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ORIGINAL ARTICLE

Biogeographical diversification of mainland Asian *Dendrobium* (Orchidaceae) and its implications for the historical dynamics of evergreen broad-leaved forests

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ABSTRACT

Aim Evergreen broad-leaved forests (EBLFs) occupy most tropical and subtropical regions of mainland Asia and exhibit high species diversity, productivity and richness. Understanding the historical dynamics of EBLFs is important for biodiversity conservation and regional carbon storage and cycling under global climate change. However, little is known about the historical dynamics of EBLFs in mainland Asia. *Dendrobium*, an epiphytic orchid genus found in EBLFs, was used to gain new insights of the historical establishment and extension of EBLFs in mainland Asia.

Location Mainland Asia.

Methods We sampled *c.* 80% of the currently recognized species of *Dendrobium* found in mainland Asia. A phylogeny was generated using maximum parsimony, maximum likelihood and Bayesian inference methods. A calibrated chronogram was obtained using a Bayesian relaxed-clock model approach. Biogeographical scenarios were investigated using the statistical dispersal–vicariance analysis and dispersal–extinction–cladogenesis methods. Ancestral states were constructed by MESQUITE and BAYESTRAITS, and diversification of *Dendrobium* was investigated by APE and LASER packages.

Results Our results indicate that Asian *Dendrobium* was present on mainland Asia since the Oligocene, after which this genus diversified in this region and dispersed into higher elevations. The ancestral habit of *Dendrobium* was epiphytic and terrestrial and lithophytic habits each have multiple, recently independent origins.

Main conclusions Our results support the hypothesis that EBLFs have been established in mainland Asia at least since the Oligocene and further suggest that the EBLFs of mainland Asia expanded into higher elevations prior to the late Cenozoic. The diversification of Asian *Dendrobium* coincided with a warmer climate during the late Oligocene and middle Miocene and the expansion of key groups (including Fagaceae, Lauraceae, Magnoliaceae and Theaceae) of EBLFs during the early Cenozoic.

Keywords

Biogeography, *Dendrobium*, evergreen broad-leaved forests, mainland Asia, molecular dating, Orchidaceae

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INTRODUCTION

Evergreen broad-leaved forests (hereafter EBLFs), including tropical rain forest, tropical seasonal rain forest and subtropical EBLFs, occupy most regions of tropical and subtropical mainland Asia and are very important terrestrial ecosystems in terms of species diversity and ecosystem functioning (Fang & Yoda, 1991; Wu, 1995; He *et al.*, 1998; Ying, 2001; Zhu, 2013). Species of several woody families, such as Fagaceae, Lauraceae, Magnoliaceae and Theaceae, dominate EBLFs, which appear to be unique in terms of their climate characteristics, forest structure and species composition (Wu, 1995). In contrast to the EBLFs of Asia, most regions around the world at similar latitudes are occupied by savanna vegetation or even hot, dry deserts (Good, 1974). Evergreen broad-leaved forests provide considerable social and ecological benefits (Ying, 2001; Piao *et al.*, 2009). Recent results have demonstrated that they play critical roles in biological conservation and regional carbon storage and cycling, and a better understanding of the establishment and the historical dynamics of the EBLFs is important for biodiversity conservation and carbon sequestration, especially with respect to global climate change (Fang *et al.*, 1998, 2014; Myers *et al.*, 2000; Ying, 2001; Zhang *et al.*, 2007; Piao *et al.*, 2009; Pan *et al.*, 2011).

Currently, there is little knowledge regarding the historical dynamics of the development of Asian EBLFs. The frequent hybridization, complicated evolutionary histories (Samuel, 1999; Wu *et al.*, 2003; Zhang *et al.*, 2014), and taxonomic confusion in keystone forest tree families, notably Fagaceae (Samuel, 1999), Lauraceae (Rohwer, 2000; Chanderbali *et al.*, 2001), Magnoliaceae (Nie *et al.*, 2008) and Theaceae (Luna & Ochoterena, 2004; Zhang *et al.*, 2014), present major obstacles in tracing the historical dynamics of EBLFs. Understanding the historical dynamics of the development of Asian EBLFs is further complicated by complex geographical events, including the uplift of the Himalayas (An *et al.*, 2001), and by climatic changes, such as the establishment of the Asian monsoon (Sun & Wang, 2005) and climate changes during the Neogene (Zhou, 1999; Jacques *et al.*, 2011).

Recent results of fossil and molecular dating analyses have indicated that the woody families that dominate EBLFs diverged after the middle Cretaceous (Friis *et al.*, 1987; Magallón & Castillo, 2009; Smith *et al.*, 2011) and diversified during the early Cenozoic (Xiang *et al.*, 2014). The prevailing view is that EBLFs in tropical region began to appear after the K-Pg boundary (e.g. Upchurch & Wolfe, 1987; Morley, 2000; Johnson & Ellis, 2002; Schuettpelz & Pryer, 2009; Wang *et al.*, 2012). Therefore, it is logical to propose the hypothesis that the EBLFs have been established in mainland Asia since the early Cenozoic.

The orchid genus *Dendrobium* (Epidendroideae, Orchidaceae) is among the largest genera of flowering plants, with 1200–1500 species that are mainly distributed in Asia and Oceania (Cribb & Govaerts, 2005; Wood, 2006; Zhu *et al.*, 2009; Pridgeon *et al.*, 2014). Of the *c.* 200 species of *Dendrobium* in mainland Asia, most are typically epiphytic and grow on the trees of EBLF keystone groups, such as Fagaceae, Lauraceae, Magnoliaceae and Theaceae (Tsi, 1999; Wood, 2006; Zhu *et al.*, 2009). Like most epiphytic orchids, *Dendrobium* species have tiny wind-dispersal seeds with a high dispersal capacity (Hamrick & Godt, 1996; Alcantara *et al.*, 2006; Philips *et al.*, 2012). Several species extend to the northern limits of EBLFs (Tsi, 1999; Wood, 2006; 2001–2013 our field observations). Therefore, *Dendrobium* species can be considered characteristic of these forests (Wood, 2006; Zhu *et al.*, 2009). The results of recent morphological and molecular analyses have indicated that *Dendrobium* s.l. is monophyletic and is divided into two clades: an Asian clade and an Australasian clade (Yukawa *et al.*, 1993, 2000; Clements, 2003, 2006; Burke *et al.*, 2008; Schuiteman, 2011; Xiang *et al.*, 2013). However, there is little knowledge about the historical biogeography and diversification of *Dendrobium*. The discovery of *Dendrobium* leaf fossils in New Zealand indicated the expansion of *Dendrobium* into Zealandia by the middle Cenozoic (Early Miocene, 23–20 Ma) (Conran *et al.*, 2009), which shed new insights on the spatial and temporal evolution of *Dendrobium*. Therefore, *Dendrobium* offers a remarkable and unique opportunity for studying the historical dynamics of the development of EBLFs in mainland Asia.

Our major aims are (1) to infer the historical biogeography and diversification of the epiphytic orchid *Dendrobium* and use this as a model to (2) infer the historical establishment and extension of EBLFs in mainland Asia and to (3) test the hypothesis that EBLFs have been established in mainland Asia since the early Cenozoic. To achieve these objectives, we first reconstructed a robust phylogenetic framework for *Dendrobium*, focusing on the mainland Asian groups and using more extensive sampling than that of any previous study. We then investigated the temporal and spatial diversification of mainland Asian *Dendrobium* by integrating phylogenetic, biogeographical and molecular dating methods.

