Mountain-associated clade endemism in an ancient frog family (Nyctibatrachidae) on the Indian subcontinent

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ABSTRACT

Night frogs (Nyctibatrachidae) form a family endemic to the Western Ghats, a hill chain along the west coast of southern India. Extant members of this family are descendants of a lineage that originated on the subcontinent during its longtime isolation in the Late Cretaceous. Because the evolutionary history of Nyctibatrachidae has always been tightly connected to the subcontinent, these tropically-adapted frogs are an ideal group for studying how patterns of endemism originated and evolved during the Cenozoic in the Western Ghats. We used a combined set of mitochondrial and nuclear DNA fragments to investigate the phylogenetic relationships of 120 ingroup specimens of all known species of Nyctibatrachidae. Our analyses indicate that, although this family had an early origin on the Indian subcontinent, the early diversification of extant nyctibatrachids happened only in the Eocene. Biogeographic analyses show that dispersal across the Palghat gap and Shencottah gap was limited, which led to clade endemism within mountain ranges of the Western Ghats. It is likely that multiple biota have been affected simultaneously by these prominent geographical barriers. Our study therefore further highlights the importance of considering the Western Ghats–Sri Lanka biodiversity hotspot as an assemblage of distinct mountain regions, each containing endemism and deserving attention in future conservation planning.

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1. Introduction

The Indian subcontinent harbours unique assemblages of ancient endemic animal and plant taxa (e.g. Biju and Bossuyt, 2003; Conti et al., 2002; Roelants et al., 2004). This endemism can be largely attributed to the unique extended geological isolation of the subcontinent during the Cretaceous or Early Tertiary (Bossuyt et al., 2006; Hedges, 2003; Thewissen, 2001). The Indian subcontinent, which was part of the southern supercontinent Gondwana (South America, Africa, Indo-Madagascar, Australia–New Guinea and Antarctica), initially detached as part of an Indo-Madagascar block in the Early Cretaceous (Sanmartin and Ronquist, 2004), subsequently to separate from Madagascar and the Seychelles plateau around 88 million year ago (Mya) (Storey et al., 1995), and 65 Mya (Courtillot et al., 1988), respectively. Although alternative hypotheses suggest less isolation than that proposed by the standard "biotic ferry" model (Hedges, 2003), most of them agree on a substantial amount of isolation of the Indian subcontinent for a

prolonged period of time (Bossuyt et al., 2006 and references therein). As a consequence, faunal and floral interchange with other continents became suppressed, allowing local evolution of endemic lineages, similar to that observed in island evolution. Molecular studies demonstrate that a substantial amount of extant plant (Conti et al., 2002) and animal (Biju and Bossuyt, 2003; Bossuyt and Milinkovitch, 2001; Wilkinson et al., 2002) taxa of the Indian subcontinent originated during that northward odyssey across the Tethys sea. Many of these ancient endemics currently living on the subcontinent, are clades of tropical species restricted to the Western Ghats, a mountain range running parallel to the west coast of Peninsular India for over 1600 km. The Western Ghats mainly consists of tropical, tropical deciduous and montane shola forests, while the surrounding Deccan plateau is a dryer, savannah-like ecosystem. For specialised, tropically-adapted organisms, these dryer plains have been a barrier for dispersal and range expansion (Karanth, 2003; Van Bocxlaer et al., 2009), thereby adding to the formation and maintenance of the unique diversity of the Western Ghats (Biju et al., 2009; Bossuyt et al., 2004).

The Western Ghats of the subcontinent's mainland and the highlands of Sri Lanka are very similar in their geology and evolutionary history. Despite being often treated as a single biodiversity

hotspot (Myers et al., 2000), the Western Ghats–Sri Lanka biodiversity hotspot contains isolated hills separated by ecologically distinct plains. Sri Lanka is currently separated from the Indian mainland by a large, oceanic barrier (the Palk Strait), but it was connected to the mainland as recently as the latest glacial periods (Rohling et al., 1998). On the mainland, the highest peaks occur in the southern part of the Western Ghats, while the altitude decreases in more northern latitudes (Fig. 1).

In general, three important discontinuities can be distinguished in the Western Ghats (Fig. 1): First, the Goa gap approximately coincides with the southern margin of the Deccan Traps (Fig. 1), a large igneous province that was formed by a series of eruptions around the KT-boundary. This massive Deccan Traps volcanism, which was most prominent around Mumbai but had lava outflows that reached as far as the east coast (Knight et al., 2003), probably had an important impact on the biodiversity at the time. Second, the origin of the 30 km wide Palghat gap, which forms the most prominent interruption of the Western Ghats, is controversial, but recent studies indicate that its topography is indicative of shearing and erosion (D' Cruz et al., 2000). Third, the Shencottah gap more to the South is narrower (7.5 km), but it forms the second deepest discontinuity of the Western Ghats.

Although the exact timing of the origin of these three discontinuities is debated, they are all estimated to have arisen around (Goa gap) or before (Palghat and Shencottah gaps) the KT-boundary (Gunnell et al., 2003; Santosh et al., 1992; Soman et al., 1990; Storey, 1995). These three barriers thus have major ecological significance for explaining evolution of endemic lineages throughout the Cenozoic. Most studies on the impact of these gaps within the Western Ghats, however, focussed on species or population-level diversification (Bahulikar et al., 2004; Deshpande et al., 2001; Robin et al., 2010; Vidya et al., 2005). To our knowledge, there are no studies using molecular data that examined how the geographical and ecological discontinuities of the Ghats have influenced the distribution and evolutionary history of biota that were continuously present on the Indian subcontinent during the whole of the Cenozoic.

Nyctibatrachidae is a frog family of 28 known species all endemic to the Indian subcontinent. The extant members of this family are descendants of a lineage that originated on the subcontinent during its longtime isolation in the Late Cretaceous (Bossuyt and Milinkovitch, 2001; Roelants et al., 2004, 2007; Wiens et al., 2009). The members of this family are classified in two genera: *Nyctibatrachus* and *Lankanectes*. *Nyctibatrachus* species occur in terrestrial, marshy habitats or are restricted to rocky torrents in montane and sub-montane evergreen or semi-evergreen forests of the Western Ghats (Biju et al., 2011). Their closest relative is the monotypic genus *Lankanectes*, which occurs only in the highlands of Sri Lanka (Bossuyt and Milinkovitch, 2001). *Lankanectes corrugatus* is an aquatic species found in many forested and shaded wetland habitats (Manamendra-Arachchi et al., 2010), and, it strikingly resembles some medium-sized *Nyctibatrachus* species. Because of their long history on the Indian subcontinent and their tropical habitat requirements, Nyctibatrachidae are an ideal group for studying the formation of patterns of endemism in the Western Ghats during the Cenozoic.

