The reanalysis of biogeography of the Asian tree frog, *Rhacophorus* (Anura: Rhacophoridae): Geographic shifts and climatic change influenced the dispersal process and diversification

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Rapid uplifts of the Tibetan Plateau and climate change in Asia are thought to have profoundly modulated the diversification of most of the species distributed throughout Asia. The ranoid tree frog genus *Rhacophorus*, the largest genus in the Rhacophoridae, is widely distributed in Asia and especially speciose in the areas south and east of the Tibetan Plateau. Here, we infer phylogenetic relationships among species and estimate divergence times, asking whether the spatiotemporal characteristics of diversification within *Rhacophorus* were related to rapid uplifts of the Tibetan Plateau and concomitant climate change. Phylogenetic analysis recovered distinct lineage structures in *Rhacophorus*, which indicated a clear distribution pattern from Southeast Asia toward East Asia and India. Molecular dating suggests that the first split within the genus date back to the Middle Oligocene (approx. 30 Ma). The *Rhacophorus* lineage through time (LTT) showed that there were periods of increased speciation rate: 14 – 12 Ma and 10 – 4 Ma. In addition, ancestral area reconstructions supported Southeast Asia as the ancestral area of *Rhacophorus*. According to results of the molecular dating, ancestral area reconstructions and LTT, we think the geographic shifts , the staged rapid rises of the Tibetan Plateau with parallel climatic changes and reinforcement of the Asian monsoons (15 Ma, 8 Ma and 4 – 3 Ma), possibly prompted a burst of diversification in *Rhacophorus*.

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ABSTRACT

Rapid uplifts of the Tibetan Plateau and climate change in Asia are thought to have profoundly modulated the diversification of most of the species distributed throughout Asia. The ranoid tree frog genus *Rhacophorus*, the largest genus in the Rhacophoridae, is widely distributed in Asia and especially speciose in the areas south and east of the Tibetan Plateau. Here, we infer phylogenetic relationships among species and estimate divergence times, asking whether the spatiotemporal characteristics of diversification within *Rhacophorus* were related to rapid uplifts of the Tibetan Plateau and concomitant climate change. Phylogenetic analysis recovered distinct lineage structures in *Rhacophorus*, which indicated a clear distribution pattern from Southeast Asia toward East Asia and India. Molecular dating suggests that the first split within the genus date back to the Middle Oligocene (approx. 30 Ma). The *Rhacophorus* lineage through time (LTT) showed that there were periods of increased speciation rate: 14 – 12 Ma and 10 – 4 Ma. In addition, ancestral area reconstructions supported Southeast Asia as the ancestral area of *Rhacophorus*. According to results of the molecular dating, ancestral area reconstructions and LTT, we think the geographic shifts, the staged rapid rises of the Tibetan Plateau with parallel climatic changes and reinforcement of the Asian monsoons (15 Ma, 8 Ma and 4 – 3 Ma), possibly prompted a burst of diversification in *Rhacophorus*.

INTRODUCTION

Abiotic factors like climatic and tectonic events, and biotic factors like inter- or intraspecific

39 interactions, competition and predation, may be the predominant driving factors during the
40 evolution and diversification of organisms (Antonelli & Sanmartín 2011; Benton 2009). These
41 factors can also affect the diversification at different temporal and geographical scales (Benton
42 2009). Understanding the processes of diversification and which factors have driven the
43 evolution and diversification of organisms, may help guide prioritization in conservation and
44 forecast the population demography under future climate conditions (Avise 2000; Frankham et al.
45 2002). It had been shown that orogenic activity during recent geological history was linked to the
46 formation of hotspots of biodiversity (Myers et al. 2000). Consequently, the study of the origin
47 and evolution of biodiversity in mountain systems has experienced a growing scientific interest
48 (Klaus et al. 2016; van der Meijden et al. 2007; Zhang et al. 2006). In Asia, the uplift of the
49 Tibetan Plateau was the most intense orogenic movement leading to the formation of several
50 biomes (Favre et al. 2015; Klaus et al. 2016; Myers et al. 2000; Yang et al. 2009). Therefore,
51 research has focused on the uplift of the Tibetan Plateau, based on a temporal (molecular dating)
52 and spatial (biogeographic) framework, which may have triggered a series of evolutionary
53 changes in different biological groups (Klaus et al. 2016), such as in plants (Gao et al. 2013;
54 Jabbour & Renner 2012; Tu et al. 2010; Wang et al. 2009), birds (Lei et al. 2014; Tietze &
55 Borthakur 2012; Tietze et al. 2013), mammals (Deng et al. 2011) and amphibians (Che et al.
56 2010; Guo et al. 2011; Li et al. 2013; Vijayakumar et al. 2016; Zhang et al. 2006).

57 The ranoid treefrog genus, *Rhacophorus*, is the largest genus in the Rhacophoridae,
58 currently containing 88 species (Frost 2016), which are widely distributed across India, China,
59 Japan, mainland South-east Asia, the Greater Sunda Islands and the Philippines (Frost 2016). A

previous study disclosed that Rhacophoridae underwent an early dispersal from India to Asia between 46 and 57 Ma, that a transient faunal exchange ceased during the Middle Eocene, and a subsequent increase of Rhacophorid dispersal events between Asia and the Indian subcontinent during the Oligocene that continued until the Middle Miocene (Li et al. 2013). Uplift of the Tibetan Plateau and a series of climatic and environmental changes led to many speciation events on a very large scale (Favre et al. 2015; Myers et al. 2000; Yang et al. 2009). *Rhacophorus* taxa are widely distributed across the areas around the Tibetan Plateau, according to previous study (Li et al. 2013), the speciation process in this genus may be linked to the uplift of Tibetan Plateau during the Miocene and Pliocene.