MATERIALS AND METHODS

Sampling and molecular data

To fully understand the diversification of mainland Asian *Dendrobium*, we sampled all sections present in mainland Asia based on previous phylogenetic results, thus representing the geographical and taxonomic diversity of the genus (Clements, 2003, 2006; Wood, 2006; Burke *et al.*, 2008; Xiang *et al.*, 2013). Approximately 80% of the species from mainland Asia were sampled, and all relevant sequences were downloaded from GenBank (last download in 11 August 2015). The matrix contained 319 species (taxonomy of *Dendrobium* following Wood, 2006; Xiang *et al.*, 2013; Pridgeon *et al.*, 2014), of which 40 species were from the Australasian group and 10 species were outgroups. Four chloroplast DNA sequences (*matK*, *rbcL*, *trnH-psbA* spacer and *trnL-F* region) and the nuclear internal transcribed spacer (ITS) region were used for analyses. Voucher information and GenBank accession numbers are listed (see Table S1 in Appendix S1 of Supporting Information).

Phylogenetic analyses

In order to assess the impact of missing data on phylogenetic reconstruction, five different matrixes of *Dendrobium* were generated and analysed using the same methods: matrix 1 with 80 taxa, each taxon with five loci (80 taxa, 5 loci), matrix 2 (91 taxa, 4–5 loci), matrix 3 (129 taxa, 3–5 loci), matrix 4 (281 taxa, 2–5 loci) and matrix 5 (319 taxa, 1–5 loci).

The alignment was performed in ClustalX 1.83 (Thompson *et al.*, 1997) and then adjusted manually in bioedit (Hall, 1999). The data were partitioned as follows: ITS1, 5.8S rDNA, ITS2, *matK*, *rbcL*, *trnH-psbA* and *trnL-F*. Phylogenetic analyses were carried out using MrBayes 3.2.1 (Ronquist & Huelsenbeck, 2003) for Bayesian inference (BI), paup v4.0b10 (Swofford, 2003) for maximum parsimony (MP) and RAxML 7.0.4 (Stamatakis, 2006) for maximum likelihood (ML).

Modeltest 3.7 (Posada & Crandall, 1998) was used to determine the best-fit model under the Akaike information criterion (AIC). The GTR+I+Γ model was selected for ITS, *rbcL* and the combined matrix while the TVM+I+G model was selected for all other loci. In the BI analysis, we used default priors and set the option *prset ratepr* as variable. The Markov chain Monte Carlo (MCMC) algorithm was run for 6,000,000 generations with one cold chain and three heated chains, starting from a random tree. The convergence was assessed using Tracer v1.5 (Rambaut & Drummond, 2007). With the first 25% trees discarded as burn-in, the 50% majority-rule consensus tree and posterior probabilities (PP) were calculated from the remaining trees.

For the MP analysis, heuristic searches were performed using 1000 random-addition-sequence replicates and tree-bisection-reconnection branch swapping with MulTrees in effect and steepest descent off. Bootstrap support values were calculated by conducting heuristic searches using 1000 replicates.

For the ML analysis, the GTR+GAMMA model was set for all partition, but all model parameters were estimated for each partition respectively. 1000 rapid bootstrap inferences were executed before a thorough ML search.

Molecular age estimation

Dendrobium divergence times were estimated in two steps. First, we used a phylogeny representing the major orchid lineages to estimate the crown group age of a monophyletic clade that included *Dendrobium*. We then used the estimated crown age to calibrate the rate corrected phylogeny of the *Dendrobium* species used in this study.

Divergence time estimation in Orchidaceae

We downloaded *matK* and *rbcL* sequences from GenBank to estimate the divergence times in Orchidaceae. Of 203 species included in the analysis, 195 represented 186 genera from five subfamilies in Orchidaceae and 8 taxa were outgroups selected from closely related families in Asparagales (see Table S2 in Appendix S1 in Supporting Information). Divergence times were estimated using a Bayesian uncorrelated relaxed-clock model implemented in beast 1.7.4 (Drummond & Rambaut, 2007). Four fossil calibration points were used: (1) the age of the oldest known monocot fossil (110–120 Ma) for the root, following Friis *et al.* (2004) and Ramírez *et al.* (2007) with a normal prior distribution (mean: 115 Ma, and a standard deviation of 3.05), for a normal distribution can be used to reflect the non-directional uncertainty on radiometric dating (Ho & Phillips, 2009); (2) 15 Ma as the minimum age for the monophyletic Goodyerinae Klotzsch (Orchidoideae, Orchidaceae) with exponential distribution (offset: 15, mean: 10) (Ramírez *et al.*, 2007); (3) 23.2 Ma as the minimum divergent age of the Asian and Australasian *Dendrobium* clades with exponential distribution (offset: 23.2, mean: 8) (Conran *et al.*, 2009; Pole, 2014); and (4) a leaf fossil of *Earina* (23.2 Ma) as the minimum stem age of *Earina* Lindl. (Epidendroideae, Orchidaceae) with exponential distribution (offset: 23.2, mean: 8) (Conran *et al.*, 2009; Pole, 2014). Except for the root age for which a normal distribution was used, an exponential distribution was used for the other three calibration points, which was decided by the characteristics of the exponential distribution and the greatest prior weight placed on node ages very close to the age of the fossil with diminishing probability to ∞ (Ho & Phillips, 2009). MCMC searches were run for 50,000,000 generations and sampled every 5000 generations. Convergence was monitored using Tracer 1.5 (Rambaut & Drummond, 2007). The effective sample sizes (ESSs) of all parameters were assessed. The maximum clade credibility tree was computed using treeAnnotator 1.7.4 (Drummond *et al.*, 2012).

Divergence time estimation in Dendrobium

A likelihood ratio test was performed to estimate whether *Dendrobium* evolved at a homogeneous rate along all branches of the phylogeny. The result rejected a constant rate for *Dendrobium* ($\delta = 1683.39$, d.f. = 319, $P < 0.001$).

Previous results indicated that there are only 'soft' conflicts between the nuclear versus plastid trees in *Dendrobium* (Xiang *et al.*, 2013). Divergence times within *Dendrobium* were therefore estimated using the combined ITS, *matK*, *rbcL*, *trnH-psbA* and *trnL-F* matrix and the following tree priors: (1) based on the results of the chronogram of Orchidaceae (above), the root age was set to a normal prior distribution with a mean of 47.56 Ma and a standard deviation of 5.75 (resulting in a 95% CI ranging from *c.* 39–57 Ma); (2) a minimum age (23.2 Ma) was used for the stem age of the

Australasian clade of *Dendrobium* (Conran *et al.*, 2009; Pole, 2014) with an exponential prior distribution (mean = 3.0, SD = 0.5). The Yule process was chosen as the speciation process, and GTR+I+Γ was used for the best-fitting evolutionary model (see Modeltest). Markov chain Monte Carlo searches were run for 100,000,000 generations sampled every 5000 generations. Convergence, ESS of parameters and the calculation of the maximum clade credibility tree followed the methods outlined above.

Diversification analyses

Birth–death likelihood (BDL) models were used to test the significance of heterogeneity or the consistency of the temporal diversification rate (Rabosky, 2006a,b). The model selection was based on the difference in the AIC scores between the best-fitting rate-constant and rate-variable models (ΔAIC_{RC}). The calculations were performed using laser 2.3 (Rabosky, 2006a).

To visualize the temporal variation in the diversification rates, semi-logarithmic lineage-through-time (LTT) plots were constructed using the R package ape 2.5-1 (Paradis *et al.*, 2004). One thousand trees were sampled randomly from the converged beast trees and used to calculate a 95% credibility interval.