Here we analysed a combined set of mitochondrial and nuclear DNA fragments to investigate the phylogenetic relationships and biogeography of Nyctibatrachidae. Our sampling includes 120 ingroup specimens of all known species in this family (Table S1) (Biju et al., 2011). We performed dating estimates and biogeographic analyses to investigate biotic exchange between the different hill ranges within the Western Ghats, and evaluated how the discontinuities in these mountains have influenced diversification in this ancient endemic frog family.

2. Material and methods

2.1. Sampling

This study includes *L. corrugatus* and 119 *Nyctibatrachus* taxa sampled across the Western Ghats over a period of 15 years. *Philautus wynaadensis*, *Meristogenys kinabaluensis* and an unidentified *Indirana* species served as outgroup taxa. Species were identified based on a recent taxonomic review of this group (Biju et al.,
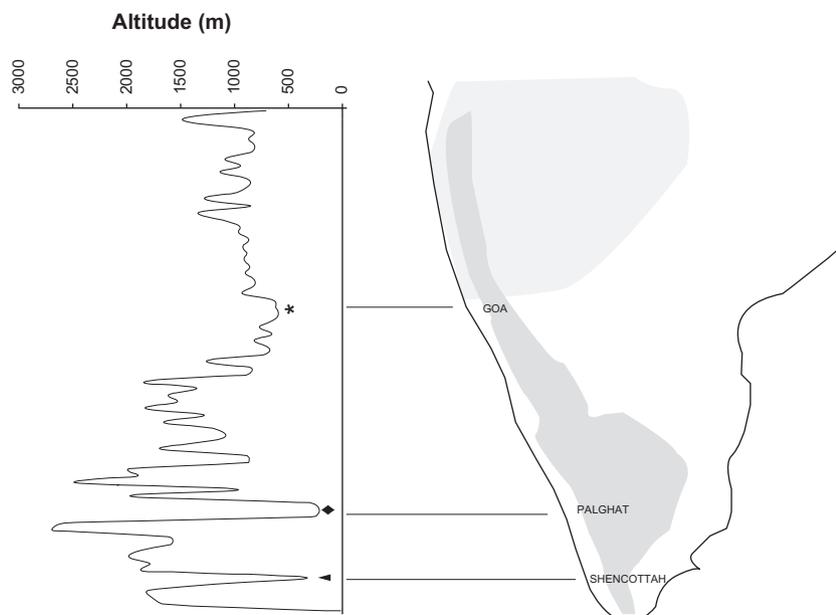

Fig. 1. Elevational profile of a cross-section through the Western Ghats. The three major discontinuities from North to South: Goa gap (∗), merely a sudden drop in altitude; the Palghat gap (♦), the largest valley; Shencottah gap (▲), a narrower valley. Adapted from (Ramesh et al., 1997) and (Robin et al., 2010). The Western Ghats are indicated in dark grey, the light grey patch corresponds to the Deccan Traps that cover the northern part of the mountain range.

2011). Sampling localities and latitudinal range per species are given in Table S1 and Fig. 2, respectively. Exact coordinates are not included for protective reasons, but they can be obtained by contacting S.D. Biju. We tried to obtain an unbiased view on *Nyctibatrachus* distribution patterns by sampling as much as possible randomly. DNA was extracted from muscle or liver tissue using a standard extraction protocol (Sambrook et al., 1989). One nuclear gene fragment and three overlapping mitochondrial DNA (mtDNA) fragments were PCR-amplified and cycle-sequenced on both strands. The nuclear gene fragment is a region of <534 bp of tyrosinase, while the combined mitochondrial DNA fragment of <1500 bp encompasses part of the 16SrRNA gene, the complete tRNA[LEU] gene and part of the ND1 gene. Primers used in this study are published elsewhere (Bossuyt and Milinkovitch, 2000; Roelants and Bossuyt, 2005; Simon et al., 1994). PCR-products were purified following an agarose gel extraction protocol (Qiagen), cycle sequenced on both strands and analysed using a GeneScan 3100 automated sequencer. Sequences were submitted to GenBank under accession numbers JN644772-JN644918.

To verify our species determination and to obtain distribution ranges for species, we first constructed a preliminary data matrix of <600 bp of the ND1 gene for all specimens. A second data set, including all fragments, yielded a <2000 bp data matrix for 30 ingroup taxa (Table S1, indicated in bold), and was used for further phylogenetic and biogeographic analyses. This larger dataset includes all known species of *Nyctibatrachus* (as recognised by Biju et al., 2011) and covers the sampling required for the biogeographic analyses performed in this paper.

