



Genetic divergence and evolutionary relationships in six species of genera *Hoplobatrachus* and *Euphlyctis* (Amphibia: Anura) from Bangladesh and other Asian countries revealed by mitochondrial gene sequences

Mohammad Shafiqul Alam^a, Takeshi Igawa^a, Md. Mukhlesur Rahman Khan^b, Mohammed Mafizul Islam^a, Mitsuru Kuramoto^c, Masafumi Matsui^d, Atsushi Kurabayashi^a, Masayuki Sumida^{a,*}

^aInstitute for Amphibian Biology, Graduate School of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashihiroshima, Hiroshima 739-8526, Japan

^bBangladesh Agricultural University, Mymensingh 2202, Bangladesh

^c3-6-15 Hikarigaoka, Munakata, Fukuoka 811-3403, Japan

^dGraduate School of Human and Environmental Studies, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

ARTICLE INFO

Article history:

Received 27 September 2007

Revised 8 April 2008

Accepted 13 April 2008

Available online 22 April 2008

Keywords:

Genetic divergence
Molecular phylogeny
Mitochondrial genes
Divergence time
Amphibia
Hoplobatrachus
Euphlyctis
Cryptic species
Bangladesh

ABSTRACT

To elucidate the species composition, genetic divergence, evolutionary relationships, and divergence time of *Hoplobatrachus* and *Euphlyctis* frogs (subfamily Dicroglossinae, family Ranidae) in Bangladesh and other Asian countries, we analyzed the mitochondrial Cyt *b*, 12S, and 16S rRNA genes of 252 specimens. Our phylogenetic analyses showed 13 major clades corresponding to several cryptic species as well as to nominal species in the two genera. The results suggested monophyly of Asian *Hoplobatrachus* species, but the position of African *Hoplobatrachus occipitalis* was not clarified. Nucleotide divergence and phylogenetic data suggested the presence of allopatric cryptic species allied to *Euphlyctis hexadactylus* in Sundarban, Bangladesh and several parapatric cryptic species in the Western Ghats, India. The presence of at least two allopatric cryptic species among diverged *Euphlyctis cyanophlyctis* in Bangladesh, India, and Sri Lanka was also suggested. In some cases, our estimated divergence times matched the paleogeological events of South and Southeast Asian regions that may have led to the divergence of *Hoplobatrachus* and *Euphlyctis* taxa. Especially, land formation at Bangladesh (15–10 Ma) may have allowed the spread of these frog taxa to Southeast Asian areas, and the aridification of central India (5.1–1.6 Ma) might have affected the gene flow of widely distributed species. The present study revealed prior underestimation of the richness of the amphibian fauna in this region, indicating the possible occurrence of many cryptic species among these groups.

© 2008 Elsevier Inc. All rights reserved.

1. Introduction

Bangladesh, located in the tropical climatic zone, features one of the world's largest deltas (Ganges–Brahmaputra river delta) formed by Miocene sedimentation and subsidence during continent–continent collision (Uddin and Lundberg, 2004) and is endowed with a rich diversity of unique flora and fauna. Biogeographically, this country is part of the Oriental region, nestled between the Indo-Himalayan and Indo-Chinese subregions of the Orient (Nishat et al., 2002). Although the amphibian fauna of the Western Ghats, India includes a large number of endemic taxa (Inger and Dutta, 1986), the available information on Bangladesh amphibian fauna lists only 22 frog species (Islam et al., 2000). A recent herpetofaunal survey reported the occurrence of some interesting species in Bangladesh for the first time (Reza, 2007),

but the genetic divergence and evolutionary aspects of the herpetofauna of Bangladesh have basically been neglected.