Ancestral area reconstructions

Based on the floristic regions delimited by Takhtajan (1986) and the extant distribution of *Dendrobium* and outgroups, four main regions were defined: A, mainland Asia (including Eastern Asia, India, Indo-China and Myanmar); B, Malesia (including Malaysia, Indonesia, Philippines, Singapore and New Guinea); C, Australasia (including Australia, New Zealand, New Caledonia and Tasmania); and D, circumpolar (for outgroups). The ancestral areas at internal nodes within the phylogenetic tree were inferred using two methods: (1) a statistical dispersal–vicariance analysis (S-DIVA) (Ronquist, 1997) implemented in rasp (Yu *et al.*, 2013) and (2) a likelihood approach using the dispersal–extinction–cladogenesis (DEC) model implemented in rasp with the source code of the C++ version of lagrange (Ree & Smith, 2008). All analyses were based on the 1001 output trees and the maximum clade credibility tree from the beast analyses. The outgroups were included in the analyses. Neither analysis was performed with constraints on the maximum number of areas per node.

Ancestral states reconstruction

We inferred the elevation range of each species of *Dendrobium* according to Zhu *et al.* (2009), Wood (2006), the Orchids Species (http://www.orchidspecies.com/, last assessed in 11 August 2015) and our field observations (see Table S1 in Appendix S1 in Supporting Information). Shifts in *Dendrobium* elevation ranges was reconstructed using the squared-change parsimony option for continuous characters using the continuous method with mesquite 2.74 (Maddison & Maddison, 2011). The maximum clade credibility tree obtained from beast was used in the analysis.

Species habit data were also obtained from the sources above (see Table S1 in Appendix S1). We defined three habit categories: epiphytic, lithophytic and terrestrial. The ancestral habit of *Dendrobium* and the direction of habit shifts were reconstructed using the MCMC approach in bayestraits (Pagel *et al.*, 2004) using a set of 1000 chronograms chosen randomly from the post-burn-in beast trees.

RESULTS

Phylogenetic analyses

Our results indicated that the backbones of the five phylogenetic topologies from analyses based on different taxon and character sampling strategies are similar, with differences only in clades that are weakly supported (Fig. 1; see Fig. S1 in Appendix S1). Matrix 5 with 319 *Dendrobium* taxa (each taxon with 1–5 loci) was therefore used in followed analyses and discussion. The aligned combined data set consisted of 7010 bp, 20% of which were parsimony-informative, and 56.1% of which contained missing data. The parsimony strict consensus tree of *Dendrobium* was largely consistent to the topologies of ML and BI analyses with differences only in weakly supported clades.

Dendrobium was divided into two main clades (MP-BS = 90, ML-BS = 100, PP = 1.00): an Asian clade and an Australasian clade (Fig. 1). The results were consistent with those of previous studies (Clements, 2003, 2006; Burke *et al.*, 2008; Xiang *et al.*, 2013). Twelve major clades were recognized within the Asian group, and all of these clades were recognized by Xiang *et al.* (2013) and Takamiya *et al.* (2014). However, the relationships among these clades are not well resolved and show a similar level of resolution to previous results (Clements, 2003, 2006; Burke *et al.*, 2008; Xiang *et al.*, 2013).

Age estimates

Family-level molecular dating indicated that Orchidaceae diverged at 93 Ma [95% highest posterior density (HPD): 83–102] and the four subfamilies (except subfamily Apostasioideae) diverged during the late Cretaceous (see Fig. S2 in Appendix S1). Divergence time analysis of *Dendrobium* using the combined data set (Fig. 2) showed that the stem age of *Dendrobium* was 30.34 Ma (95% HPD: 26.92–34.03), with the split between the Asian and Australasian clades occurring at 28.17 Ma (95% HPD: 25.1–31.63). The crown age of the Asian clade was 25.20 Ma (95% HPD: 20.96–29.31). Given the inferred credibility intervals of the divergence times, our estimates indicated that 10 clades/sections within the mainland Asian *Dendrobium* (i.e. all except clade VII and VIII) arose or diverged between the late Oligocene and the middle Miocene (Fig. 2; Table 1).