2.2. Alignment and phylogenetic analyses

Alignments for all individual gene fragments were created with ClustalX 1.81 using the default settings, (Thompson et al., 1997) and manually optimised in MacClade 4.06 (Maddison and Maddison, 2000) using secondary structures for ribosomal DNA and amino acid sequences for nuclear DNA. To investigate the potential decay of phylogenetic signal due to saturation, observed transitions and transversions were plotted against uncorrected
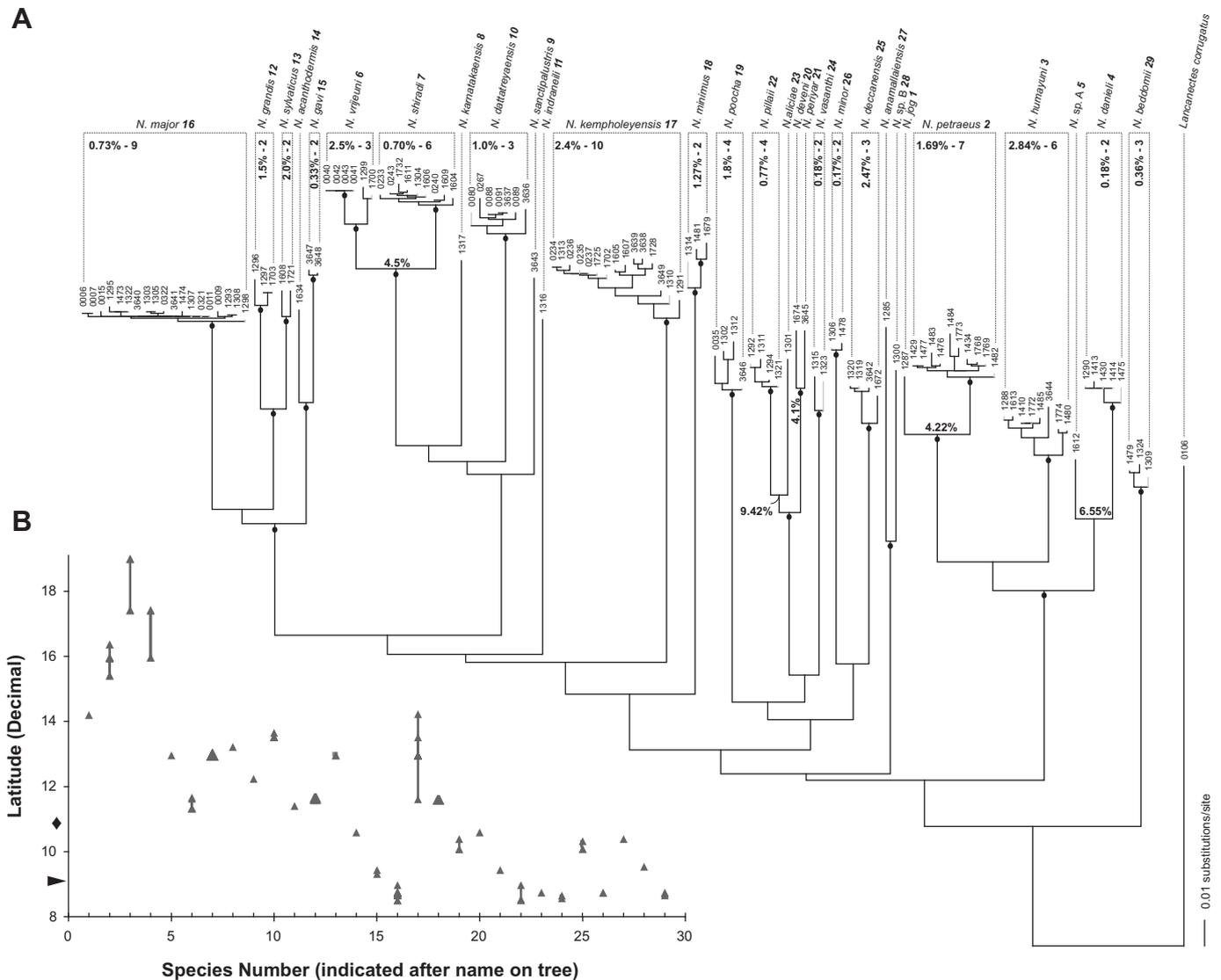
Fig. 2. (A) Neighbour Joining tree for 120 Nyctibatrachidae. Numbers of OTU's are cross-referenced in Table S1. The tree includes *Lankanectes corrugatus*, and 2 undescribed and 27 known *Nyctibatrachus* species. Percentages indicate the maximum interspecific uncorrected pairwise divergence, while the adjacent number indicates the number of unique haplotypes for that species. Bold numbers next to the species names are referenced in Fig. 2B. Dots indicate Neighbour Joining bootstrap values of 100. (B) Latitudinal distribution per species. Species numbers correspond to Fig. 2A. The latitude of sampling localities (see Table S1) is indicated by triangles. Connecting lines are for clarity and do not necessarily imply a continuous distributional range. The latitudinal positions of the Palghat gap (♦) and Shencottah gap (▲) are indicated.

pairwise distances and distances corrected according to the F84 model of base substitution (Rodriguez et al., 1990). To investigate species diversity and to verify species identification, a Neighbour Joining (NJ) tree with F84 + G distance correction was calculated using the software PAUP* (Swofford, 2003). The monophyly of haplotypes sampled from each species was assessed by analysing 1000 bootstrap replicates under the same model. Phylogenetic relationships were reconstructed using maximum parsimony (MP), Bayesian and maximum likelihood (ML) searches. MP searches were executed with PAUP* in 10,000 replicates with all characters unordered and equally weighted, and using tree bisection reconnection (TBR) branch swapping. Bayesian and likelihood analyses were performed with the GTR + G + I model of DNA substitution, which received the best Akaike information score by Modeltest 3.06 (Posada and Crandall, 1998). Bayesian analyses were done with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003), using a mixed model with three partitions (RNA-coding mtDNA, protein-coding mtDNA and the nuclear gene). Two runs of four MCMC chains each were executed in parallel for five million generations, with a sampling interval of 500 generations and a burn-in corresponding to the first one million generations. Convergence of the parallel runs was confirmed by split frequency standard deviations (<0.001), and by potential scale reduction factors (PSRF; ∼1.0) for all model parameters using the software Tracer v1.3 (Drummond and Rambaut, 2007). Posterior probabilities for clades were obtained by combining the post-burn-in trees from parallel runs in a single consensus tree. Heuristic ML searches were performed with PAUP* 4.0b10 (Swofford, 2003) and RAxML 7.0.4 (Stamatakis, 2006, 2008) using respectively the concatenated and a gene-based partitioned dataset. For the reconstruction with PAUP*, heuristic searches were performed with substitution rates, gamma-shape parameter and proportion of invariable sites estimated from Neighbour Joining trees. These parameters were re-estimated from the best ML tree found so far and the tree was submitted to additional rounds of TBR swapping; this procedure was repeated several times. For the reconstruction with RAxML, the same partition was used as for the Bayesian analysis. The RAxML search was performed specifying 200 alternative runs on distinct random starting trees. Clade support was assessed by analysing 1000 "rapid" bootstrap replicates under the likelihood criterion using RAxML 7.0.4 (Stamatakis, 2006, 2008).