Among the amphibian fauna reported from Bangladesh, *Hoplobatrachus* and *Euphlyctis* frogs were the most common species, and during the 1980s Bangladesh was a major world supplier of frogs. The Bangladesh Government eventually banned the exporting of frogs in order to maintain the country's natural resources and ecological balance. As for the genus *Hoplobatrachus*, *H. tigerinus* (Indian bullfrog) is one of the most widely distributed species in Bangladesh, whereas the distribution of *H. crassus* (Jerdon's bullfrog) is not clear due to insufficient data (Islam et al., 2000). These two species are also distributed in other Asian countries such as India, Nepal, Bhutan, and Sri Lanka (Frost, 2007). Two more species belonging to the genus *Hoplobatrachus* are distributed in other countries: *H. chinensis* in Myanmar, China, Thailand, and Malaysia, and *H. occipitalis* in several African countries (Frost, 2007). As for the genus *Euphlyctis*, *E. cyanophlyctis* (Indian skipper frog) and *E. hexadactylus* (Indian green frog) are known from Bangladesh

* Corresponding author. Fax: +81 82 424 0739.

E-mail address: msumida@hiroshima-u.ac.jp (M. Sumida).

(Islam et al., 2000). The type localities of these two species are not clear, but Frost (2007) and Bauer (1998) suggested that they might be in Tranquebar and Pondichéry located in Southeast India near Sri Lanka. They also show wide distribution in other Asian countries: *E. cyanophlyctis* in India, Pakistan, Afghanistan, Nepal, Sri Lanka, Myanmar, and Vietnam, and *E. hexadactylus* in India, Pakistan, and Sri Lanka (Frost, 2007). Among them, *E. cyanophlyctis* from the northwestern highlands of Pakistan was recognized as a subspecies, *E. cyanophlyctis microspinulata* (Khan, 1997). Two more species belonging to the genus *Euphlyctis* are distributed in other Asian countries: *E. ghoshi*, known only from its type locality (Manipur, India), and *E. ehrenbergii*, inhabiting the southwestern Arabian Peninsula (Saudi Arabia and Yemen) (Frost, 2007).

It is well known that the genus *Hoplobatrachus* is the sister taxon to the genus *Euphlyctis* (Kosuch et al., 2001; Grosjean et al., 2004; Kurabayashi et al., 2005; Frost et al., 2006). The species of these two genera were formerly regarded as members of the genus *Rana*. However, Dubois (1987, 1992) suggested that the genus *Rana* was a phylogenetically heterogeneous group, and transferred many species from *Rana* to other genera including *Hoplobatrachus* and *Euphlyctis*. Although several studies have been performed for phylogenetic analyses of higher taxa including these genera (Bossuyt et al., 2006; Kosuch et al., 2001; Roelants et al., 2004; Vences et al., 2003), there has been no investigation regarding detailed species composition, genetic relationships and phylogeographic patterns among *Hoplobatrachus* and *Euphlyctis* groups in Bangladesh and neighboring countries.

The increasing utilization of molecular data has led to the reorganization of amphibian taxonomy (Biju and Bossuyt, 2003; Borkin et al., 2004; Bossuyt et al., 2006; De la Riva et al., 2000; Frost et al., 2006; Meegaskumbura et al., 2002) and the discovery of many cryptic species (Bickford et al., 2006; Fouquet et al., 2007a,b; Köhler et al., 2005; Stuart et al., 2006). Recent analyses of molecular and allozyme data on samples from Asian countries suggested the underestimation of diversity of amphibian fauna in this region as well as among these groups (Kurabayashi et al., 2005; Djong et al., 2007a,b; Kuramoto et al., 2007; Sumida et al., 2007; Islam et al., 2008). Inger (1999) suggested that additional samplings in South Asia would undoubtedly increase the number of species known from each area and illuminate detailed information on the distribution of species.

In order to elucidate the genetic diversity and phylogenetic relationships among *Hoplobatrachus* and *Euphlyctis* groups from Bangladesh and neighboring countries, we performed molecular phylogenetic analyses using mitochondrial Cyt *b* and 12S and 16S rRNA gene data from 252 frog specimens. Based on the results, we showed the possible existence of several cryptic species in these frog groups. We also estimated the divergence times among these taxa to determine the paleogeological events that had caused these divergences.