To gain a better understanding of the diversification processes in biomes around the Tibetan Plateau, we herein provide a historical biogeographic pattern of *Rhacophorus*. In the present study, we collected all the sequences datasets of Asian *Rhacophorus* that have been reported in addition to newly sequenced DNA from *Rhacophorus* specimens collected from the Dabie Mountains in Anhui, China. We infer the phylogenetic relationships within the genus and estimate the divergence times. Further, the correlation between diversification events within *Rhacophorus* and the geographic shifts in the Tibetan Plateau are explored.

MATERIALS AND METHODS

Ethical Statement

The collection of samples was performed within a long-term investigation project on

amphibians of Dabie mountains. This investigation project and the sample collection were approved by Anhui Tianma National Nature Reserve, Anhui Province, China. The relevant document of field permit was provided in the supplementary material.

Data Collection

For the phylogenetic analyses, sequences of about half the species of *Rhacophorus* were used in combination with sequences of two outgroup species, *Polypedates megacephalus* (Rhacophoridae, *Polypedates*) and *Spinomantis peraccae* (Mantellidae, *Spinomantis*) (Li et al. 2013; Li et al. 2012a; Li et al. 2012b). Sequence data were obtained from GenBank (the GenBank Accession numbers are given in Table S1). In total, there were 149 individuals of 57 species of *Rhacophorus* involved (Fig. 1 and Table S1). All the taxonomic revisions within *Rhacophorus* were follow previous studies (Biju et al. 2013; Li et al. 2013; Orlov et al. 2012).

Sampling, DNA Extraction, PCR Amplification, and Sequencing

Between 2012 to 2015, nine specimens of *R. zhoukaiyae* were collected from the Dabie Mountains, China (Pan et al. 2017). Muscle tissue from each individual was sampled and preserved in 100% ethanol for DNA extraction. Total DNA was extracted from the samples using a standard proteinase K/phenol-chloroform protocol (Sambrook et al. 1989). An EasyPure PCR Purification Kit (TransGene) was used to purify the DNA extractions. The sequences of 12S and 16S ribosome RNA (rRNA) of *R. zhoukaiyae* were collected from Pan et al. (2017). In

addition, we also amplified and sequenced five nuclear gene fragments with the indicated primer pairs (Table S2), including brain-derived neurotrophic factor (BDNF), proopiomelanocortin (POMC), recombination activating gene 1 (RAG-1), rhodopsin exon 1 (RHOD) and tyrosinase exon 1 (TYR) (Bossuyt & Milinkovitch 2000; Li et al. 2009; van der Meijden et al. 2007; Vieites et al. 2007; Wiens et al. 2005). Polymerase chain reactions (PCR) were performed using a reaction mixture (25 μL) containing 1 μL genomic DNA (concentration 10 – 50 ng/μL), 2.5 μL 10×buffer, 1 μL of 2.5 mM MgSO4, 2 μL of 2 mM dNTPs, 1 U *Taq* polymerase (Meridian Bioscience, Singapore) and 0.3 mM of each of the primers. Pure molecular biology grade water was added to reach the appropriate volume. The amplification protocol included an initial denaturation step of 95°C for 5 min; this was followed by 32 cycles of denaturation at 95°C for 30 s, primer annealing at 51°C – 57°C for 30 s, and an extension at 72°C for 40 s – 80 s, with a final extension at 72°C for 10 min. PCR products were purified using an EasyPure PCR Purification Kit (TransGene) and sequenced using previous primers and the BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) following the manufacturer's instructions on an ABI Prism 3730 automated sequencer. All the sequences obtained in this study were deposited into GenBank (Table S1). For the analyses, the sequences were trimmed to match data downloaded from GenBank, then all the sequences were aligned automatically using Clustal X version 1.83 (Thompson et al. 1997), followed by visual confirmation and manual adjustments. Nucleotide sites with ambiguous alignments were removed from the analyses, and gaps were analyzed as missing data.

118 **Phylogenetic Analyses**

119 Two different datasets were generated for the different analyses. Dataset 1 was used for a
120 phylogenetic analysis of *Rhacophorus* by Maximum Likelihood (ML) and Bayesian methods,
121 was comprised of the 12S and 16S rRNA gene together with the complete t-RNA for the valine
122 sequence of the *Rhacophorus* species and the outgroups (Table S1). Dataset 2 contained more
123 genes (12S, 16S, Val, BDNF, POMC, RAG-1, RHOD, TYR) of more individual and species
124 than Dataset 1 (Table S1). However, it was only used to calculate a Bayesian consensus tree. The
125 best-fit model of evolution was calculated by MrModeltest 1.0 b under the AIC criterion
126 (Nylander 2003). ML analyses were performed in RAxML version 8 (Stamatakis 2014) and a
127 general time reversible model of nucleotide substitution under the Gamma model of rate
128 heterogeneity (i.e., GTRCAT). Support for the internal branches for the best-scoring tree was
129 evaluated via the bootstrap test with 1,000 iterations. A Bayesian inference of phylogeny was
130 performed using the MrBayes 3.1.2 software program (Huelsenbeck & Ronquist 2005), using the
131 best-fit substitution model. Two Markov Chain Monte Carlo (MCMC) models were run to
132 provide additional confirmation of the convergence of posterior probability distributions.
133 Analyses were run for 3,000,000 generations. Chains were sampled every 1000 generations. The
134 first 25% of the total trees were discarded as "burn-in" and the remaining trees were used to
135 generate a majority-rule consensus tree and to calculate Bayesian posterior probabilities.