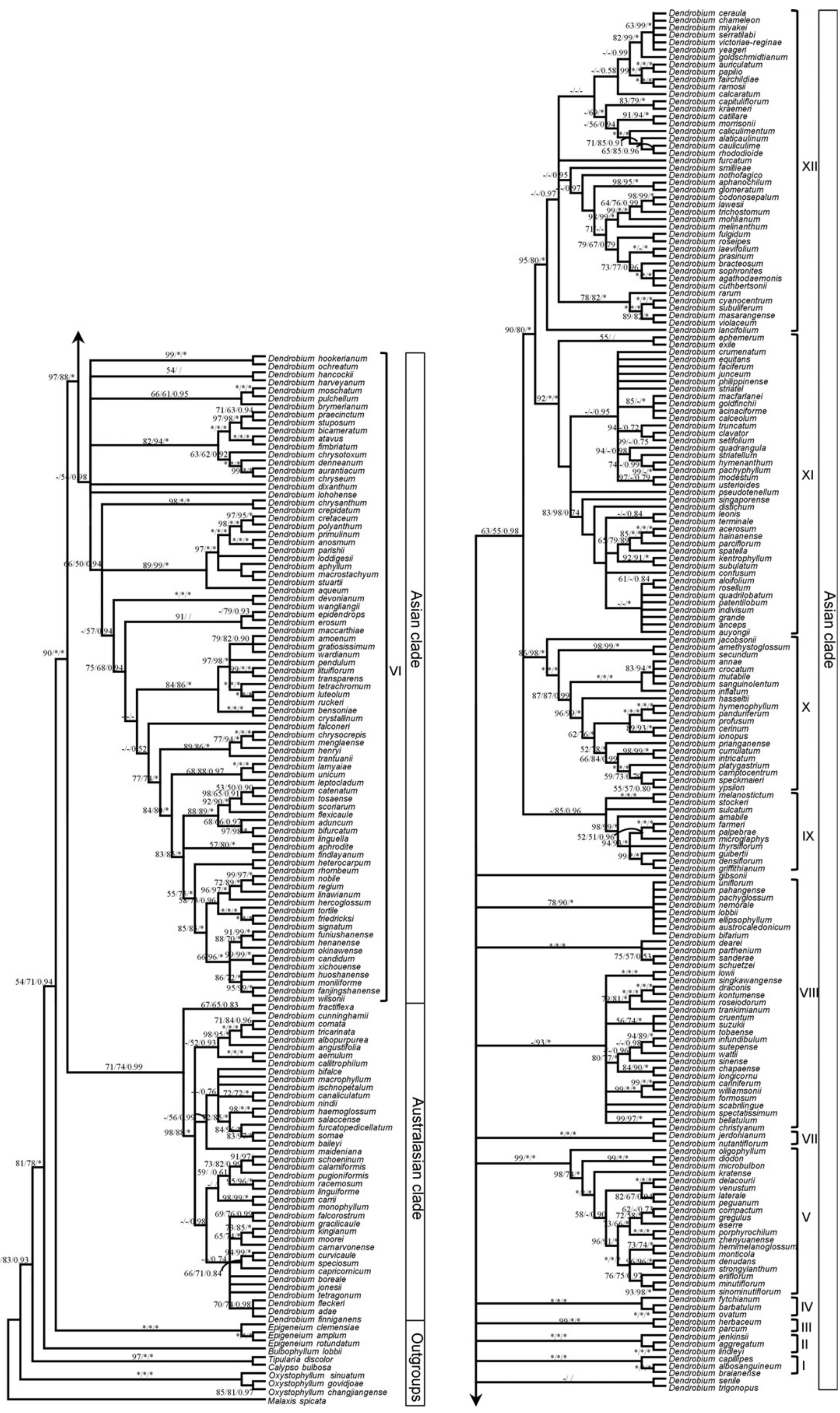

Dendrobium hookerianum
Dendrobium ochreatum
Dendrobium hancockii
Dendrobium harveyanum
Dendrobium moschatum
Dendrobium pulchellum
Dendrobium brymerianum
Dendrobium praecinctum
Dendrobium stuposum
Dendrobium bicameratum
Dendrobium atavus
Dendrobium fimbriatum
Dendrobium chrysotoxum
Dendrobium denneanum
Dendrobium aurantiacum
Dendrobium chryseum
Dendrobium dixanthum
Dendrobium lohohense
Dendrobium chrysanthum
Dendrobium crepidatum
Dendrobium cretaceum
Dendrobium polyanthum
Dendrobium primulinum
Dendrobium anosmum
Dendrobium parishii
Dendrobium loddigesii
Dendrobium aphyllum
Dendrobium macrostachyum
Dendrobium stuartii
Dendrobium aqueum
Dendrobium devonianum
Dendrobium wangliangii
Dendrobium epidendrops
Dendrobium erosum
Dendrobium maccarthiae
Dendrobium amoenum
Dendrobium gratiosissimum
Dendrobium wardianum
Dendrobium pendulum
Dendrobium lituiflorum
Dendrobium transparens
Dendrobium tetrachromum
Dendrobium luteolum
Dendrobium ruckeri
Dendrobium bensoniae
Dendrobium crystallinum
Dendrobium falconeri
Dendrobium chrysocrepis
Dendrobium menglaense
Dendrobium henryi
Dendrobium trantuanii
Dendrobium lamyaiae
Dendrobium unicum
Dendrobium leptocladum
Dendrobium catenatum
Dendrobium tosaense
Dendrobium scoriarum
Dendrobium flexicaule
Dendrobium aduncum
Dendrobium bifurcatum
Dendrobium linguella
Dendrobium aphrodite
Dendrobium findlayanum
Dendrobium heterocarpum
Dendrobium rhombeum
Dendrobium nobile
Dendrobium regium
Dendrobium linawianum
Dendrobium hercoglossum
Dendrobium tortile
Dendrobium friedricksi
Dendrobium signatum
Dendrobium funiushanense
Dendrobium henanense
Dendrobium okinawense
Dendrobium candidum
Dendrobium xichouense
Dendrobium huoshanense
Dendrobium moniliforme
Dendrobium fanjingshanense
Dendrobium wilsonii
Dendrobium fractiflexa
Dendrobium cunninghamii
Dendrobium comata
Dendrobium tricarinata
Dendrobium albopurpurea
Dendrobium angustifolia
Dendrobium aemulum
Dendrobium callitrophilum
Dendrobium bifalce
Dendrobium macrophyllum
Dendrobium ischnopetalum
Dendrobium canaliculatum
Dendrobium nindii
Dendrobium haemoglossum
Dendrobium salaccense
Dendrobium furcatopedicellatum
Dendrobium somae
Dendrobium baileyi
Dendrobium maideniana
Dendrobium schoeninum
Dendrobium calamiformis
Dendrobium pugioniformis
Dendrobium racemosum
Dendrobium linguiforme
Dendrobium carrii
Dendrobium monophyllum
Dendrobium falcorostrum
Dendrobium gracilicaule
Dendrobium kingianum
Dendrobium moorei
Dendrobium carnarvonense
Dendrobium curvicaule
Dendrobium speciosum
Dendrobium capricornicum
Dendrobium boreale
Dendrobium jonesii
Dendrobium tetragonum
Dendrobium fleckeri
Dendrobium adae
Dendrobium finniganens
Epigeneium clemensiae
Epigeneium amplum
Epigeneium rotundatum
Bulbophyllum lobbii
Tipularia discolor
Calypso bulbosa
Oxystophyllum sinuatum
Oxystophyllum govidjoae
Oxystophyllum changjiangense
Malaxis spicata
VI
Asian clade
Australasian clade
Outgroups
Dendrobium ceraula
Dendrobium chameleon
Dendrobium miyakei
Dendrobium serratilabi
Dendrobium victoriae-reginae
Dendrobium yeageri
Dendrobium goldschmidtianum
Dendrobium auriculatum
Dendrobium papilio
Dendrobium fairchildiae
Dendrobium ramosii
Dendrobium calcaratum
Dendrobium capituliflorum
Dendrobium kraemeri
Dendrobium catillare
Dendrobium morrisonii
Dendrobium caliculimentum
Dendrobium alaticaulinum
Dendrobium cauliculime
Dendrobium rhododioide
Dendrobium furcatum
Dendrobium smillieae
Dendrobium nothofagico
Dendrobium aphanochilum
Dendrobium glomeratum
Dendrobium codonosepalum
Dendrobium lawesii
Dendrobium trichostomum
Dendrobium mohlianum
Dendrobium melinanthum
Dendrobium fulgidum
Dendrobium roseipes
Dendrobium laevifolium
Dendrobium prasinum
Dendrobium bracteosum
Dendrobium sophronites
Dendrobium agathodaemonis
Dendrobium cuthbertsonii
Dendrobium rarum
Dendrobium cyanocentrum
Dendrobium subuliferum
Dendrobium masarangense
Dendrobium violaceum
Dendrobium lancifolium
Dendrobium ephemerum
Dendrobium exile
Dendrobium crumenatum
Dendrobium equitans
Dendrobium faciferum
Dendrobium junceum
Dendrobium philippinense
Dendrobium striatel
Dendrobium macfarlanei
Dendrobium goldfinchii
Dendrobium acinaciforme
Dendrobium calceolum
Dendrobium truncatum
Dendrobium clavator
Dendrobium setifolium
Dendrobium quadrangula
Dendrobium striatellum
Dendrobium hymenanthum
Dendrobium pachyphyllum
Dendrobium modestum
Dendrobium usterioides
Dendrobium pseudotenellum
Dendrobium singaporense
Dendrobium distichum
Dendrobium leonis
Dendrobium terminale
Dendrobium acerosum
Dendrobium hainanense
Dendrobium parciflorum
Dendrobium spatella
Dendrobium kentrophyllum
Dendrobium subulatum
Dendrobium confusum
Dendrobium aloifolium
Dendrobium rosellum
Dendrobium quadrilobatum
Dendrobium patentilobum
Dendrobium indivisum
Dendrobium grande
Dendrobium anceps
Dendrobium auyongii
Dendrobium jacobsonii
Dendrobium amethystoglossum
Dendrobium secundum
Dendrobium annae
Dendrobium crocatum
Dendrobium mutabile
Dendrobium sanguinolentum
Dendrobium inflatum
Dendrobium hasseltii
Dendrobium hymenophyllum
Dendrobium panduriferum
Dendrobium profusum
Dendrobium cerinum
Dendrobium ionopus
Dendrobium prianganense
Dendrobium cumulatum
Dendrobium intricatum
Dendrobium platygastrium
Dendrobium camptocentrum
Dendrobium speckmaieri
Dendrobium ypsilon
Dendrobium melanostictum
Dendrobium stockeri
Dendrobium sulcatum
Dendrobium amabile
Dendrobium farmeri
Dendrobium palpebrae
Dendrobium microglaphys
Dendrobium thyrsiflorum
Dendrobium guibertii
Dendrobium densiflorum
Dendrobium griffithianum
Dendrobium gibsonii
Dendrobium uniflorum
Dendrobium pahangense
Dendrobium pachyglossum
Dendrobium nemorale
Dendrobium lobbii
Dendrobium ellipsophyllum
Dendrobium austrocaledonicum
Dendrobium bifarium
Dendrobium dearei
Dendrobium parthenium
Dendrobium sanderae
Dendrobium schuetzei
Dendrobium lowii
Dendrobium singkawangense
Dendrobium draconis
Dendrobium kontumense
Dendrobium roseiodorum
Dendrobium trankimianum
Dendrobium cruentum
Dendrobium suzukii
Dendrobium tobaense
Dendrobium infundibulum
Dendrobium sutepense
Dendrobium wattii
Dendrobium sinense
Dendrobium chapaense
Dendrobium longicornu
Dendrobium canniferum
Dendrobium williamsonii
Dendrobium formosum
Dendrobium scabrilingue
Dendrobium spectatissimum
Dendrobium bellatulum
Dendrobium christyanum
Dendrobium jerdonianum
Dendrobium nutantiflorum
Dendrobium oligophyllum
Dendrobium diodon
Dendrobium microbulbon
Dendrobium kratense
Dendrobium delacourii
Dendrobium venustum
Dendrobium laterale
Dendrobium peguanum
Dendrobium compactum
Dendrobium gregulus
Dendrobium eserre
Dendrobium porphyrochilum
Dendrobium zhenyuanense
Dendrobium hemimelanoglossum
Dendrobium monticola
Dendrobium denudans
Dendrobium strongylanthum
Dendrobium eriiflorum
Dendrobium minutiflorum
Dendrobium sinominutiflorum
Dendrobium fytchianum
Dendrobium barbatulum
Dendrobium ovatum
Dendrobium herbaceum
Dendrobium parcum
Dendrobium jenkinsii
Dendrobium aggregatum
Dendrobium lindleyi
Dendrobium capillipes
Dendrobium albosanguineum
Dendrobium braianense
Dendrobium senile
Dendrobium trigonopus
XII
XI
X
IX
VIII
VII
V
IV
III
II
I
Asian clade