2.3. Dating estimates

We performed dating estimates using two different relaxed molecular clock models: a Bayesian, multi-gene with correlated rates implemented in Multidivtime (Thorne and Kishino, 2002) and a multi-gene relaxed molecular clock model with uncorrelated rates implemented in BEAST v1.6.1 (Drummond and Rambaut, 2007). The ML tree topology obtained with PAUP* was used as input for the analyses. Since the fossil record of Indian frogs is deficient for Nyctibatrachidae and geological evidence on the Western Ghats is not in consensus, we based our priors and time calibrations on results of previous large-scale studies (Bossuyt et al., 2006; Van Bocxlaer et al., 2006) that contained *Nyctibatrachus* species, and which are in general agreement with other independent

studies (Roelants et al., 2007; Wiens et al., 2009). To reduce the risk of over-constraining the resulting timetree, we only used minimum time constraints in the multidivtime analyses and minimum and soft maximum constraints in the BEAST analyses. An overview of calibration points used for BEAST and Multidivtime analyses is given in Table 1 (BEAST minimum and soft maximum A–D and Multidivtime minimum B–D). To evaluate the individual influence of all calibration points, we performed additional analyses each time excluding a different calibration point. Furthermore, to be extra conservative, we used different root priors for the BEAST and Multidivtime analyses.

2.3.1. Multidivtime

Branch lengths in base substitutions were estimated for three fragments (RNA-coding mtDNA, protein-coding mtDNA and the nuclear gene) with the program ESTBRANCHES using the Multidivtime-inherent F84 + G model and parameters estimated by PAUP*. Proper approximation of the optimal branch lengths was verified by comparing the resulting log-likelihood values with those estimated by PAUP*. Optimised branch lengths with their variance–covariance matrices were used as input for the program Multidivtime, which calculates 95% credibility intervals for node ages, based on relaxed-clock model priors and calibration points. The prior on the split between Nyctibatrachidae and (Ranidae, Rhacophoridae) was set to 79.25 ± 15.45 Mya (Van Bocxlaer et al., 2006). The single MCMC chain was run for 1.1 million generations, with a sampling frequency of one per 100 generations and a burn-in corresponding to the first 100,000 generations.

2.3.2. Beast

Analyses were performed under the GTR + G + I model of DNA evolution with input model parameters estimated for the same three fragments (RNA-coding mtDNA, protein-coding mtDNA and the nuclear gene) by PAUP*. The uncorrelated lognormal distribution was used to describe the relaxed molecular clock model. The branch lengths for the starting chronogram were obtained by running an initial BEAST analysis with a birth–death speciation prior on the branching process. For the final analysis, operators for the birth–death model were switched off. The root prior on the split between Ranixalidae and (Nyctibatrachidae, Ranidae and Rhacophoridae) was set as following the lognormal distribution. The minimum bound of the distribution was set to 68.3 Myr; the soft maximum bound of the distribution was set to 100.2 Myr (Van Bocxlaer et al., 2006). We used a conservative approach by defining a soft maximum bound, which makes the sampling of time estimates beyond the maximum bound probable within 5%. The mean and standard deviation of the distribution were chosen so that 95% of the probability lies within the minimum and maximum bounds; the median was set as the arithmetical median of the interval. The same approach was used to calibrate four internal nodes (Table 1). The MCMC chain was run twice for 100 million generations, with a sampling frequency of one per 1000 generations. Burn-in and convergence of the runs were determined with Tracer v1.3 (Drummond and Rambaut, 2007). An effective sampling size of at least 2700 was obtained for all estimated parameters.

Table 1
List of calibration points used in this study. For the multidivtime analyses we only used minimum time constraints (B–D); in the BEAST analyses, we used the minimum and soft maximum time constraint (A–D) as following a lognormal distribution.

	Min (Myr)	Soft Max (Myr)	Node	References
A	63.8	94.7	split between Nyctibatrachidae and (Ranidae, Rhacophoridae)	Van Bocxlaer et al. (2006)
B	60.7	90.7	split between Ranidae and Rhacophoridae	Van Bocxlaer et al. (2006)
C	38.8	78.8	split between *Nyctibatrachus* and *Lankanectes*	Bossuyt et al., 2006 and Van Bocxlaer et al. (2006)
D	13.056	28.62	split between *N. dattatreyaensis* and *N. major*	Bossuyt et al., 2006

2.4. Biogeographic analyses

Ancestral distributions were estimated using a statistical dispersal-vicariance model for evolution of geographic range as implemented in sDIVA (Ronquist, 1997; Yu et al., 2010). This method reconstructs ancestral ranges while accounting for both phylogenetic uncertainty and multiple solutions in DIVA optimisation (Yu et al., 2010). We defined four biogeographic units in the Western Ghats and treated Sri Lanka as a single unit. The division in the Western Ghats was based on physical barriers in the mountain range (Fig. 1), as discussed in the introduction: the Shencottah gap around 9°N latitude, the Palghat gap around 11°N latitude, and the Goa gap around 14°N. Analyses were done on the 16,000 post burn-in trees obtained with MrBayes, and taxa were coded by sampling locality (Table S1). Ancestral ranges were assumed to include no more than two areas (maxarea = 2), which is reasonable given our knowledge of extant species being confined to a single area. We performed two analyses, one using four biogeographic units (real valleys in the Ghats + Sri Lanka), and a second using the five biogeographic units (adding the Goa gap). Additionally, in a second round, we assumed a maximum of three areas (maxarea = 3).

We also performed analyses using a likelihood model for geographic-range evolution implemented in Lagrange (Ree and Smith, 2008). Because the Western Ghats form a linear mountain range, we assumed dispersal to occur only between adjacent areas. The reconstructions were done on the ultrametric tree obtained with multidivtime, and rate parameters for dispersal and extinction were estimated. The same analyses were performed as in sDIVA, i.e., using the same biogeographic units (four and five units) and the same maximum ancestral areas (maxarea = 2 and maxarea = 3).