136 **Divergence Time Analyses**

137 To estimate divergence times of *Rhacophorus*, we applied a Bayesian MCMC method with
138 mitochondrial genes (Dataset 1), which employs a relaxed molecular clock approach, as
139 implemented in BEAST 1.7.4 (Drummond et al. 2012). We assumed a relaxed uncorrelated log
140 normal model of lineage variation and a Yule Process prior to the branching rates based on the
141 GTR + I + G model as recommended by MrModeltest 1.0 b (Nylander 2003). Four replicates
142 were run for 10,000,000 generations with tree and parameter sampling every 1,000 generations.
143 The first 25% of samples were discarded as burn-in. All parameters were assessed by visual
144 inspection using Tracer v. 1.5 (Rambaut & Drummond 2007). The tree was generated and
145 visualized with TreeAnnotator v. 1.6.1 (Rambaut & Drummond 2010) and FigTree v. 1.3.1
146 (Rambaut 2009), respectively. Calibration points were taken from Li et al. (2013) (Table 1). In
147 addition, to visualizing the temporal accumulation of species, a log-transformed lineage-through-
148 time (LTT) (Nee et al. 1994) plot was constructed and compared with the null distribution for the
149 LTT line simulated under the empirical pure-birth model. For visualizing diversification rate
150 changes, we plotted the number of newly appearing species against the fixed time intervals of 2
151 million years (Ma) (Venditti et al. 2010).

152 **Ancestral Area Reconstructions**

153 Ancestral area reconstructions were inferred by the program RASP 3.2 (Yu et al. 2015) for
154 speciational evolution in phylogenetic trees, using the Bayesian Binary MCMC (BBM) method

155 (Ronquist & Huelsenbeck 2003) and the statistical dispersal-vicariance method (S-DIVA) (Yu et
156 al. 2010). To reconstruct ancestral areas on the basis of the topography, the distributional range
157 of Asian *Rhacophorus* was divided into four regions, W, X, Y and Z (Fig. 2). W represents
158 Southeast Asia, including the Indochinese Peninsula, Sundaland and the south margin of the
159 Tibetan Plateau, X contains the Hengduan mountains and the mountains around the Sichuan
160 Basin, Y refers to South China and Japan and Z represents India (Fig. 2). The tree data sets and
161 the condensed tree were generated by BEAST 1.7.4 (Drummond et al. 2012). The distribution of
162 each species was collected from http://maps.iucnredlist.org. For all analyses, the maximum
163 number of ancestral areas at each node was constrained to three. The frequencies of an ancestral
164 range at a node were averaged over all trees and each alternative ancestral range at a node was
165 weighted by the frequency of occurrence for the node.
166

167 **RESULTS**

168 **Molecular Phylogenetic Analyses**

169 The aligned mtDNA gene fragments from *Rhacophorus* consisted of 1935 bp nucleotide
170 positions before trimming (Dataset 1). After trimming, 1851 nucleotide positions were retained
171 for genealogical reconstructions. The fragments contained 934 constant and 917 potentially
172 phylogenetically informative characters. The ML or BI phylogenetic approaches based on
173 Dataset 1 resulted in virtually identical topology, and all terminal clades obtained relatively high-

174 supporting values (Fig. S1). The genus *Rhacophorus* was supported as monophyletic containing
175 four major clades (Fig. S1). For further probing of the dispersal process and diversification of the
176 Asian tree frog, the molecular dating and ancestral area reconstructions were carried out. The
177 phylogenetic tree, collected from the molecular dating, showed three distinct clades (A, B and C)
178 in the genus of *Rhacophorus* (Fig. 2). There were some difference in the species distribution
179 areas among the three clades. Species in clade A were mostly distributed in Southeast Asia and
180 East Asia, species in Clade B were distributed in Southeast Asia and India, and species in lineage
181 C only found in Southeast Asia. Clade A contained six groups, A1 to A6 (Fig. 2). The
182 phylogenetic tree, based on Dataset 2, was largely consistent with the results from Dataset 1 (Fig.
183 S2). However, there were some minor differences between them, such as the polyphyletic of
184 clade B and C in Fig. S2. But, generally, it did not affect the results of ancestral area
185 reconstructions of *Rhacophorus*.
186

187 **Molecular Dating, Ancestral Area Reconstructions and Lineage Through Time**

188 Dating analyses based on Dataset 1 suggested that the most recent common ancestor
189 (MRCA) of *Rhacophorus* dates back to 29.51Ma (median value; 95% of the highest posterior
190 density [HPD] = 25.00 – 34.07Ma) (Table 1 and Fig. 3).The MRCA of Clade A and Clade B was
191 estimated at 27.38Ma (95% HPD = 22.44 – 32.17 Ma). The MRCA of Clade A was 21.56 Ma
192 (95% HPD = 17.92 – 25.22 Ma) and the MRCA of Clade B was 26.73 Ma (95% HPD = 21.56 –
193 31.83 Ma).

194 Ancestral area reconstructions from S-DIVA and BBM analyses were largely similar with
195 some minor differences (Fig. 2). All analyses supported Southeast Asia (Area W, Fig. 2) as the
196 ancestral area of *Rhacophorus* and most speciation events were attributed to dispersal. The
197 empirical LTT plot of *Rhacophorus* showed that, after a lengthy period of constant
198 diversification, the diversification rate of the genus had increased during the middle Pliocene.
199 The cumulative curve of species-birth per time interval showed that the diversification of
200 *Rhacophorus* fluctuated through time, especially during 14 – 12 Ma and 10 – 4 Ma (Fig. 4).