2. Materials and methods

2.1. Specimens

A total of 252 individuals consisting of four species of the genus *Hoplobatrachus* (*H. tigerinus*, *H. crassus*, *H. chinensis*, and *H. occipitalis*) and two species of the genus *Euphlyctis* (*E. cyanophlyctis* and *E. hexadactylus*) were used in the present study (Table 1 and Fig. 1). Among them, 201 individuals were collected from 17 localities in Bangladesh, 46 individuals from 20 localities in India, Nepal, Sri Lanka, Thailand, Laos, and Vietnam, and three individuals of *H. occipitalis* were commercially obtained from Tanzania. Species identification was based on Dubois (1992) and Frost (2007) classifications. Details of specimens are shown in Electronic Supplement 1.

2.2. DNA extraction

Total genomic DNA for PCR was extracted from the clipped toes of each specimen using a DNA extraction kit (DNeasy Tissue Kit, QIAGEN) according to the manufacturer's instructions. The extracted DNA solutions were used to amplify partial fragments of Cyt *b* and 12S and 16S rRNA genes by polymerase chain reaction (PCR).

2.3. PCR and sequencing

PCR amplification was performed on partial sequences of Cyt *b* (564 bp), 12S rRNA (689 bp), and 16S rRNA (517 bp) genes. These segments corresponded to the sites 16,785–17,348, 4474–5163, and 6251–6765, respectively, in the *Fejervarya limnocharis* complete mtDNA sequence (Accession No. AY158705, Liu et al., 2005). The following sets of primers were used for PCR amplification: Cytb Fow-1-1 (Sano et al., 2005) and Cytb Rev-1 (Kurabayashi, unpublished) for Cyt *b* gene, FS01 and RFR60 for 12S rRNA gene (Sumida et al., 1998), and F51 and R51 for 16S rRNA gene (Sumida et al., 2002). The sequences of the primers are available from Electronic Supplement 2. PCR mixtures were prepared with the TaKaRa Ex Taq™ Kit (TaKaRa Bio Inc.) as recommended by the manufacturer's protocol. Cyt *b* and 12S and 16S rRNA segments were amplified by 35 cycles, each cycle consisting of denaturation for 10 s at 98 °C, annealing for 30 s at 47.5 °C (10 cycles), 45.0 °C (10 cycles) and 42.5 °C (15 cycles), and extension for 1 min 20 s at 72 °C. The PCR products were purified by ethanol precipitation. The amplified Cyt *b* and 12S and 16S rRNA gene segments were directly sequenced for both strands using the BigDye Terminator Cycle Sequencing Kit (ABI) with automated DNA Sequencer (3100-Avant, ABI). The resultant sequences were deposited in the DDBJ database under Accession Nos. AB274044–AB274170, AB273137–AB273176, AB272583–AB272608, AB290594–AB290612, and AB290412–AB290434 (Table 1).

2.4. Selection of haplotypes

We found 146 haplotypes in Cyt *b* from 252 individuals, and these 146 samples were used for sequencing of 12S and 16S rRNA genes. To reduce computational time, we used a small data set containing 28 haplotypes (Table 1) taken from all lineages for combined analysis of Cyt *b*, 12S, and 16S rRNA genes (Table 1). As outgroups, data on *F. limnocharis*, *Buergeria buergeri*, *Mantella madagascariensis*, and *Microhyla okinavensis* (Accession Nos. AY158705, AB127977, AB212225, and AB303950, respectively) were used from the DDBJ database (Liu et al., 2005; Sano et al., 2004; Kurabayashi et al., 2006; Igawa et al., 2008).

2.5. Phylogenetic analyses

The nucleotide sequences of each gene (Cyt *b* and 12S and 16S rRNA) were aligned using the ClustalW program (Thompson et al., 1994). Gaps and ambiguous areas were excluded using Gblocks Ver. 0.91b (Castresana, 2000) with default parameters (3, 203, and 65 sites) were deleted for Cyt *b* and 12S and 16S rRNA genes, respectively. We then combined the data on these three genes. Before combining the nucleotide sequences of the three genes, we conducted the partition homogeneity test [parsimony method by Farris et al. (1995) as implemented in PAUP*4.0b10 (Swofford, 2003)] to check whether all of the sequences were suitable for combination.