3. Results and discussion

3.1. Phylogeny of Nyctibatrachidae

Alignment of a fragment of the mitochondrial ND1 gene for 120 ingroup and 3 outgroup specimens yielded a data matrix of 628 basepairs (bp), of which 600 bp could be unambiguously aligned. A Neighbour Joining tree using the F84 + gamma model was in agreement with species delimitation as recognised in a recent taxonomic review of the family (Biju et al., 2011) (Fig. 2A). The maximum intra-specific uncorrected pairwise divergence for our ND1 fragment ranges from 2.84% in *Nyctibatrachus humayuni* to 0.17% in *Nyctibatrachus minor*. The lowest interspecific uncorrected pairwise divergences observed for this fragment were 4.1% between *Nyctibatrachus deveni* and *Nyctibatrachus periyar*, 4.2% between *Nyctibatrachus jog* and *Nyctibatrachus petraeus* and 4.5% between *Nyctibatrachus vrijeuni* and *Nyctibatrachus shiradi*. Our analyses suggest that many species have a narrow latitudinal range: none of the species' distribution ranges surpasses the Palghat or Shencottah gap (Fig. 2B, indicated with ♦ and ▲ respectively). Only one species, *Nyctibatrachus kempholeyensis*, which largely occurs south of the Goa gap, is also found around the border (Fig. 2B, species number 17). Our study thus confirms the importance of geographical gaps in maintaining local distributions in today's species (Robin et al., 2010).

To investigate the deeper evolutionary history of this family, we created an expanded dataset for phylogenetic and biogeographic analyses of 30 ingroup and 3 outgroup taxa, consisting of 2172 bp (1640 bp mitochondrial- and 532 bp nuclear DNA), of which 1910 bp could be unambiguously aligned. Of these, 1119 were constant and 610 sites were parsimony informative. The obtained fragment sequences showed a maximum pairwise

divergence for Nyctibatrachidae and *Nyctibatrachus* of respectively 9.4% and 6.7% (Tyrosinase gene) to 30.4% and 27.5% (ND1 gene) when uncorrected, and of 10.5% and 7.13% (Tyrosinase gene) to 40.3% and 35.3% (ND1 gene) when GTR-corrected. None of the fragments showed saturation. Maximum Parsimony (MP) analyses of the total dataset produced a single tree (tree length 3331, tree not shown), which is very similar to the Likelihood and Bayesian trees. Maximum likelihood (ML) analyses of the total dataset using PAUP* yielded a single ML tree (Fig. 3, −lnL = 16469.54620, with gamma shape parameter = 0.518546, and proportion of invariable sites = 0.426758). The Bayesian consensus phylogram (not shown) is the same as the ML tree obtained with PAUP*.

Overall, most branches in our trees are well-supported, but the early diversification of *Nyctibatrachus* receives low support. The consensus of all analyses is a trichotomy of three major lineages: one that gave rise to a clade of species occurring mostly north of the Palghat gap, but with some southern species nested (Fig. 3, clade A, southern species: *Nyctibatrachus acanthodermis*, *Nyctibatrachus gavi* and *Nyctibatrachus major*), one that gave rise to a clade exclusively occurring south of the Palghat gap (Fig. 3, clade C), and a lineage that gave rise to *Nyctibatrachus minimus* (Fig. 3, indicated with B). The subsequent diversification of clade A receives an overall high support and contains the strongly supported *N. humayuni*, *Nyctibatrachus sanctipalustris*, and *N. major* species groups (Fig. 3). Clade C groups most small and medium-sized species and contains the strongly supported *Nyctibatrachus aliciae* species and *Nyctibatrachus beddomii* species groups.

Nyctibatrachus is currently lumped with the genus *Nannobatrachus*, but they were originally recognised as distinct genera (Boulenger, 1882). These genera were considered morphologically distinct based on differences in toe webbing, tongue position, separation of the metatarsals and the shape of the sternum (Boulenger, 1882). Although not explicitly stated, Boulenger and several other authors have considered *Nyctibatrachus* to group the larger species (such as the *N. humayuni* and *N. major* species group), while *Nannobatrachus* (hence the name) grouped the smaller species. We recover with high support non-monophyly of the species formerly referred to as *Nannobatrachus* (i.e., *N. anamalaiensis*, *N. beddomii* and *N. kempholeyensis*) (Fig. 3, indicated with *), but find high support for monophyly of the *N. beddomii* group (Fig. 3). These results provide molecular support for the merging of *Nyctibatrachus* and *Nannobatrachus* (Dubois, 1986; Schaffer, 1988).

3.2. Delayed radiation of extant Nyctibatrachus species

To understand the tempo of nyctibatrachid diversification, we used our ML tree to calculate dating estimates for species diversification in this family. Analyses with Multidivtime and BEAST using different calibration sets yielded similar node ages (Table 2, Table S2 and Fig. S1). Removal of individual time constraints in most cases yielded highly congruent mean dating estimates with respect to the total set of calibration points (Fig. S2). The largest differences were observed at the stem origin of *Nyctibatrachus*, with exclusion of calibration point C (on the *Meristogenys-Philautus* split) yielding the largest reduction in divergence age (Multidivtime: 9.3 Myr younger).