201 **DISCUSSION**

202 **The Dispersal Process of *Rhacophorus* and Its Spread toward East Asia and India**

203 Previous studies have indicated that the diversification of Rhacophoridae was closely linked
204 to the India-Asia collision (57 Ma – 35 Ma) (Li et al. 2013). Southeastern Asia houses three
205 globally significant hot spots divided by sharp, yet porous biogeographic boundaries (Evans et al.
206 2003; Favre et al. 2015; Schmitt et al. 1995; Wallace 1860). Studies have shown that the
207 dynamics of the formation of biodiversity in Southeastern Asia is assumed to be interrelated with
208 many geological events and a unique climatic history,. Events such as the continuing processes
209 of volcanic uplift and the emergence of many new islands in Indo-Australian Archipelago during
210 the Miocene-Pliocene (Fig. 3iii) (Esselstyn et al. 2009; Hall 1996; Hall 1998; Hall 2002; Lohman
211 et al. 2011), the rapid uplifts of the Tibetan Plateau (Shi et al. 1999), repeated sea level
212 fluctuations during the Pleistocene (Bird et al. 2005; Esselstyn et al. 2009; Hall 1998; Heaney

1985; Heaney 1986; Jansa et al. 2006; Voris 2000) and the onset of the Asian monsoon system (An et al. 2001; Qiang et al. 2001; Sun & Wang 2005; Zhisheng et al. 2001). Many phylogeographical studies of plants and animals support this assumption (Deng et al. 2011; Klaus et al. 2016; Shi et al. 1999), such as those on *Lilium* (Gao et al. 2013), Delphinieae (Jabbour & Renner 2012), Hyoscyameae (Tu et al. 2010), Mandragoreae (Tu et al. 2010), *Saussurea* (Wang et al. 2009), birds (Lei et al. 2014; Tietze & Borthakur 2012; Tietze et al. 2013; Yang et al. 2009), Hynobiidae (Zhang et al. 2006), lizards (Guo et al. 2011) and Spiny Frogs (Che et al. 2010), so the diversification and speciation in *Rhacophorus* may also be related to the special geological formations and the climatic history.

The phylogenetic analysis shows that *Rhacophorus* is composed of multiple lineages. In the phylogenetic tree with timescale, calculated by BEAST, *Rhacophorus* is composed of three major clades, A, B and C (Fig. 2). Among these clades, Clade C was the basal branch of *Rhacophorus*, which contained ten species from Southeast Asia, and the age of the MRCA of *Rhacophorus* was estimated at 29.51 Ma (i.e., 95% CI, 25 – 34.07 Ma, Fig. 2 and Table 1). The MRCA of Clades B and Clade A was 27.38 Ma (95% CI, 22.44 Ma – 32.17 Ma) during the Oligocene (Fig. 2, Table 1). The members of Clades A and B are mainly distributed in the south of the Tibetan Plateau margin, India and Eastern Asia (Fig. 1). Clade A contained six groups which were distributed in three areas: Southeast Asia (group A1), the south of the Tibetan Plateau margin (group A2) and an Eastern Asia (group A3 to A6) (Fig. 1, 2). The MRCA of Clade A occurred 21.56 Ma ago (95% CI, 17.92 – 25.22 Ma; Fig. 2) and the time of the split of different groups was estimated at 14.09 Ma (A2 vs A3 ~A6, 95% CI, 10.96 – 17.41 Ma), 11.39

234 Ma (A3 vs A4 ~ A6, 95% CI, 8.89 – 14.16 Ma), 8.56 Ma (A4 vs A5 ~ A6, 95% CI, 6.43–10.88
235 Ma) and 5.33 Ma (A5 vs A6, 95% CI, 3.92 – 6.99 Ma) respectively (Table 1). In addition, the
236 LTT plot analysis indicated an increased diversification rate during two periods (14 – 12 Ma and
237 10 – 4 Ma) (Fig. 4). Basically, the above mentioned phylogeographical information reflected the
238 trend of diversification and the speciation process. Obviously, the distribution of these species
239 expanded continuously from Southern Asia to India and Eastern Asia, reaching as far as Japan
240 (Fig. 2).

241 During the Oligocene and Miocene the uplift progressed, causing the extension of the
242 Tibetan Plateau (Harrison et al. 1992; Mulch & Chamberlain 2006). The start of the uplift of the
243 northern Tibetan Plateau occurred at about 30 Ma BP (Sun & Wang 2005) or slightly earlier
244 (Wang et al. 2012b). Then, the eastern parts of the Tibetan Plateau likely reached an elevation
245 comparable to the present-day elevation in the Mid to Late Miocene (from 15 to 5 Ma) (Axelrod
246 1997; Currie et al. 2005; Jacques et al. 2011; Spicer et al. 2003; Tapponnier et al. 2001; Valdiya
247 1999; Zhang et al. 2013). The southeastern edge of the Tibetan Plateau, the Hengduan mountain
248 range, experienced rapid uplift only after the Miocene (5.33 Ma), reaching a peak elevation
249 shortly before the Late Pliocene (5.33 – 2.66 Ma) (Li & Fang 1999; Mulch & Chamberlain 2006;
250 Sun et al. 2011; Zheng et al. 2000), which separated several major rivers that ran in parallel (the
251 Yangtze, Mekong, and Salween valleys) (Clark et al. 2004). This series of rapid Tibetan Plateau
252 uplifts dramatically changed the terrain and landform in this area, which resulted in speciation,
253 especially in animal groups (Che et al. 2010; Deng et al. 2011; Gao et al. 2013; Jabbour &
254 Renner 2012; Lei et al. 2014; Li et al. 2013; Shi et al. 1999; Tietze & Borthakur 2012; Tietze et