Figure 1 Strict consensus maximum parsimony tree of *Dendrobium* based on 319 species and five loci. Numbers on branches are maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) support values respectively. Asterisk (*) indicates support value at a node is 100%, dash (–) indicates support value at a node < 50%, and blank indicates that a node does not appear in the ML or BI trees.

Diversification analyses

The BDL analysis rejected the null hypothesis of temporally homogeneous diversification rates within the Asian clade ($\Delta AIC_{RC} = 5.1$, $P < 0.001$). The yule3rate model, which included two diversification rate shifts and no extinction, was found to best fit the data and suggest that *Dendrobium* started to diversify with a rate $r_1 = 0.2177$ sp Ma^{-1}. A decrease in the diversification rate to $r_2 = 0.1493$sp Ma^{-1} occurred at $ts_1 = 5.21$ Ma (95% HPD: 2.70–7.21), followed by a further decrease at $ts_2 = 0.33$ Ma (95% HPD: 0.02–1.35) to $r_3 = 0.0225$ sp Ma^{-1} (Fig. 3). The LTT plots also indicated that *Dendrobium* did not exhibit a constant speciation rate: an acceleration in speciation of the Asian lineages occurred from the late Oligocene to the middle Miocene with a slight decrease after the late Miocene.

Ancestral area reconstruction

The reconstructions obtained for the major clades of interest using the DEC model and S-DIVA were highly congruent (Table 1). The optimal ancestral area reconstructions under S-DIVA are shown in Fig. 2. Our results suggest that *Dendrobium* most likely originated in mainland Asia and then diverged into Malesia and Australasia (Fig. 2). The most recent common ancestor (MRCA) of the Asian group was likely located in mainland Asia (Fig. 2, Table 1). The MRCAs of clades X, XI and XII originated in Malesia (Fig. 2), which have been connected to mainland Asia since the Eocene (Lomolino *et al.*, 2005).

The reconstruction of *Dendrobium* elevation ranges (Fig. 4) illustrated that the early lineages were distributed primarily in low-elevation regions (< 1000 m) and then spread to higher elevations (≥ 1000 m) in both the Asian and Australasian clade. Of the 309 *Dendrobium* species sampled, 94.84% are typically epiphytic, 4.19% are lithophytic, and 0.97% are terrestrial. An epiphytic habit was the ancestral habit of *Dendrobium*, the lithophytic evolved independently 11 times in four clades and terrestrial habit evolved 3 times (see Fig. S3 in Appendix S1).

DISCUSSION

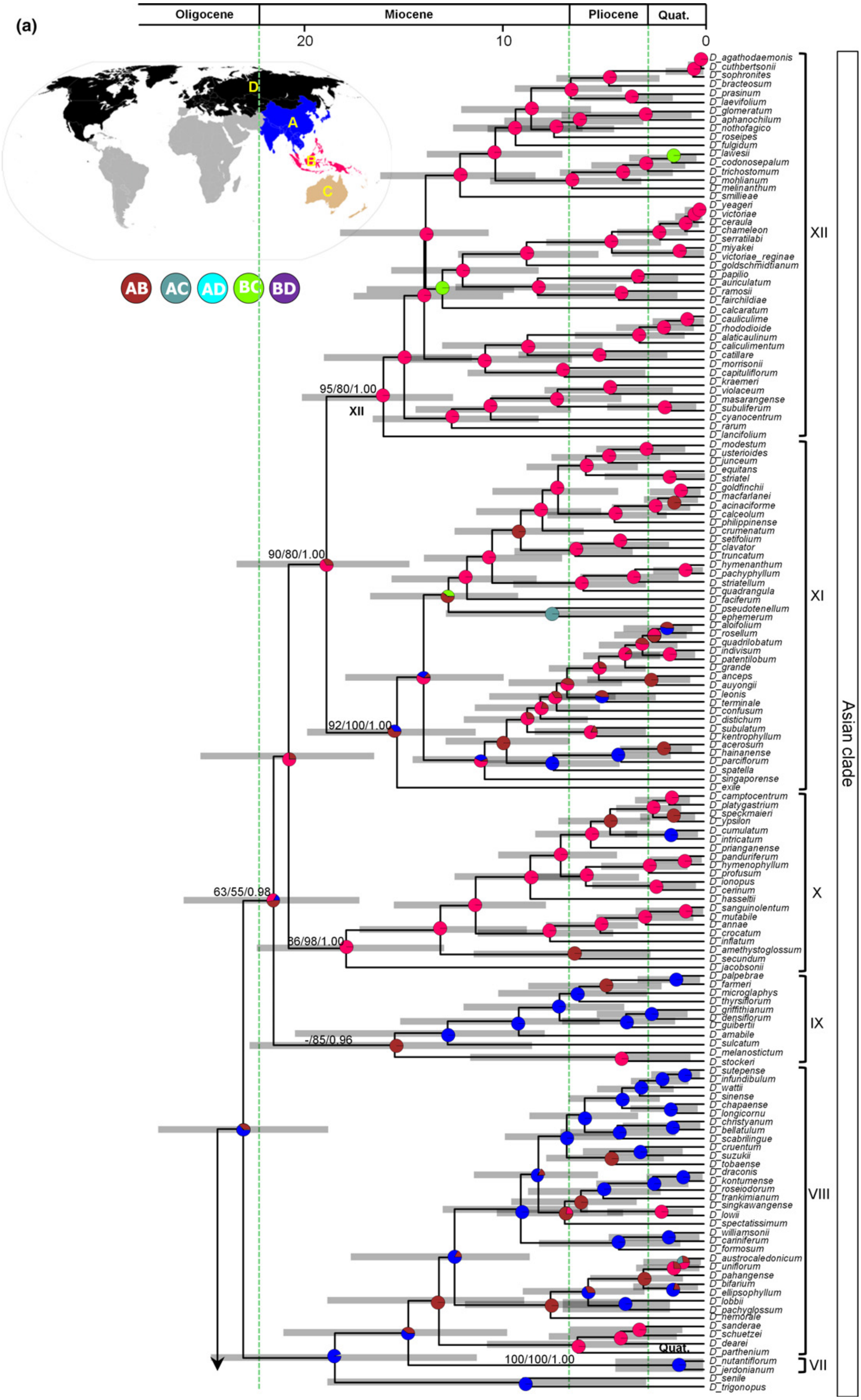
According to our results, the crown age of Orchidaceae diverged at 93 Ma (95% HPD: 83–102), and it is younger than the crown age estimated by Janssen & Bremer (2004) (111 Ma), but older than the estimates of Ramírez *et al.* (2007) and Bouetard *et al.* (2010) [76 ± 5 and 83 Ma (95% HPD: 68–104) respectively]. Four previous studies estimated the divergence times in Orchidaceae subfamilies (Ramírez *et al.*, 2007; Gustafsson *et al.*, 2010; Guo *et al.*, 2012; Givnish *et al.*, 2015). Our results for the divergence of the subfamilies are older than the results of Ramírez *et al.* (2007) and Gustafsson *et al.* (2010), but close to those of Guo *et al.* (2012) and Givnish *et al.* (2015) (see Table S3 in Appendix S1). The latter four studies of Orchidaceae subfamilies had different sampling strategies (60, 70, 200 and 201 taxa respectively), whereas we included 203 taxa. Our results indicated that similar sampling strategies have yielded similar molecular phylogenetic inferences and molecular dating.