The estimate on the stem origin of Nyctibatrachidae is placed in the Mid- to Late Cretaceous, a result consistent with previous studies (Bossuyt et al., 2006; Bossuyt and Milinkovitch, 2001; Roelants et al., 2007; Wiens et al., 2009). Furthermore, our analyses place the divergence of the Western Ghats endemic *Nyctibatrachus* from the Sri Lankan *Lankanectes* in the Late Cretaceous to Early Paleocene (Fig. 4, Table 2), a divergence time consistent with previous studies covering higher-level amphibian taxon sampling (Roelants et al., 2007; Wiens et al., 2009). However, the major diversification of extant members of the genus *Nyctibatrachus* in the Western
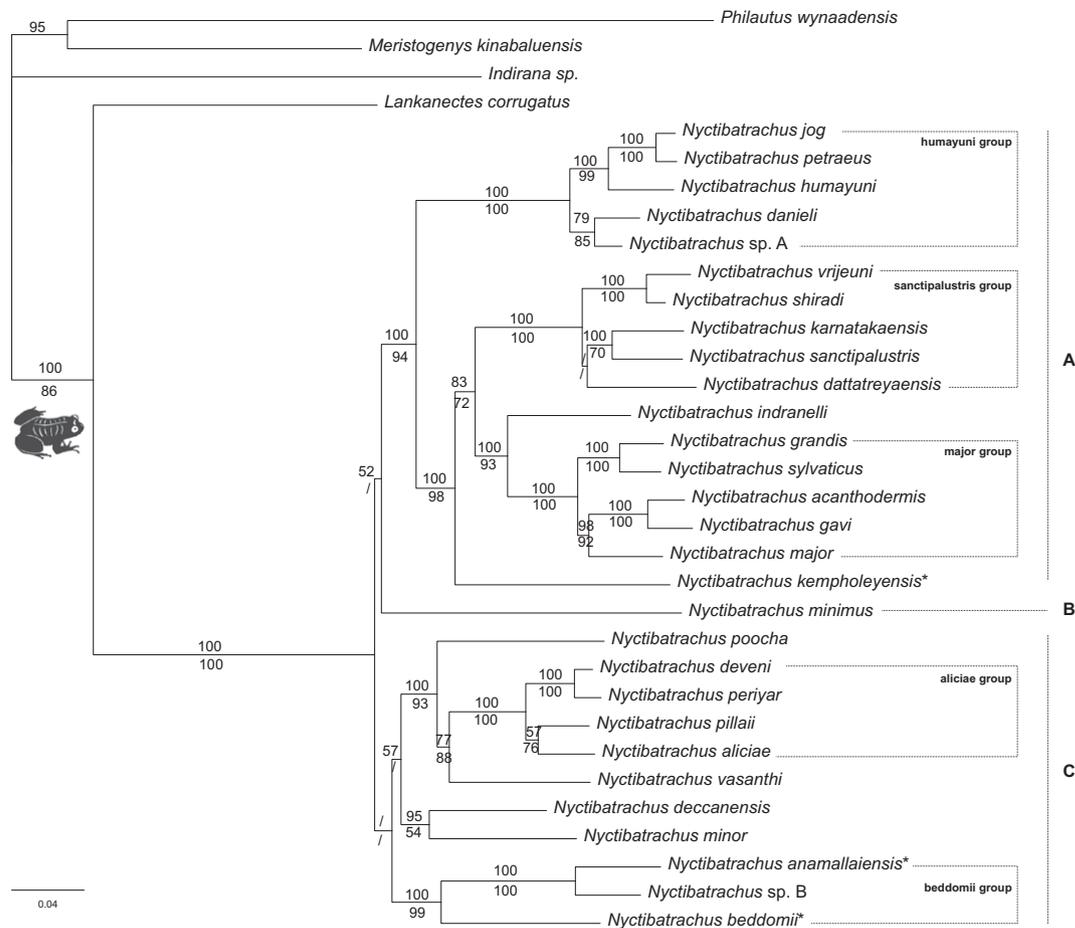
Fig. 3. Maximum Likelihood tree for 30 taxa of Nyctibatrachidae and three outgroup species. Numbers above and below the branches indicate Bayesian Posterior Probabilities and RaxML bootstrap values, respectively. Groups and letters are referenced in the text, *indicate species formerly recognised as *Nannobatrachus*.

Table 2
Dating estimates (Mya) for biogeographically relevant nodes. Mean and 95% interval are given for the seven nodes that are cross-referenced in Fig. 4. Ancestral distributions were estimated using sDIVA, and their implication for dispersal across the gaps is indicated.

No.	Multidivtime		Beast		Divergence
	Mean	95% Interval	Mean	95% Interval	
N.a.	73.5	[56.7, 98.1]	72.0	[63.4, 81.5]	Split between *Lankanectes* and *Nyctibatrachus*
1	37.6	[25.6, 55.1]	44.2	[37.6, 51.9]	Crown origin of *Nyctibatrachus*, north and south of the Palghat gap
2	12.3	[7.5, 19.3]	15.1	[11.9, 18.9]	North and south of the Palghat gap
3	25.7	[16.6, 38.4]	29.1	[23.0, 36.2]	North and south of the Shencottah gap
4	25.5	[16.5, 38.0]	29.7	[22.4, 37.6]	North and south of the Shencottah gap
5	27.6	[17.9, 41.1]	31.2	[24.7, 38.7]	North and south of the Shencottah gap
6	10.9	[6.5, 17.2]	13.1	[10.1, 16.7]	North and south of the Shencottah gap
7	11.1	[6.5, 17.7]	12.1	[9.0, 16.1]	North and south of the Shencottah gap

Ghats is estimated only much later, around the Eocene (Fig. 4, Table 2). This indicates that, between the origin of crown Nyctibatrachidae and their major radiation of extant members, there is a substantial gap where diversification is absent or not apparent (Fig. 4, Table 2, between nodes 1 and 2, Multidivtime: 32.4 ± 4.5 Myr and BEAST: 28.4 ± 1.2 Myr). This long branch indicates a period in which: (1) Nyctibatrachidae did not substantially diverge for several tens of years and/or (2) offshoot lineages eventually went through an extinction episode (Roelants et al., 2004). Two elements in the evolution of the Indian subcontinent's paleo-environment potentially explain these observations: First, accretion of the Indian subcontinent to the Eurasian mainland caused the gradual uplift of the Himalayan mountain range, which initiated a dramatic shift in the subcontinent's climate and vegetation. Second, limited diversification or extinction may have been locally induced or reinforced by volcanic eruptions that occurred in the north of the Western Ghats. Paleontological evidence from intratrappean sediments around the periphery of the Deccan Plateau suggest that volcanic eruptions on the Indian subcontinent not only occurred around the KT boundary, but may have continued locally in the Eocene and Oligocene (Beane et al., 1986).