al. 2013; Tu et al. 2010; Wang et al. 2009; Zhang et al. 2006). Zhang et al. (2006) found that the origin and phylogenetic divergence of the Hynobiidae had a correlation to the uplift of the Tibetan Plateau (Zhang et al. 2006). The phylogenetic history of Paini (Anura: Dicroglossidae) illuminates a critical aspect of the timing of geological events, especially for the uplift of the Tibetan Plateau (Che et al. 2010). On the other hand, the Tibetan Plateau and its adjacent mountain ranges acted as an orographic barrier to atmospheric circulation in Asia and consequently contributed to the formation of the Asian monsoon system, which was one of the major climatic changes in this region (Early Miocene, 24 Ma) due to the Tibetan Plateau's considerable size and altitude (Guo et al. 2008; Kutzbach et al. 1993; Liu & Yin 2002; Ruddiman & Kutzbach 1991; Song et al. 2010; Sun & Wang 2005; Tang et al. 2013; Zhisheng et al. 2001). In the following millions of years, the East Asian monsoon intensified three times (~15 Ma, ~8 Ma and 4 – 3 Ma) (An et al. 2001; Jacques et al. 2011; Molnar et al. 2010; Song et al. 2010; Sun & Wang 2005; Valdiya 1999; Wan et al. 2007; Zhisheng et al. 2001). The development of the Asian monsoon system directly gave birth to the warm and humid climate in the south of China (Sun & Wang 2005), which was maybe favorable for the geographical spread and speciation of amphibians (Che et al. 2010; Thorn & Raffaėlli 2001; Wu et al. 2013; Zhang et al. 2006). In addition, the climate oscillations that began about 2.8 million years ago, in the Late Pliocene (Deng et al. 2011), also provided the chance for diversification and speciation of many species (Zhang et al. 2000), such as birds (Lei et al. 2014), the Tibetan woolly rhino (*Coelodonta thibetana*) (Deng et al. 2011) and stream-dwelling frog (*Feirana quadranus*) (Wang et al. 2012a). Molecular dating suggested that the TMRC of Clade A and Clade B was during the Oligocene

(22.44 Ma – 32.17 Ma) (Fig. 3, Table 1). At same time, ancestral area reconstructions supported Southeast Asia (W) as the ancestral area of *Rhacophorus* and the dispersal events happened from ancestral area of Clade A and Clade B (Fig. 2, node b). In addition, the land and sea in the Indo-Australian Archipelago changed greatly during this period (Lohman et al. 2011), which may promote the dispersal events from Southeast Asia. In Clade A, the time of the split of subgroups was estimated from 14.09 to 5.33 Ma (Table 1). In addition, the time of most nodes in Clade B also occurred during this period (Fig. 3). Based on the LTT plot analysis, there were two increased diversification rate periods (14 – 12 Ma, 10 – 4 Ma) in *Rhacophorus* (Fig. 4). The series of Tibetan Plateau rapid uplifts (from 15 to 2.66 Ma) dramatically changed the landscape, which resulted in the diversification of species or speciation in this area (Che et al. 2010; Deng et al. 2011; Gao et al. 2013; Jabbour & Renner 2012; Lei et al. 2014; Li et al. 2013; Shi et al. 1999; Tietze & Borthakur 2012; Tietze et al. 2013; Tu et al. 2010; Wang et al. 2009; Zhang et al. 2006) and the biotic interchange between the Indian subcontinent and mainland Asia (Klaus et al. 2016). In addition, the intensified East Asian monsoon (~15 Ma, ~8 Ma and 4 – 3 Ma) directly gave birth to the warm and humid climate in the south of China, which was favorable for the geographical spread and speciation of amphibians (Che et al. 2010; Thorn & Raffaėlli 2001; Wu et al. 2013; Zhang et al. 2006). Obviously, the diversification events in *Rhacophorus* were in line with the time frame of the orogenic movement and climatic histories, especially the staged rapid uplift of the Tibetan Plateau and the enhanced Asian monsoon system (Figs. 2 and 3). Therefore, we think that the diversification and speciation events in Clade A and Clade B, are related to the staged uplift of the Tibetan Plateau and the subsequent chain-reaction events, such as the

297 establishment of the Asian monsoon system, which facilitated the radiations and speciation of
298 amphibians (Che et al. 2010; Thorn & Raffaėlli 2001; Wu et al. 2013; Zhang et al. 2006).
299 Overall, the evolutionary history of *Rhacophorus* originated approx 30 Ma Bp (Oligocene).
300 Basically, it is the dispersal process from its ancestral area, Southeast Asia, toward India and
301 East Asia. During the process, *Rhacophorus* diversified by multiple factors, such as geographic
302 shifts, the staged rapid rises of the Tibetan Plateau with parallel climatic changes, the
303 reinforcement of the Asian monsoons (15 Ma, 8 Ma and 4–3 Ma) and alternating glacial-
304 interglacial oscillations.
305

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Figure 1(on next page)

Sample sites of *Rhacophorus* species used in this study.