Phylogenetic analysis based on the combined data was performed by maximum likelihood (ML), maximum parsimony (MP), and Bayesian inference (BI) methods. In all analyses, *M. okinavensis* was used as the outgroup; the sister taxon relationship of Microhylidae (+ Afrobatrachia) and ranids (= Natanaura sensu Frost et al., 2006) was well corroborated (e.g., van der Meijden et al.,

Table 1
Specimens used and haplotypes of nucleotide sequences of the mitochondrial DNA genes

Species	Collecting station		No. of frogs used	Haplotype abbreviation ^a	No. of haplotypes (Accession Number)			GPS information
	Country	Locality			Cyt b	12S	16S	
<i>Hoplobatrachus tigerinus</i>	Bangladesh	BAU Campus, Mymensingh	29	Htig-Ba1	25 (AB274044–AB274068)	5 (AB273137–AB273141)	2 (AB272583, AB272584)	24° 44' 50" N, 90° 24' 24" E
		Shambhuganj, Mymensingh	20	Htig-Ba2	11 (AB274069–AB274079)	2 (AB273142–AB273143)	1 (AB272585)	24° 44' 59" N, 90° 27' 36" E
		Fulbaria, Mymensingh	11	—	2 (AB274112, AB274113)	—	—	24° 37' 60" N, 90° 16' 0" E
		Fulpur, Mymensingh	11	—	4 (AB274114–AB274117)	—	—	24° 57' 0" N, 90° 21' 0" E
		Churkhai, Mymensingh	9	—	3 (AB274125–AB274127)	—	1 (AB272591)	24° 38' 27" N, 90° 24' 32" E
		Netrokona	7	—	5 (AB274128–AB274132)	—	1 (AB272592)	24° 52' 48" N, 90° 43' 48" E
		Kishoreganj	1	—	1 (AB274136)	—	—	24° 25' 60" N, 90° 46' 60" E
		Jamalpur	16	Htig-Ba3	5 (AB274080–AB274084)	2 (AB273144, AB273145)	1 (AB272586)	24° 55' 12" N, 89° 57' 36" E
		Jagannathganj, Jamalpur	14	Htig-Ba4	11 (AB274101–AB274111)	3 (AB273150–AB273152)	2 (AB272589, AB272590)	24° 45' 0" N, 89° 49' 0" E
		Rangpur	15	—	8 (AB274092–AB274099)	2 (AB273147, AB273148)	—	25° 36' 0" N, 89° 15' 0" E
		Pabna	6	Htig-Ba5	2 (AB274133, AB274134)	1 (AB273155)	1 (AB272593)	24° 19' 48" N, 89° 0' 0" E
		Nawabganj	2	—	1 (AB274135)	—	—	24° 43' 48" N, 88° 12' 0" E
		Borguna	11	—	7 (AB274118–AB274124)	2 (AB273153, AB273154)	—	22° 9' 3" N, 90° 7' 35" E
	Sundarban, Khulna	15	Htig-Ba6	7 (AB274085–AB274091)	1 (AB273146)	1 (AB272587)	22° 21' 0" N, 89° 18' 0" E	
	Sylhet	15	Htig-Ba7	1 (AB274100)	1 (AB273149)	1 (AB272588)	24° 55' 12" N, 92° 0' 0" E	
	India	Padil, Mangalore, Western Ghats	2	Htig-In1	2 (AB274137, AB274138)	1 (AB273156)	1 (AB272594)	12° 52' 09" N, 74° 52' 57" E
		Bajipe, Mangalore, Western Ghats	5	Htig-In2	4 (AB274139–AB274140, AB290595–AB290596)	3 (AB273157, AB290423–AB290424)	1 (AB290412)	12° 57' 46" N, 74° 53' 27" E
		Karnoor, Western Ghats	2	—	2 (AB274141, AB274142)	—	—	12° 45' 03" N, 74° 54' 54" E
		Shirva, Western Ghats	2	—	1 (AB290594)	2 (AB290421, AB290422)	—	13° 19' 28" N, 74° 49' 18" E
<i>Hoplobatrachus chinensis</i>	Thailand	Nong Khai	2	Hchi-Th1	2 (AB274144, AB274145)	2 (AB273159, AB273160)	2 (AB272596, AB272597)	17° 54' 18" N, 102° 44' 48" E
		Ko Chang	2	Hchi-Th2, 3	2 (AB274146, AB274147)	1 (AB273161)	1 (AB272598)	12° 03' 05" N, 102° 20' 58" E
		Phang Nga	1	Hchi-Th4	1 (AB290606)	1 (AB290431)	1 (AB290416)	08° 26' 23" N, 98° 31' 05" E
	Laos	—	2	—	2 (AB290598, AB290599)	2 (AB290426, AB290427)	—	—
		Luang Prabang Province	1	—	1 (AB290600)	1 (AB290428)	—	20° 00' 06" N, 102° 40' 28" E
	—	Long Nai, Phongsaly Province	2	Hchi-La	2 (AB290601, AB290602)	1 (AB290429)	1 (AB290417)	21° 39' 15" N, 102° 12' 41" E
	Vietnam	Huu Lien	4	Hchi-Ve	3 (AB290603–AB290605)	1 (AB290430)	2 (AB290414, AB20415)	21° 40' 13" N, 106° 23' 09" E