The observation of congruent patterns of delayed diversification in other plant and animal taxa from the Indian subcontinent would provide valuable insights on paleo-environmental conditions during the first half of the Cenozoic. Previous studies so far indicate that this might be the case in two other ancient endemic frog genera of the subcontinent: i.e., *Micrixalus* and *Indirana* (Roelants et al., 2004).
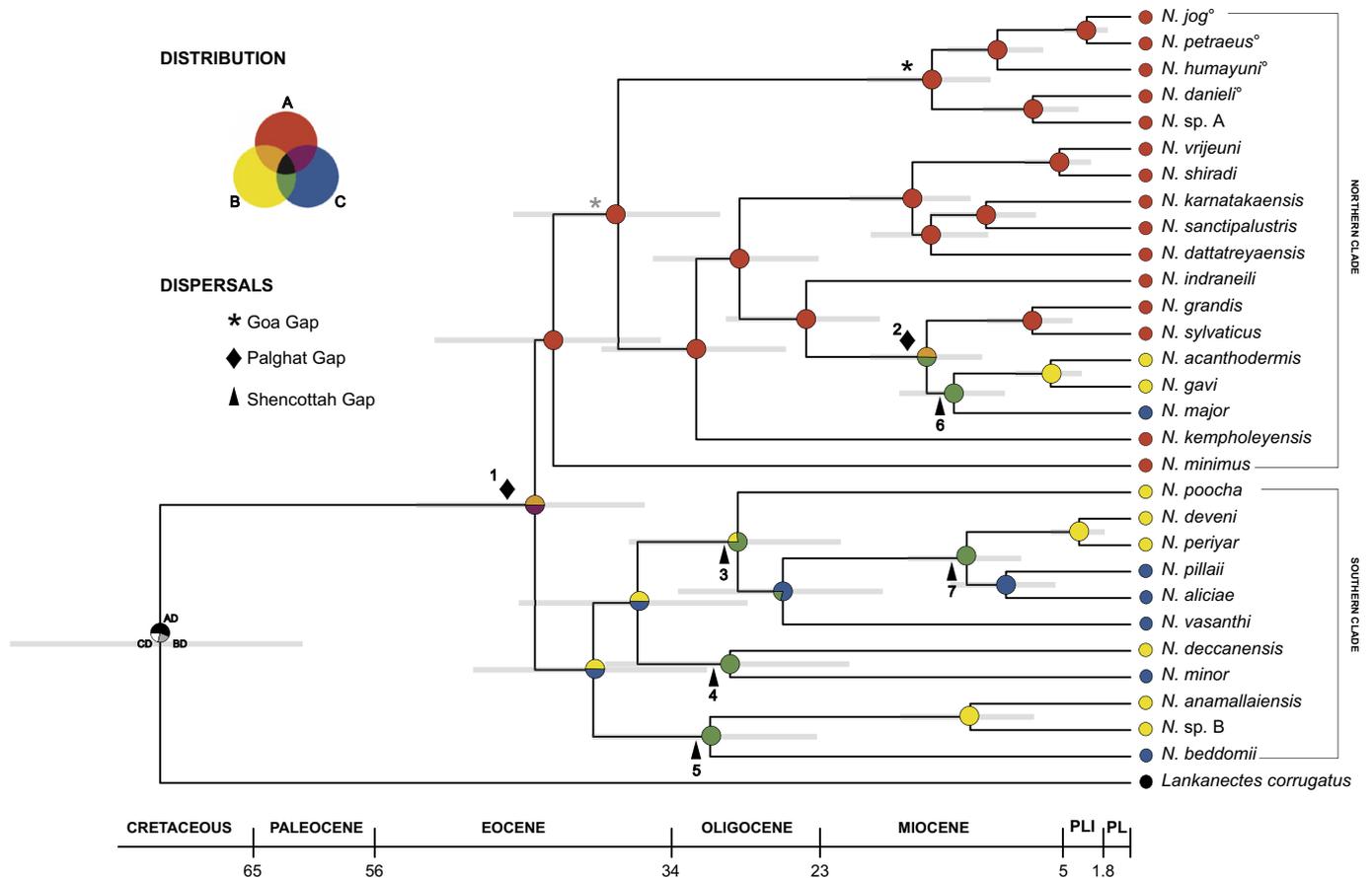
Fig. 4. Molecular timescale and biogeography of Nyctibatrachidae. The molecular timescale was obtained with BEAST using the ML tree topology obtained with PAUP* (same as Bayesian tree topology). Grey bars indicate 95% intervals. The distribution of species in the Western Ghats mountain ranges is indicated by the primary pigment colours; from north to south: red (A, north of the Palghat gap), yellow (B, between Palghat gap and Shencottah gap), and blue (C, south of the Shencottah gap), while combinations are indicated with secondary pigment colours (see legend). The letter D indicates Sri Lanka. Ancestral distributions were calculated from 16,000 post burn-in trees of our Bayesian analyses using sDiva (Yu et al., 2010). Pie charts indicate the percentage of the trees that showed a particular distribution for that node (internal nodes of the tree) or the current distribution (leaves of the tree). Numbers indicate dispersal events that are cross-referenced in the main text and Table 2.

3.3. Mountain-range clade endemism

We used the 16,000 post burn-in trees of our Bayesian analyses to reconstruct ancestral distributions using Statistical Dispersal-Vicariance Analysis (sDiva) (Yu et al., 2010). Additionally, we used Lagrange to estimate ancestral distributions and dispersals in our timetree. These analyses (Fig. 4 and S3) give very similar results (see Supplementary info) and we here further discuss the sDIVA analyses. These suggest that the ancestor of all extant Nyctibatrachidae (split between *Nyctibatrachus* and *Lankanectes*) had a distribution that spanned Sri Lanka and part of the Indian subcontinent (Fig. 4, sDiva $P = 0.44$ for south of the Palghat + Sri Lanka, sDiva $P = 0.56$ for north of the Palghat + Sri Lanka). Because, on this linear and broad scale, continuous ranges are generally much more likely than disjunct distributions, we hypothesise that the ancestor of extant nyctibatrachids lived in the southern hill ranges of the Indian subcontinent, and that the environmental conditions in the Palk Strait (strait between mainland India and the northernmost part of Sri Lanka) were favourable for dispersal between the mainland and the island. This hypothesis is in line with the idea that the southern (and central) parts of the Western Ghats have been refugia during the Late Paleocene and Early Eocene (Prasad et al., 2009).