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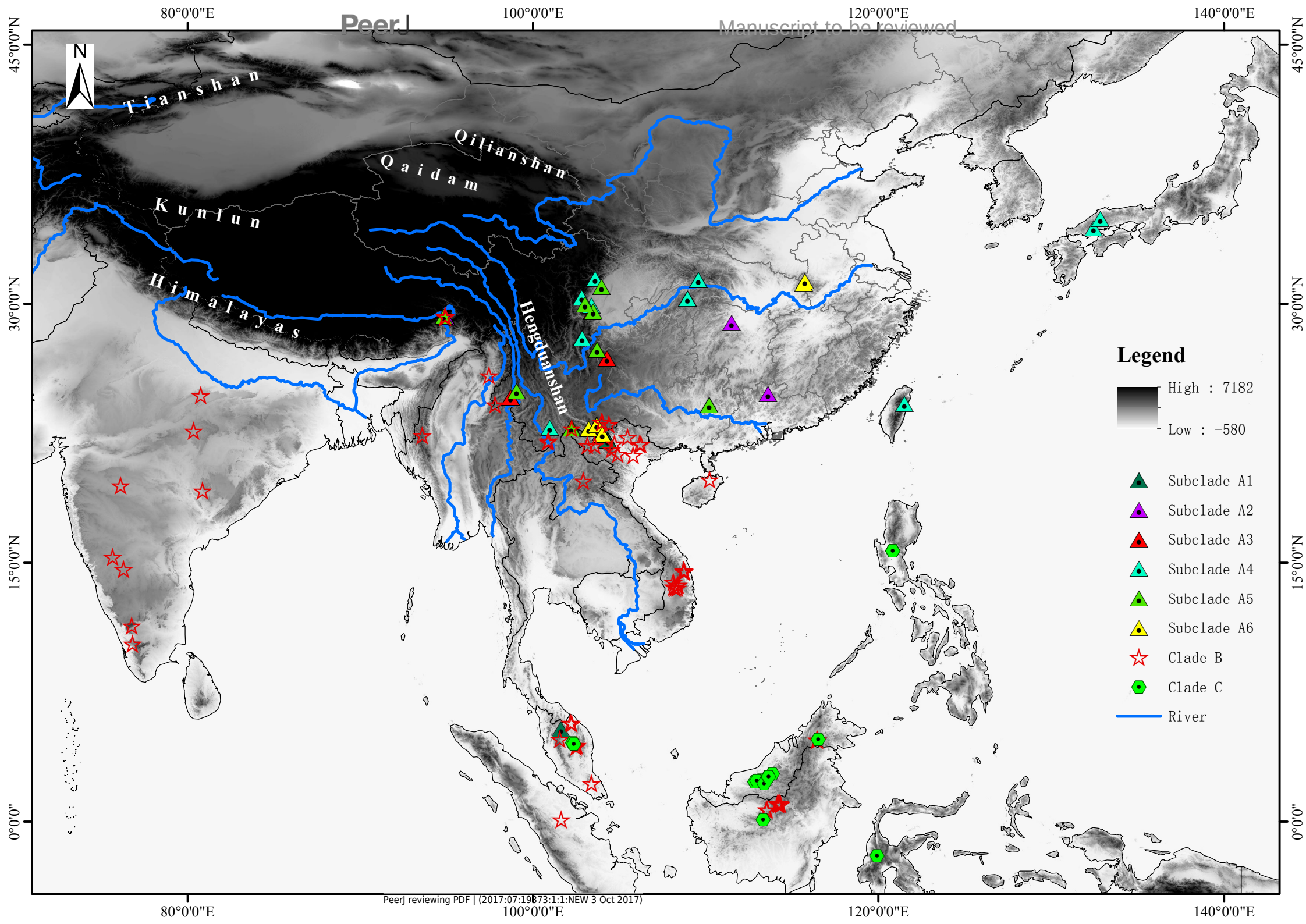

Figure 2(on next page)

Chronogram and ancestral area reconstructions of *Rhacophorus* with outgroup species, *Polypedates megacephalus* and *Spinomantis peraccae* based on Dataset 1.

Branches in the tree are proportional to absolute ages (Ma). Node charts showed the relative probabilities of alternative ancestral distributions obtained by integrating the statistical dispersal-vicariance analysis (S-DIVA; above branches) and a Bayesian Binary MCMC method (BBM; below branches), and the first two areas with highest probability are shown corresponding to their relative probability on the area of one circle. Areas are divided for reconstructing ancestral areas. (W) Southeast Asia, including the Indochinese Peninsula, Sundaland, and the south margin of the Tibetan Plateau; (X) Hengduan mountains and the mountains around the Sichuan Basin; (Y) South China and Japan; (Z) India.

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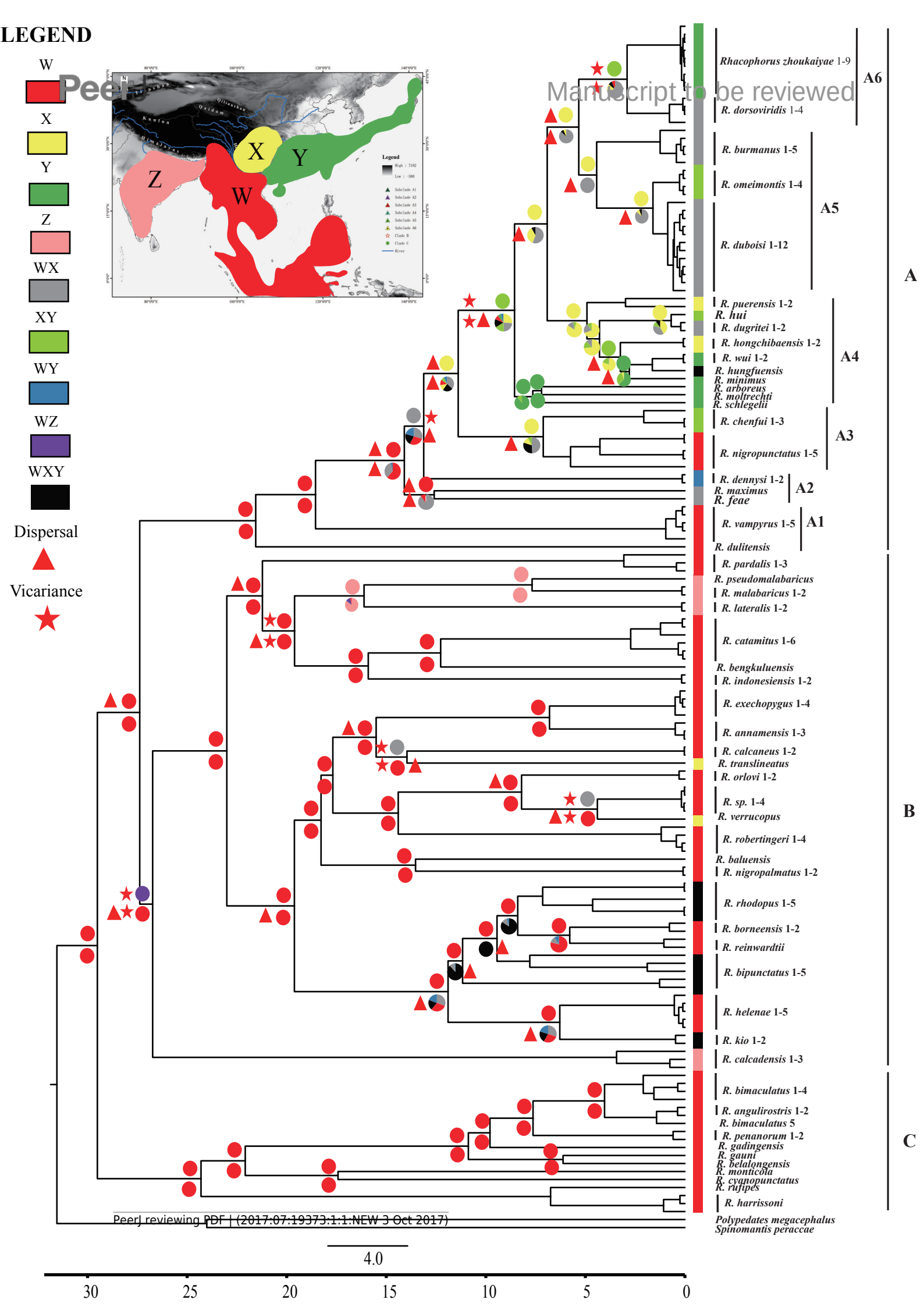