(continued on next page)

Table 1 (continued)

Species	Collecting station		No. of frogs used	Haplotype abbreviation ^a	No. of haplotypes (Accession Number)			GPS information	
	Country	Locality			Cyt b	12S	16S		
<i>Hoplobatrachus crassus</i>	Bangladesh	Sundarban, Khulna	1	Hcra-Ba	1 (AB274143)	1 (AB273158)	1 (AB272595)	22° 21' 0" N, 89° 18' 0" E	
	India	Assam	1	Hcra-In	1 (AB290597)	1 (AB290425)	1 (AB290413)	26° 09' 56" N, 92° 50' 29" E	
<i>Hoplobatrachus occipitalis</i>	Tanzania	—	4	Hocc-Ta1, 2	4 (AB274148–AB274150, AB290607)	3 (AB273162–AB273163, AB290432)	2 (AB272599, AB272600)	—	
<i>Euphlyctis cyanophlyctis</i>	Bangladesh	BAU Campus, Mymensingh	6	Ecya-Ba1, 2	5 (AB274151–AB274155)	2 (AB273164, AB273165)	2 (AB272601, AB272602)	24° 44' 50" N, 90° 24' 24" E	
		Bailor, Mymensingh	6	—	1 (AB274156)	—	—	24° 37' 20" N, 90° 24' 10" E	
	India	Gajni, Shergpur	2	—	1 (AB274157)	—	—	25° 0' 0" N, 90° 0' 0" E	
		Padil, Mangalore, Western Ghats	1	Ecya-In	1 (AB274158)	1 (AB273166)	2 (AB272603, AB272604)	12° 52' 09" N, 74° 52' 57" E	
		Bajipe, Mangalore, Western Ghats	1	—	1 (AB274160)	1 (AB273167)	—	12° 57' 46" N, 74° 53' 27" E	
		Madikeri, Western Ghats	1	—	1 (AB274159)	—	—	12° 25' 11" N, 75° 44' 21" E	
		Karnoor, Western Ghats	3	—	2 (AB274161, AB274162)	2 (AB273168, AB273169)	—	12° 45' 03" N, 74° 54' 54" E	
		Assam	1	—	1 (AB290611)	—	1 (AB290420)	26° 09' 56" N, 92° 50' 29" E	
		Nepal	—	2	—	1 (AB290608)	—	—	—
		Sri Lanka	—	2	Ecya-Sr1, 2	2 (AB290609, AB290610)	2 (AB290433, AB20434)	2 (AB290418, AB20419)	—
<i>Euphlyctis hexadactylus</i>	Bangladesh	Sundarban, Khulna	3	Ehex-Ba	1 (AB274163)	1 (AB273170)	1 (AB272605)	22° 21' 0" N, 89° 18' 0" E	
	India	Adyar, Mangalore, Western Ghats	7	Ehex-In1, 2	5 (AB274164–AB274168)	5 (AB273171–AB273175)	2 (AB272606, AB272607)	12° 52' 12" N, 74° 55' 12" E	
		Bajipe, Mangalore, Western Ghats	3	—	2 (AB274169, AB290612)	—	—	12° 57' 46" N, 74° 53' 27" E	
		Mudigere, Western Ghats	1	Ehex-In3	1 (AB274170)	1 (AB273176)	1 (AB272608)	13° 08' 04" N, 75° 38' 28" E	
Total			252		146	54	35		

^a Haplotypes used for combined data set.

