while the impacts of temperature on the diversification rate in plants are usually based on the observed negative relationships between latitude and diversification rate (Jansson & Davies, 2008; Svenning, Borchsenius, Bjorholm, & Balslev, 2008). Temperature can also indirectly impact diversification via its interaction with biotic and/or abiotic

factors (Fine, 2015; Gillman & Wright, 2014). For example, interactions between temperature and water availability, and between temperature and seasonality have been well recognized to affect the diversity (Archibald, Bossert, Greenwood, & Farrell, 2010; Hawkins et al., 2003). In our current study, the palaeoenvironmental change is likely to have affected the diversification rates in *Primulina* by temperature and seasonality with monsoon.

Many studies have hypothesized that the East Asian monsoons were associated with species diversification in East Asia (Chung et al., 2014; Guo et al., 2016; Wang et al., 2012), but no direct test between the Asian monsoons and diversification rate was tried until our current study. Our past environmental correlation analysis revealed that the speciation rate of *Primulina* was tightly linked to the strength of the East Asian monsoons. Karst is a land area made up of limestone, which can be dissolved in water. The onset of the East Asian summer monsoons around 20–15 Ma, which is characterized by a higher precipitation, provided ideal conditions for the development of karst regions (Liu, 1997; Zhang, 1989). With the abundant precipitation, the dissolution rates in karst area are ten to hundred times higher in the tropical and subtropical regions than that in other regions (Lu, 1986). We hypothesize that the monsoons have facilitated the dissolution and weathering of karst and, therefore, helped to provide suitable habitats for calciphiles such as species of *Primulina*. As the strength of monsoons exhibits remarkable variability over historical time (An et al., 2001), the opening of available habitats for calciphiles depended on the strength of the monsoons. When monsoons underwent substantial intensification, weathering of karst was intensive and resulted in more habitats facilitating diversification; in contrast, when the monsoon entered its weakened stage, fewer habitats for speciation were caused by less