Figure 3(on next page)

Biogeographical history of *Rhacophorus*.

(i) Time-calibrated phylogeny of the genus *Rhacophorus* inferred from the mitochondrial dataset with an outgroup species, *Polypedates megacephalus* and *Spinomantis peraccae*. The light-blue bars through the nodes indicate 95% credibility intervals. Detailed time estimates for nodes with letter labels are given in Table 1; (ii) Climatic sequence of events including a global average delt ^{18}O curve (right-hand axis) derived from benthic foraminifera which mirrors the major global temperature trends from the Paleocene to the Pleistocene (red line) [modified from Zachos et al. (2001), Zachos et al. (2008) and Favre et al. (2015)]. The establishment of ice sheets in the Northern Hemisphere is indicated by grey to black bars on top. The onset and development of the monsoon is symbolised by a blue polygon and its intensification by grey bars (I , II and III) (Wan et al. 2007; Jacques et al. 2011). The climate oscillations during the Quaternary are represented by a grey bar (IV) (Deng et al. 2011); (iii) Geological sequences of events are related to the diversification of *Rhacophorus* including the reconstructions historical land and sea in Southeast Asia and a graphical representation of the extent of the uplift of the TP through time. ① and ② show two Cenozoic reconstructions of land and sea in the Indo-Australian Archipelago [modified from Lohman et al. (2011)]. Red shading in ③ and ④ indicates the portion of the TP that had achieved altitudes comparable to the present day for each given time [modified from Mulch & Chamberlain, (2006) and Favre et al. (2015)].

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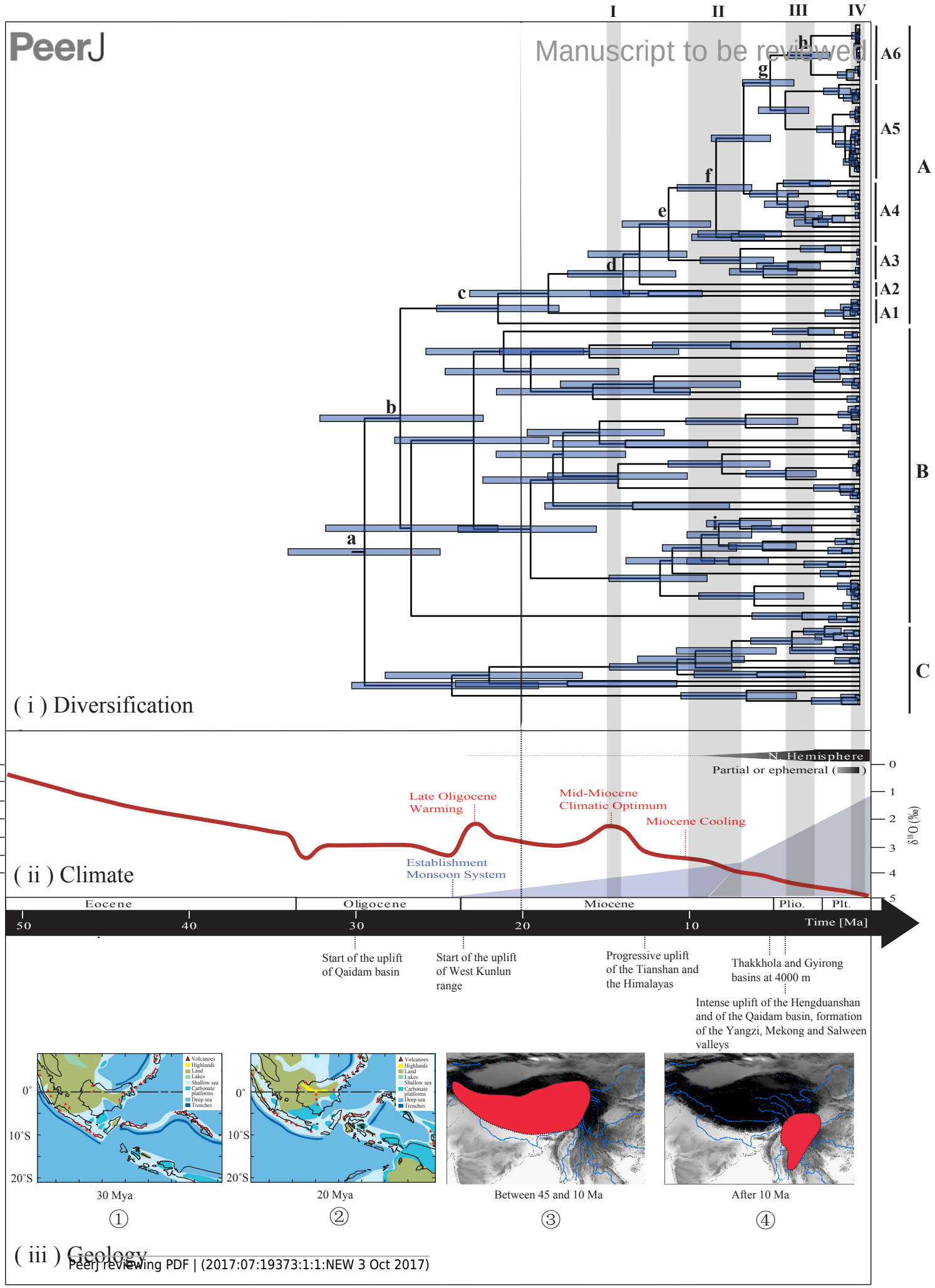

Figure 4(on next page)

Visualization of diversification rate shifts of *Rhacophorus*.