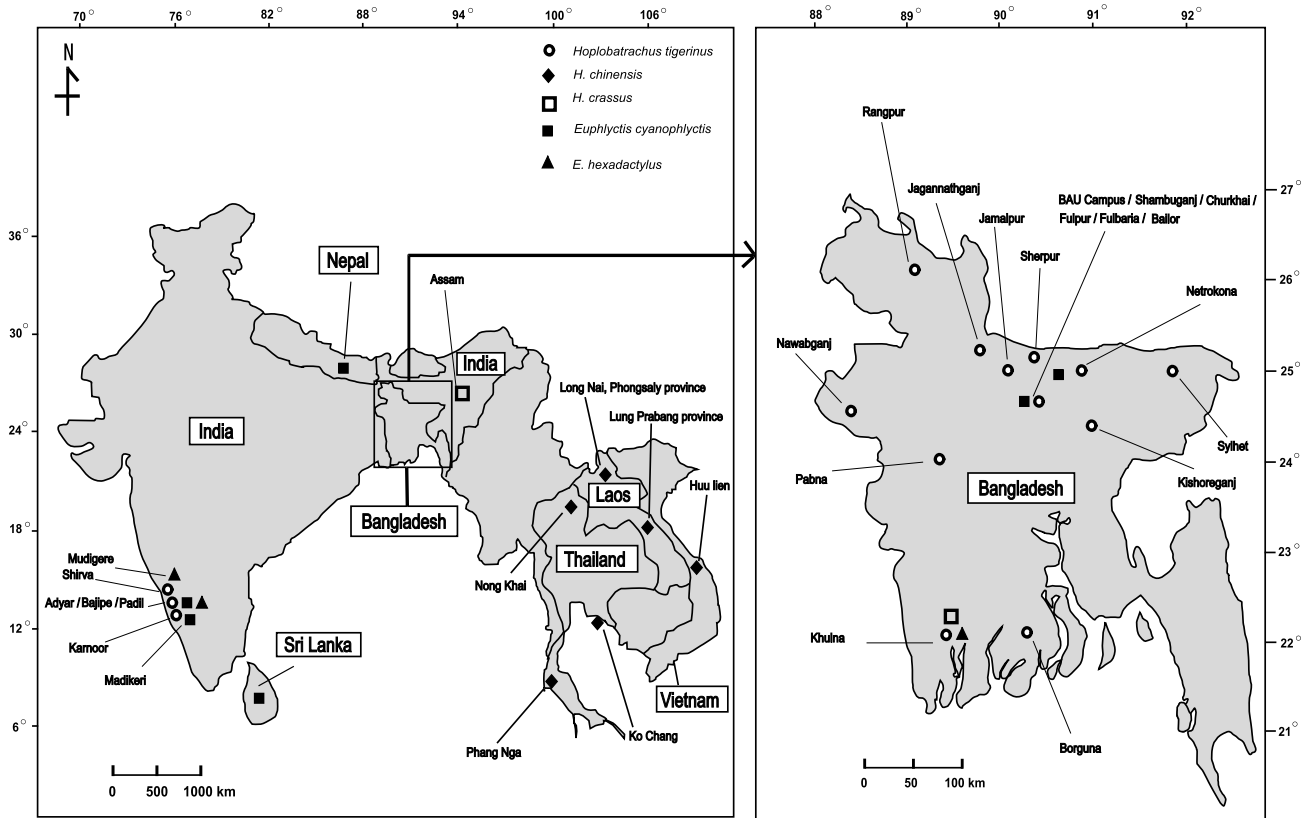


Fig. 1. Map showing collection localities of *Hoplobatrachus* and *Euphlyctis* species from Bangladesh and other Asian countries.