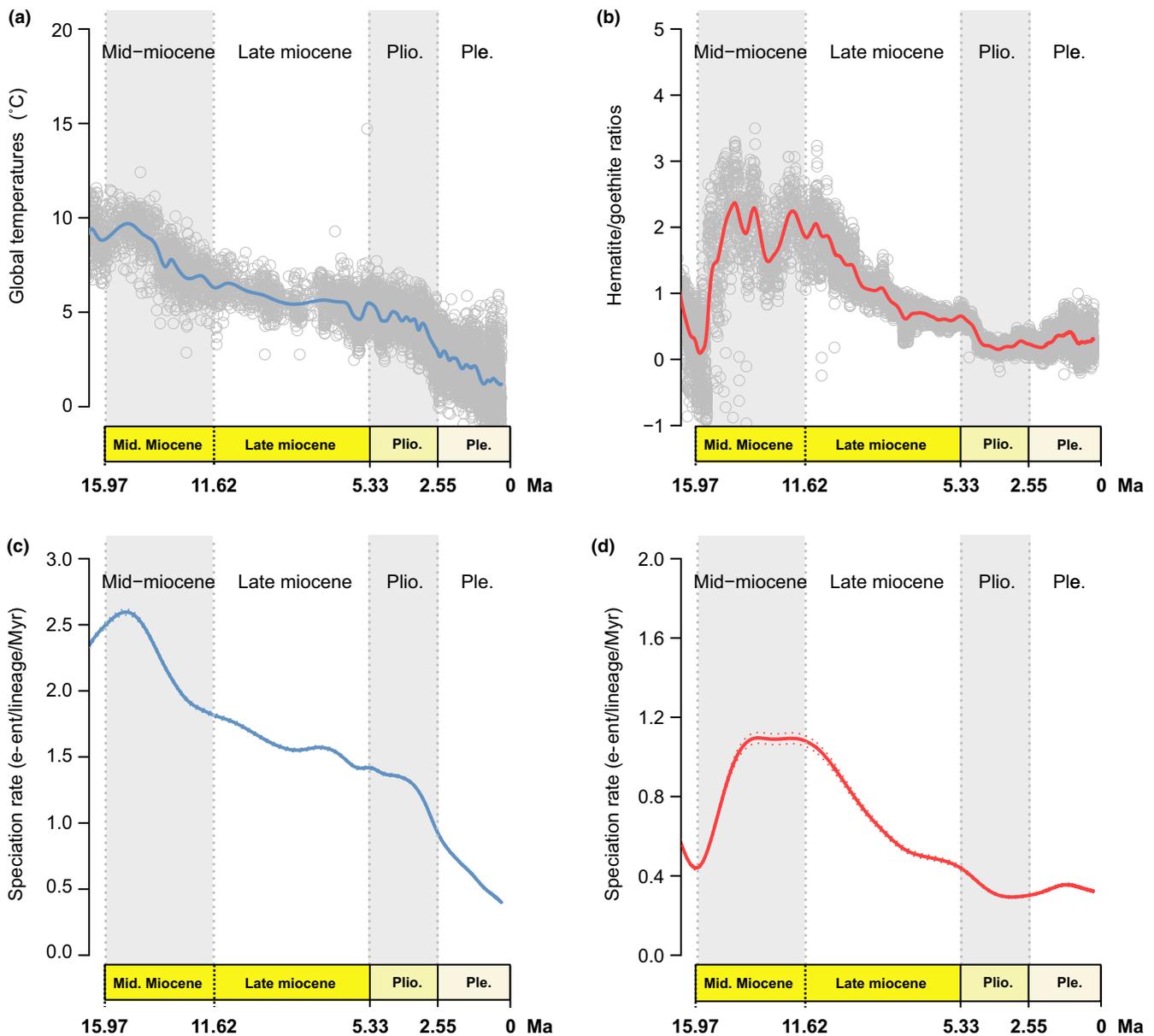


FIGURE 4 Diversification pattern of *Primulina*. (a) Past fluctuations of temperatures from the mid-Miocene (data plotted from Zachos et al., 2008). (b) Past fluctuations of East Asian monsoons from the mid-Miocene (data plotted from Clift et al., 2014). (c,d) Speciation rates through time for *Primulina* obtained from the relationship between diversification with palaeotemperatures (c) and East Asian monsoons (d), estimated using the approach of Condamine et al. (2013). The best model indicates a positive correlation between speciation and past temperatures and East Asian monsoons, respectively. Plio. = Pliocene; Ple. = Pleistocene

precipitation. In this context, the initial explosive burst of speciation in *Primulina* is consistent with a scenario of environment-driven burst speciation, which proposes that rapid climate change can lead to pulses of high speciation rate and decrease after the period ends (Moen & Morlon, 2014). Indeed, the East Asian monsoons have had a profound impact on terrestrial ecosystem turnover (Jia, Peng, Zhao, & Jian, 2003). Therefore, the burst of speciation in *Primulina* may be explained by the fact that the palaeoenvironmental change in the mid-Miocene produced numerous extinctions of pre-existing taxa (Sun & Wang, 2005; Wang, 1996; Xu, Wang, Du, & Zhang, 2000), with profound restructuring of the flora and subsequently resulted in

isolation up of new landforms and geographic opportunities for *Primulina* (Kang et al., 2014).

East Asian climate change was mostly controlled by the monsoon from the early Miocene (Lu & Guo, 2014). In spite of different hypotheses on the formation and evolution of the East Asian monsoons (Liu & Dong, 2013; Lu et al., 2010; Molnar, Boos, & Battisti, 2010), the global cooling might provide the best explanation for the formation of the monsoon in East Asia during late Cenozoic (Lu & Guo, 2014; Miao et al., 2012, 2016). Then, it is not surprising the diversification rates of *Primulina* were both positively associated with global cooling and Asian monsoons. Our results also, from another

TABLE 4 Results of MuSSE analyses for the *Primulina* with 100 random trees. In bold is displayed the best fit model for the analyses. NP, number of parameter in each model; logL, the log-likelihood of the model; AICc, the corrected Akaike information criterion; λ , speciation rate at present; μ , extinction rate at present; and q, transition rates. 1, Karst soil; 2, Danxia soil; 3, Acid soil

Model	NP	logL	AICc	λ_1	λ_2	λ_3	μ_1	μ_2	μ_3	q12	q13	q21	q23	q31	q32
All equal	3	-531.128	1068.386	0.2424	—	—	0	—	—	0.0122	—	—	—	—	—
λ free	5	-530.101	1070.531	0.2496	0.1269	0.1730	0	—	—	0.0123	—	—	—	—	—
μ free	5	-530.986	1072.301	0.2457	—	—	0	0.0107	0.0590	0.0138	—	—	—	—	—
q free	8	-518.786	1054.377	0.2423	—	—	0	—	—	0.0158	0.0027	0.2779	0	0.2020	0.0010
λ and μ free	7	-530.091	1074.805	0.2499	0.1281	0.1729	0	0.0210	0	0.0125	—	—	—	—	—
λ and q free	10	-504.071	1029.385	0.1070	0.6420	0.2913	0	—	—	0.0258	0.0091	0.6979	0.0182	0.1784	0.0859
μ and q free	10	-518.786	1058.816	0.2423	—	—	0	0	0	0.0158	0.0027	0.2780	0	0.2020	0.0010
All free	12	-504.094	1033.972	0.1091	0.6731	0.2300	0	0	0	0.0330	0.0013	0.7569	0.0216	0.0707	0.0924

side, exemplified that the influence of East Asian monsoons was synchronous with global temperature changes.