(A) Lineage-through-time plot (logarithmic scale) and 95% confidence intervals of lineage diversification; (B) Cumulative curve of diversification rate per million years. The dashed line represents the period of rapid diversification in *Rhacophorus*.

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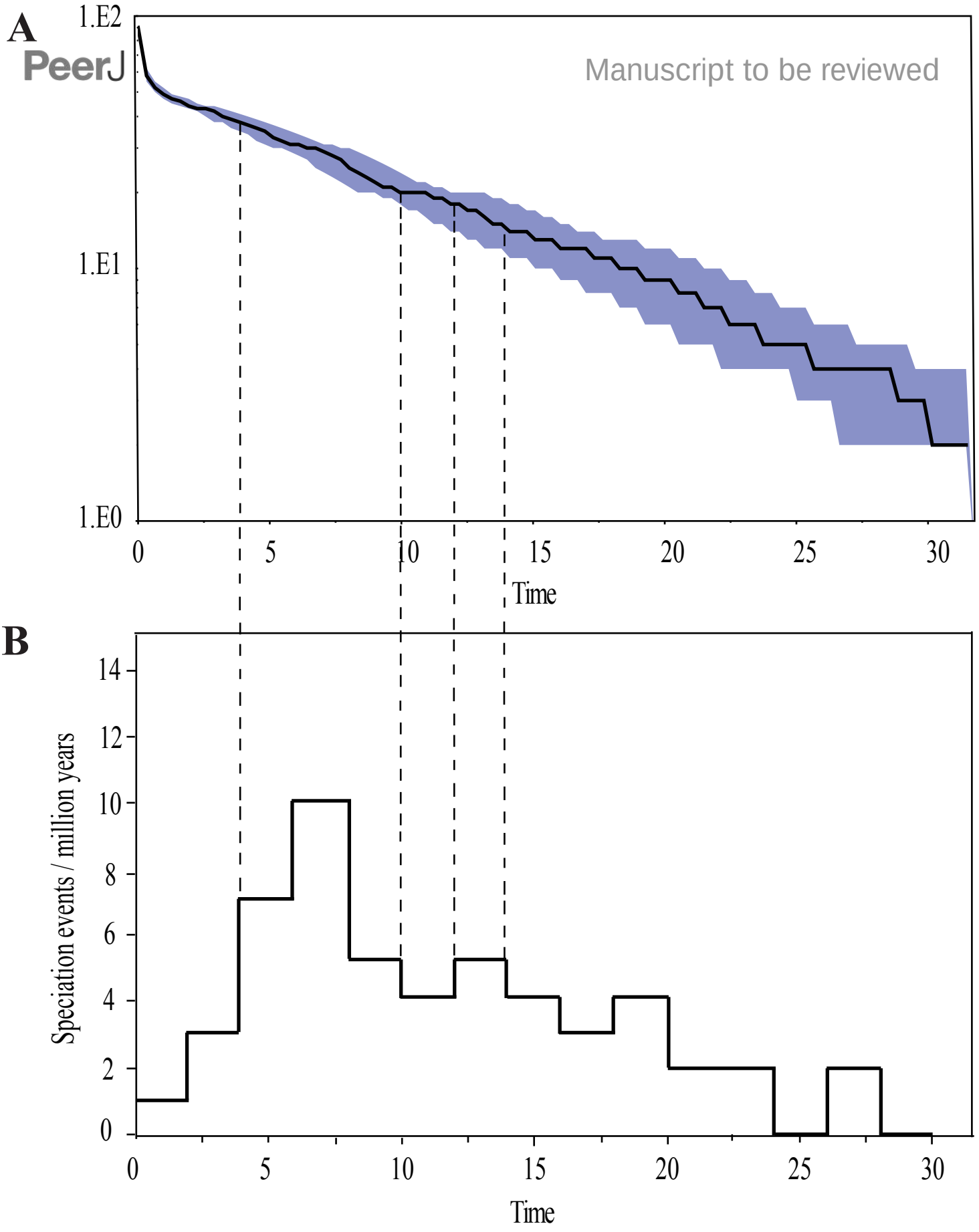

Table 1(on next page)

Detailed results of molecular dating using BEAST 1.7.4, and the calibration points.

Labels for nodes correspond to Fig. 3. Unit: one million years. The abbreviation of time to most recent common ancestor is TMRC.

Table 1. Detailed results of molecular dating using BEAST 1.7.4, and the calibration points.

Labels for nodes correspond to Fig. 3. Unit: one million years. The abbreviation of time to most recent common ancestor is TMRC.

Node	TMRC	Mean (95%)	Mean(95%) (Li et al. 2013)
Root	---	33.27 (25.11-40.20)	36.5 (31.2-40.9)
a	Clade A, B, C	29.51 (25.34-34.07)	30.6 (25.2-34.7)
b	Clade A, B	27.38 (22.44-32.17)	---
c	Clade A	21.56 (17.92-25.22)	21.6 (17.5-25.1)
d	Groups A2-A6	14.09 (10.96-17.41)	---
e	Groups A3-A6	11.39 (8.89-14.16)	---
f	Groups A4-A6	8.56 (6.43-10.88)	---
g	Groups A5, A6	5.33 (3.92-6.99)	---
h	Ggroup A6	2.9 (1.78-4.29)	---
i	---	8.4 (6.43-10.29)	8.6 (5.5-9.8)