Our analyses suggest that the most recent common ancestor of extant members of the genus *Nyctibatrachus* was distributed north and south of the Palghat gap, and that *Nyctibatrachus* crossed that gap for the first time in the Eocene (Fig. 4, dispersal 1). sDIVA indicates that this could have been either northward or southward, but based on an ancestral southern distribution (see above), the most parsimonious explanation for dispersal is South to North. Whatever the direction, the dispersal event led to the divergence of two clades of species that are now largely confined to opposite sides of the gap (Fig. 4, red dots in the northern clade and yellow, green and blue dots in the southern clade). This clade endemism on both sides of the Palghat gap probably indicates a limited period of optimal conditions in the Palghat gap (and surrounding lower areas) for tropically-adapted amphibians to cross. Species from the southern clade never successfully crossed the Palghat gap again, while only a single northern ancestor dispersed southward across the Palghat gap in the Miocene (Fig. 4, dispersal 2; sDiva $P = 1.0$). The lowest and widest gap of the Western Ghats played an important role in isolating tropically-adapted organisms throughout a large part of the Cenozoic.

Our analyses suggest that dispersal across the Shencottah pass (Fig. 4, dispersals 3–7) happened four times in the southern clade and once in the northern clade, and that this happened in two main periods. The first period includes the three earliest dispersal events (Fig. 4, dispersals 3–5), for which our dating estimates show a strong overlap of the 95% credibility intervals (Table 2), and which are estimated to have happened around the Early Oligocene.

A second period includes a dispersal event in both the northern clade (Fig. 4, dispersal 6) and the southern clade (Fig. 4, dispersal 7), which are estimated around the Middle Miocene. The combined observations of two time frames of dispersal across the Shencottah gap might indicate that climatic and vegetation conditions for dispersal of nyctibatrachids across this gap were favourable for at least two periods of time, and potentially less favourable otherwise. Range expansion and dispersal across the Shencottah Pass happened more frequently than across the Palghat gap in Nyctibatrachidae. Given that the Palghat gap is the widest and lowest gap of the Western Ghats mountain range, a reduction of humidity (aridification) on the Deccan plateau is likely to affect this area first, before affecting higher elevations. The Shencottah gap is considerably more narrow and higher, and the effect of climate change is therefore expected to be apparent only during more extreme environmental changes.

Besides the two valleys that incise the Western Ghats, the mountain range also largely drops in altitude at the Goa gap (Fig. 1). To understand the impact of that elevational drop at the southern periphery of the Deccan basalt flows, we performed an additional sDiva analysis including an extra biogeographic unit, splitting our northernmost unit north and south of the Goa gap. These analyses reveal that colonisation of the region north of this gap happened only once, but there are multiple solutions for the timing. *Nyctibatrachus* could have reached the northern part of the Western Ghats rather early in its evolution (Fig. 4, grey*, sDiva = 0.42), or later, in the Late Miocene at the latest (Fig. 4, black*, sDiva P = 0.58). However, the radiation of extant *Nyctibatrachus* members of the group in the northernmost part of the Western Ghats (i.e., the *N. humayuni* species group) is relatively young (Middle to Late Miocene) (Fig. 4). It is possible that the proposed small up-burst of basalt flows that continued until the Eocene and Oligocene (Beane et al., 1986) made it more difficult to colonise this area, but further evidence is needed.

Species of Nyctibatrachidae can be roughly categorised by their principal occurrence in one of two forest niches for this family: torrents and leaf litter (Biju et al., 2011 and references therein). Species occurring north of the Palghat gap contain a majority of larger species, and are mostly inhabitants of torrents or smaller streams (Biju et al., 2011 and references therein). These species lay their eggs on plants overhanging streams, and tadpoles fall in the water to continue their further development and metamorphosis (e.g. Kunte, 2004). Species occurring south of the Palghat gap contain many of the smaller species and are often leaf-litter inhabiting, using small forest puddles for their reproduction. We hypothesise that torrentially-adapted *Nyctibatrachus* species often disperse along the river systems and their tributaries, which may positively affect the speed of dispersal and possibly also the speciation rate. Stream-associated dispersal may be most effective during larval stages, since tadpoles can be transported long distances by strong currents during the monsoon. More fine-scale sampling and data are certainly necessary, but the largest distribution ranges in our sampling are indeed of torrential species, and there is a tendency for torrentially-adapted clades to harbour more species. However, since the river systems on the Indian subcontinent are often East–West oriented, this restriction may also have reduced range expansion of torrentially-adapted species in the North to South direction (i.e., from one river system to another).

4. Conclusion

Our study shows that Nyctibatrachidae, being an ancient family with a Late Cretaceous origin on the Indian subcontinent, had its major diversification only later, starting in the Late Eocene. However, this finding makes the endemism in this ancient lineage no less significant: in addition to the early divergence of the genus *Lankanectes* on Sri Lanka, there is a clear geographic structure that arose early in the diversification of extant *Nyctibatrachus* species, leading to endemic species and clades on different mountain regions within the Western Ghats. Restricted dispersal of tropically adapted animals (i.e., occurring in the moist forests of the Western Ghats Mountain range and not in the dryer Savannah like plains) on the Indian subcontinent has already been demonstrated between Sri Lanka and the Western Ghats (Bossuyt et al., 2004). For example, it has been shown that caecilians and uropeltid snakes colonised Sri Lanka only once and subsequently remained and diversified on the island. Other groups, such as tree frogs and freshwater crabs, also reached the island once and are characterised by a single back dispersal to the mainland later in their evolution. These patterns, and especially the latter ones, are similar to the pattern we find in Nyctibatrachidae: they crossed the Palghat gap on the mainland early in their diversification and returned only once across that major gap. Although these similarities should be interpreted with caution, they suggest that there have been periods with optimal conditions for (tropical) biota to expand their range on the Indian subcontinent, while other periods were characterised by less optimal conditions in lower areas and gaps. In Nyctibatrachidae, successful dispersal across the Palghat gap was limited to a single event from the Late Eocene untill now. The subcontinent paleoclimate of that period shows a trend of gradual transition to a monsoon-dominated climate, which caused a shift towards more open savannah-like ecosystems on the Deccan plateau, and probably also in the major gaps of the mountain ranges on the Indian subcontinent (Conti et al., 2002). We hypothesise that these paleo-environmental changes have restrained dispersal across the major gaps of the Indian subcontinent to a few species adapted to drier conditions, leading to local radiation of the majority of tropically-adapted organisms on their respective mountain ranges. Our study therefore further highlights the importance of considering the Western Ghats–Sri Lanka biodiversity hotspot as an assemblage of distinct regions, each deserving separate attention in conservation planning.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2011.11.027.

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