The concatenated alignment (matrix 5, including 319 taxa and 1–5 loci for each taxon) contains 56.1% missing data, but it appears that this has limited impact on phylogenetic reconstruction as the backbone of the topology is consistent with that for other matrices with fewer taxa but less missing data (Fig. 1; Fig. S1 in Appendix S1) and also for other studies (Clements, 2003, 2006; Burke *et al.*, 2008; Xiang *et al.*, 2013; Takamiya *et al.*, 2014).

Dendrobium most likely originated in mainland Asia during the early Oligocene (30.34 Ma, 95% HPD: 26.92–34.03) (Fig. 2, Table 1). The results of the divergence time analysis indicated that the Asian clade started to diverge at 25.20 Ma (95% HPD: 20.96–29.31), soon after the initial origin of Asian *Dendrobium* in the middle Oligocene (28.17 Ma, 95% HPD: 25.10–31.63) (Fig. 2, Table 1). There were two important expansion events in the Asian group. First, in addition to the diversification of clades I–VII in Asian clade, the ancestor of clades X–XII dispersed from the mainland Asia into Malesia, and clades VIII and XI diversified in mainland Asia and the Malesia (Fig. 2). Second, Asian *Dendrobium* spread from low altitudes to higher altitudes from the beginning of the Miocene (Fig. 4).

The test for heterogeneity of the temporal diversification rate identified a model with no extinction and two rate shifts that fit the LTT plot (Fig. 3). The first rate change was at 5.21 Ma (95% HPD: 2.70–7.21), which was during the early Pliocene. The second rate change, a decrease at 0.33 Ma,

Figure 2 Timing of the diversification of *Dendrobium*. Chronogram derived from the maximum clade credibility tree estimated by using the uncorrelated exponential method in BEAST. Grey bars indicate 95% highest posterior density (HPD) intervals of the age estimates. The red star and orange star represent the root and fossil calibration node respectively (see Materials and methods). Clades I–XII represent the sections/clades recognized in Xiang *et al.* (2013) and Takamiya *et al.* (2014). Biogeographical region abbreviations: A, Mainland Asia; B, Malesia; C, Australasia; D, circumpolar.


(a)
Oligocene
Miocene
Pliocene
Quat.
20
10
0
D
A
E
C
AB
AC
AD
BC
BD
95/80/1.00
XII
90/80/1.00
92/100/1.00
63/55/0.98
86/98/1.00
-/85/0.96
Quat.
100/100/1.00
D_agathodaemonis
D_cuthbertsonii
D_sophronites
D_bracteosum
D_prasinum
D_laevifolium
D_glomeratum
D_aphanochilum
D_nothofagico
D_roseipes
D_fulgidum
D_lawesii
D_codonosepalum
D_trichostomum
D_mohlianum
D_melinanthum
D_smillieae
D_yeageri
D_victoriae
D_ceraula
D_chameleon
D_serratilabi
D_miyakei
D_victoriae_reginae
D_goldschmidtianum
D_papilio
D_auriculatum
D_ramosii
D_fairchildiae
D_calcaratum
D_cauliculime
D_rhododioide
D_alaticaulinum
D_caliculimentum
D_catillare
D_morrisonii
D_capituliflorum
D_kraemeri
D_violaceum
D_masarangense
D_subuliferum
D_cyanocentrum
D_rarum
D_lancifolium
D_modestum
D_usterioides
D_junceum
D_equitans
D_striatel
D_goldfinchii
D_macfarlanei
D_acinaciforme
D_calceolum
D_philippinense
D_crumenatum
D_setifolium
D_clavator
D_truncatum
D_hymenanthum
D_pachyphyllum
D_striatellum
D_quadrangula
D_faciferum
D_pseudotenellum
D_ephemerum
D_aloifolium
D_rosellum
D_quadrilobatum
D_indivisum
D_patentilobum
D_grande
D_anceps
D_auyongii
D_leonis
D_terminale
D_confusum
D_distichum
D_subulatum
D_kentrophyllum
D_acerosum
D_hainanense
D_parciflorum
D_spatella
D_singaporense
D_exile
D_camptocentrum
D_platygastrium
D_speckmaieri
D_ypsilon
D_cumulatum
D_intricatum
D_prianganense
D_panduriferum
D_hymenophyllum
D_profusum
D_ionopus
D_cerinum
D_hasseltii
D_sanguinolentum
D_mutabile
D_annae
D_crocatum
D_inflatum
D_amethystoglossum
D_secundum
D_jacobsonii
D_palpebrae
D_farmeri
D_microglaphys
D_thyrsiflorum
D_griffithianum
D_densiflorum
D_guibertii
D_amabile
D_sulcatum
D_melanostictum
D_stockeri
D_sutepense
D_infundibulum
D_wattii
D_sinense
D_chapaense
D_longicornu
D_christyanum
D_bellatulum
D_scabrilingue
D_cruentum
D_suzukii
D_tobaense
D_draconis
D_kontumense
D_roseiodorum
D_trankimianum
D_singkawangense
D_lowii
D_spectatissimum
D_williamsonii
D_cariniferum
D_formosum
D_austrocaledonicum
D_uniflorum
D_pahangense
D_bifarium
D_ellipsophyllum
D_lobbii
D_pachyglossum
D_nemorale
D_sanderae
D_schuetzei
D_dearei
D_parthenium
D_nutantiflorum
D_jerdonianum
D_senile
D_trigonopus
XII
XI
X
IX
VIII
VII
Asian clade