Our results revealed that *Primulina* experienced a steady decline of speciation towards the present following the mid-Miocene burst. Early bursts followed by slowdowns in diversification are often interpreted as competition for resources as ecological niches are filled (Moen & Morlon, 2014). However, most species of *Primulina* have allopatric distributions and calciphiles growing on limestone habitats where population density is low (i.e., low α diversity) (Hao et al., 2015). Hence, it is unlikely that plants in this group experienced strong competition for ecological resources and niche differentiation. Alternatively, bursts of speciation with subsequent slowdowns could also be caused by geographic processes (Pigot, Phillimore, Owens, & Orme, 2010). In this scenario, the ancestral geographic range could have been subjected to successive vicariant events leading to new geographic ranges of increasingly smaller size. The successively smaller range sizes would in turn reduce the probability of speciation, and cause a decline in speciation rates (Moen & Morlon, 2014). During the tectonic evolution of karst system in southern China, the exogenic forces involved in erosion processes include flowing water, weathering and gravity effects. Among them, flowing water plays the most significant role in sculpting the karst area, especially in the humid southeastern China (Peng, Ren, & Pan, 2015). With the high precipitation accompanied by East Asian monsoons, the karst area in southern China was divided into isolated regions with smaller size and followed by lower diversification rates for *Primulina*. Recent studies in *Primulina* (Gao et al., 2015) and *Begonia* (Chung et al., 2014) in karst areas of southern China also lend support to the role of geographic isolation in species diversification.

No studies have investigated soil type shifts within a phylogenetic framework in plants from southern China. We detected different speciation rates among the three soil types. It is difficult to determine the cause responsible for the varied speciation rates among soil habitats. One possible explanation could be that the Danxia landforms are subjected to more geographic opportunities for

Primulina, which may have resulted in increased population divergence and speciation. This indicates that edaphic heterogeneity also contributed to species diversification in *Primulina*. Nevertheless, soil type shifts are relatively infrequent in *Primulina* and no obvious effect on the overall speciation rate within the whole genus.

Additionally, our diversification and historical biogeography analyses in *Primulina* are consistent with an “ancient cradle model” of diversity (Couvreur, Forest, & Baker, 2011), in which lineages experienced an early and rapid speciation in response to favourable climatic conditions followed by a decelerating speciation rate through time. On the other hand, the estimated extinction rate in *Primulina* is constantly low through the diversification processes, which is a typical characteristic of the “museum model” as preserving old lineages (Stebbins, 1974). It seems likely that the evolutionary history of *Primulina* can be best explained by a combined pattern of both “the cradle and the museum” models, as suggested for tropical organisms (McKenna & Farrell, 2006). Indeed, floristic studies have identified southern China as both “the cradle and the museum” of plant diversity (López-Pujol, Zhang, Sun, Ying, & Ge, 2011). In particular, karst landforms generated complex microhabitats, which have been recognized as palaeoreugia for plants and animals (Chung et al., 2014; Oliver et al., 2017). The low extinction rate could be ascribed to the lack of glaciation and extremely rugged low mountains, which may have promoted the long-term persistence of lineages (Carnaval et al., 2014; Dynesius & Jansson, 2014).

In summary, we found that global temperature change is probably the primary driver of diversification in *Primulina*. However, we assert that temperature fluctuations also played critical roles in the development of the East Asian monsoons and the karst habitats. Thus, the monsoons and edaphic characteristics are probably also strongly linked to diversification in *Primulina*. We detected that *Primulina* underwent an early burst of speciation followed by a decline in speciation rate, and this is consistent with what we know global temperature patterns and the strength of the East Asian monsoons. We suggest that many species of *Primulina* may have evolved in southern China allopatrically

due to topographical heterogeneity (e.g., cradle hypothesis) and been preserved there during the diversification processes (e.g., museum hypothesis). We suggest that the framework of this investigation can motivate additional empirical case studies on the flora of southern and eastern Asia to directly test correlations between diversification rates and global temperature changes, including combined effects with the East Asian monsoons.

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DATA ACCESSIBILITY

The data including the matrix used for phylogeny reconstruction (Figure 2), the temperature data from Cenozoic and the data for the East Asian Monsoons from 17 Ma to produce Figures 3 and 4, and the script used to generate the tree by clade matrix are available at the Dryad Digital Repository [<http://dx.doi.org/10.5061/dryad.fq4h3>].

DNA sequences: GenBank Accession nos. (KY393439–KY397429) are shown in Table S2.

AUTHOR CONTRIBUTIONS

All authors contributed to discuss the results and editing of the manuscript. M.K. conceived and designed the research. H.H.K., F.L.C. and M.K. contributed to writing the manuscript. H.H.K., F.L.C. and M.K. contributed to data analysis. A.J.H. contributed to incongruence analysis. J.L.C. conducted the laboratory work. M.K., B.P. and V.S.H. contributed to sample collection. M.M. contributed to phylogenetic analysis.

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