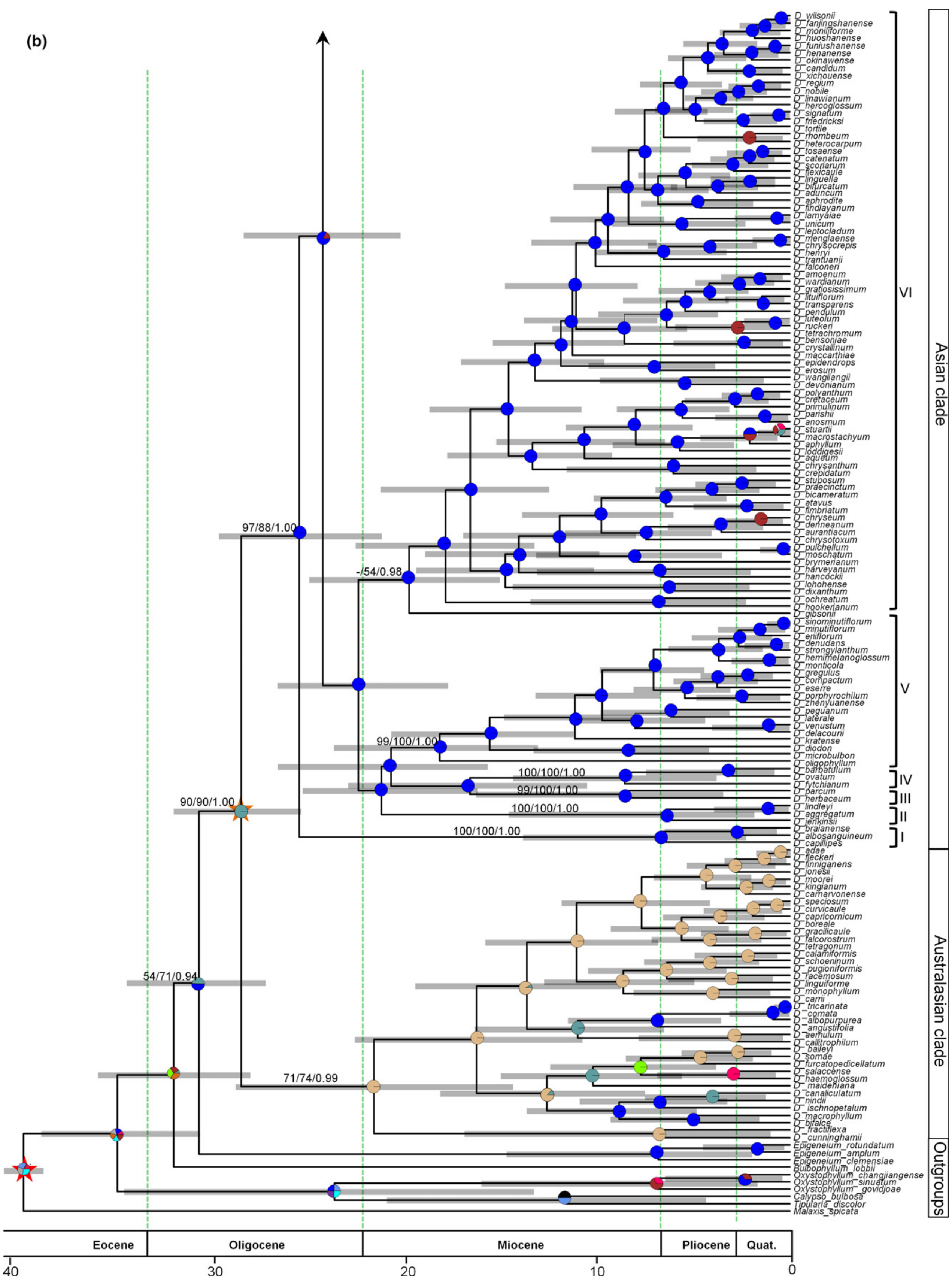


Figure 2 Continued.

Table 1 Clade support under maximum parsimony, maximum likelihood and Bayesian inference (MP, ML and PP respectively), divergence age estimates and ancestral area reconstructions for *Dendrobium*. RP, relative probability; MP, maximum probability. Clades names follow Figs 1 and 2; area codes follow Fig. 2 as follows: A, Mainland Asia; B, Malesia; C, Australasia; D, circumpolar.

			Likelihood-DEC (RP)			S-DIVA (MP)		
Clade	Clade support (MP/ML/PP)	BEAST (95% HPD) (Ma)	1	2	3	1	2	3
Dendrobium stem	54/71/0.94	30.34 (26.92–34.03)	A|A: 0.70	AC|A: 0.14		A: 0.51	AC: 0.49	
Dendrobium crown	90/100/1.00	28.17 (25.1–31.63)	A|A: 0.49	C|A: 0.31		AC		
I stem	NA	25.20 (20.96–29.31)	A|A: 0.87			A		
I crown	100/100/1.00	6.63 (2.29–13.74)	A|A: 0.85	A|AB: 0.15		A		
II stem	NA	20.99 (16.05–25.01)	A|A: 1			A		
II crown	100/100/100	6.39 (1.87–14.47)	A|A: 0.84	A|AB: 0.15		A		
III/IV stem	NA	16.45 (10.43–22.70)	A|A: 0.99			A		
III crown	99/100/1.00	8.52 (3.81–14.24)	A|A: 0.85	AB|A: 0.13		A		
IV crown	100/100/100	8.48 (3.47–16.14)	A|A: 1			A		
V stem	NA	20.49 (15.54–26.29)	A|A: 1			A		
V crown	99/100/1.00	18.00 (12.96–23.43)	A|A: 1			A		
VI stem	NA	22.17 (17.58–26.32)	A|A: 1			A		
VI crown	-/54/0.98	19.57 (14.91–24.68)	A|A: 1			A		
VII/VIII stem	NA	14.60 (9.74–20.74)	A|A: 0.49	AB|A: 0.34	B|A: 0.17	A: 0.63	AB: 0.37	
VII crown	100/100/1.00	13.10 (8.89–18.58)	AB|B: 0.72	B|B: 0.17	A|B: 0.10	B		
VIII crown	NA	1.56 (0.08–4.42)	A|A: 1			AB		
IX stem	NA	21.22 (17.01–25.65)	B|AB: 0.54	B|B: 0.22		AB:0.47	B:0.33	A:0.20
IX crown	-/85/0.96	15.26 (8.5–22.42)	A|B: 0.66	AB|B: 0.23	B|B: 0.12	AB		
X stem	NA	20.49 (16.47–24.84)	B|AB: 0.79	AB|B: 0.20		B		
X crown	86/98/1.00	17.66 (12.84–22.06)	B|B: 0.99			B		
XI/XII stem	90/80/1.00	18.63 (14.54–23.05)	B|B:0.688	AB|B:0.182	A|B:0.115	B:0.57	AB:0.43	
XI crown	92/100/1.00	15.16 (11.28–19.59)	A|AB:0.754	A|A:0.107		B:0.57	AB:0.43	
XII crown	95/80/1.00	15.82 (12.4–19.83)	B|B: 0.999			B		

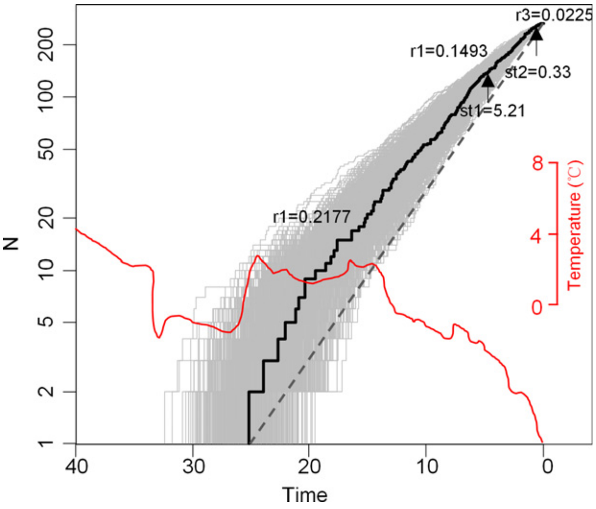


Figure 3 Semi-logarithmic lineage-through-time (LTT) plots and the rate shifts in Asian *Dendrobium* under the best rate-variable model. Grey lines represent the LTT plots for 1000 trees randomly selected from the BEAST analysis. The black line shows the maximum clade credibility tree. The red line represents the temperature curve since the Oligocene. The arrows highlight the two rate shifts identified, st_1 and st_2 are the time of the rate shift (in Ma) and r_1, r_2 and r_3 the three speciation rates inferred (in sp Ma^{-1}).

appeared as a flattening of the LTT plot as the time approached the present (Fig. 3) but might reflect sampling gaps as many species are younger than 5 Ma. Clearly, a high speciation rate has characterized the diversification of Asian *Dendrobium* through time.

Many factors, such as pollinators, mycorrhizal fungi and epiphytic habitats (e.g. EBLFs), may have affected the diversification of Orchidaceae (including *Dendrobium*) (Darwin, 1877; van der Cingel, 1995; Cozzolino & Widmer, 2005). Although few studies have focused on the roles of mycorrhizal fungi and the pollinators of this genus, recent results have indicated a low specificity of mycorrhizal fungi in some species of *Dendrobium* and in epiphytic orchids more generally (Xing *et al.*, 2013). Moreover, while several pollination mechanisms have been illustrated in *Dendrobium*, including autogamy, deceptive pollination and possible sleeping site rewards (Brodmann *et al.*, 2009; Kowalkowska & Margonska, 2012; Pang *et al.*, 2012), it seems that most *Dendrobium* species rely on deceptive pollination (van der Cingel, 2001; Jersáková *et al.*, 2006), which indicates that EBLFs provide rewards for deceived pollinators. The habitat of *Dendrobium*, is therefore likely crucial for supporting *Dendrobium* and their pollinators.

Furthermore, most species of *Dendrobium* in mainland Asia are strictly epiphytic plants in EBLFs and our reconstruction of habit indicates that the epiphytic state is ancestral in *Dendrobium*. The historical establishment and extension of EBLFs would have provided the necessary and potential habitats for the diversification of *Dendrobium* in mainland Asia and the diversification of *Dendrobium* is

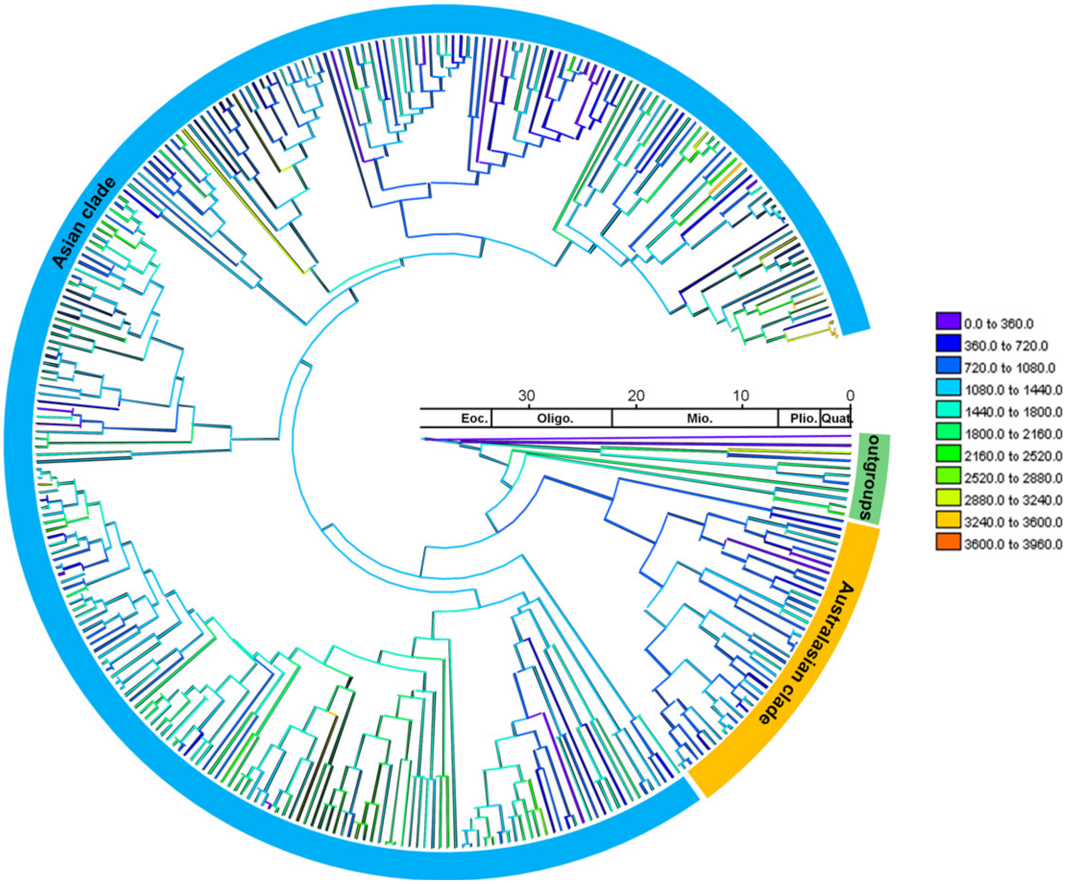


Figure 4 Ancestral reconstruction of *Dendrobium* elevation ranges. The order of the taxa is the same as in Fig. 2. Eoc., Eocene; Oligo., Oligocene; Mio., Miocene; Plio., Pliocene; Quat., Quaternary.

likely to reflect the minimum age of the establishment and extension of EBLFs. Our results indicated that Asian *Dendrobium* originated during the middle Oligocene, diversified mainly in mainland Asia and spread to Malesia by dispersal, followed by considerable expansion northwards and colonization of habitats up to at least 1400 m elevation during the late Oligocene (Fig. 4). This coincided with a warming climate during the late Oligocene to middle Miocene (see Zachos *et al.*, 2001). Fossil evidence has indicated that the EBLFs spread northwards to high latitudes and migrated to high altitudes during the warming climate of the Cenozoic (Zhou, 1999; Jacques *et al.*, 2011; Wang & Shu, 2013; Wen *et al.*, 2013). Furthermore, the distribution and diversity of several keystone families of EBLFs, such as Fagaceae, expanded widely during the early Cenozoic (Friis *et al.*, 1987, 2011; Zhou *et al.*, 2003). Molecular dating analyses indicated that Fagaceae evolved during the late Cretaceous and diversified during the early Cenozoic (Xiang *et al.*, 2014). Based on the findings of the present study, we suggest that the EBLF ecosystem in mainland Asia may have been present at higher altitudes and higher latitudes since the early Miocene.

CONCLUSION

Dendrobium, an epiphytic component of EBLFs, diverged at the beginning of the Oligocene and then radiated rapidly until the middle Miocene. The MRCA of Asian *Dendrobium* likely existed in mainland Asia, and then diversified *in situ* and spread northwards and into higher altitudes during the Miocene. A burst of diversification of the Asian *Dendrobium* group occurred during the late Oligocene to middle Miocene, which coincided with global warming. Moreover, the diversification of key groups of EBLFs (including Fagaceae, Lauraceae, Magnoliaceae and Theaceae) in the early Cenozoic provided new niches for *Dendrobium*. Based on these results, our multiple lines of evidence collectively support the hypothesis that EBLFs have been established in mainland Asia since the beginning of the Oligocene.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Additional tables (Tables S1–S3) and figures (Figs S1–S3).

Appendix S2 Nexus file for Orchidaceae dating.

BIOSKETCH

Xiaoguo Xiang is an assistant professor at the State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany (CAS). Her research is focused on understanding the temporal and spatial evolution of orchids by integrating phylogenetic, biogeographical, dating estimation and diversification approaches.

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