2005; Van Bocxlaer et al., 2006; Igawa et al., 2008). MP analysis was performed using PAUP*4.0b10 (Swofford, 2003). A heuristic search with 100 replicates of random sequence addition and TBR branch swapping was used, and all sites were of equal weighting. Clade support under MP was evaluated using 2000 replicates of nonparametric bootstrapping (nBP). For BI and ML analysis, appropriate substitution models (GTR + G + I) were chosen using the Akaike information criterion (AIC) as implemented in Modeltest 3.7 (Posada and Crandall, 1998). ML analysis based on the combined data was performed using PAUP* with heuristic search and TBR swapping. Nonparametric BP under ML was calculated using PHYML 2.4.4 (Guindon and Gascuel, 2003) with 1000 replicates. BI analysis was performed using MrBayes Ver. 3.1.2 (Ronquist and Huelsenbeck, 2003). The following settings were used for BI analysis: number of Markov chain Monte Carlo (MCMC) generations = 15×10^5 , sampling frequency = 10. The burn-in size was determined by checking the convergence of $-\log$ likelihood ($-\ln L$) values and the first 1×10^5 generations were discarded. The statistical support of the BI tree was evaluated by Bayesian posterior probability (BPP). The sequence divergence was computed with MEGA Ver. 4.0 (Tamura et al., 2007).

Alternative phylogenetic hypotheses among *Hoplobatrachus* and *Euphlyctis* were compared using the approximately unbiased (AU), Kishino–Hasegawa (KH), and Shimodaira–Hasegawa (SH) tests as implemented in CONSEL Ver. 0.1i (Shimodaira and Hasegawa, 2001). Site-wise $\ln L$ values were calculated using PAML (Yang, 1997) and used as input for the program.

2.6. Divergence-time estimation

For divergence-time estimation, we used the MultiDivtime software package (Thorne and Kishino, 2002). To focus on species-level divergence in the analysis, we decreased the number of OTUs

based on the results from the previous ML and BI analyses. Because of ambiguous phylogenetic positions of *H. occipitalis*, we separately conducted divergence-time estimation based on three alternative tree topologies; i.e., *H. occipitalis* + *Hoplobatrachus*, *H. occipitalis* + *Euphlyctis*, and polytomy of *H. occipitalis*, *Hoplobatrachus*, and *Euphlyctis*. In all estimations, we optimized the parameters for estimation using 'baseml' in the PAML package. Then, the branch lengths of the initial trees and the divergence times were estimated using the 'estbranches' and 'multidivtime' programs in the MultiDivtime package. In the analyses, as a reference point for divergence estimation, we applied the divergence between Mantellidae and Rhacophoridae 92.6–53.6 million years ago (Ma) (Bossuyt and Milinkovitch, 2001). We also applied the divergence between *Hoplobatrachus* and *Euphlyctis* 30–25 Ma, estimated by two recent studies (Bossuyt et al., 2006; Roelants et al., 2004).

3. Results

3.1. Haplotypes and sequence divergence

A total of 102 (93 from Bangladesh and 9 from India) haplotypes were found in *H. tigerinus* taxa ($N = 182$ from Bangladesh and $N = 11$ from India) in *Cyt b* genes. We found 25 and 13 haplotypes in 12S and 16S rRNA genes, respectively, in *H. tigerinus* (Table 1). The high number of *Cyt b* haplotypes was due to the huge number of silent mutations at the third codon position of this gene, and the same situation was observed in other taxa. In the *H. tigerinus* Bangladesh populations, we found seven haplotypes (Htig-Ba1 ~ Htig-Ba7) (Fig. 2A). A very low level of nucleotide divergence was observed for each gene among these haplotypes (average divergence is 0.6%, 0.2%, and 0.3% for *Cyt b*, 12S, and 16S rRNA genes, respectively) (Table 2). However, between Bangladesh and Indian haplotypes, there was a degree of nucleotide divergence (9.3%,

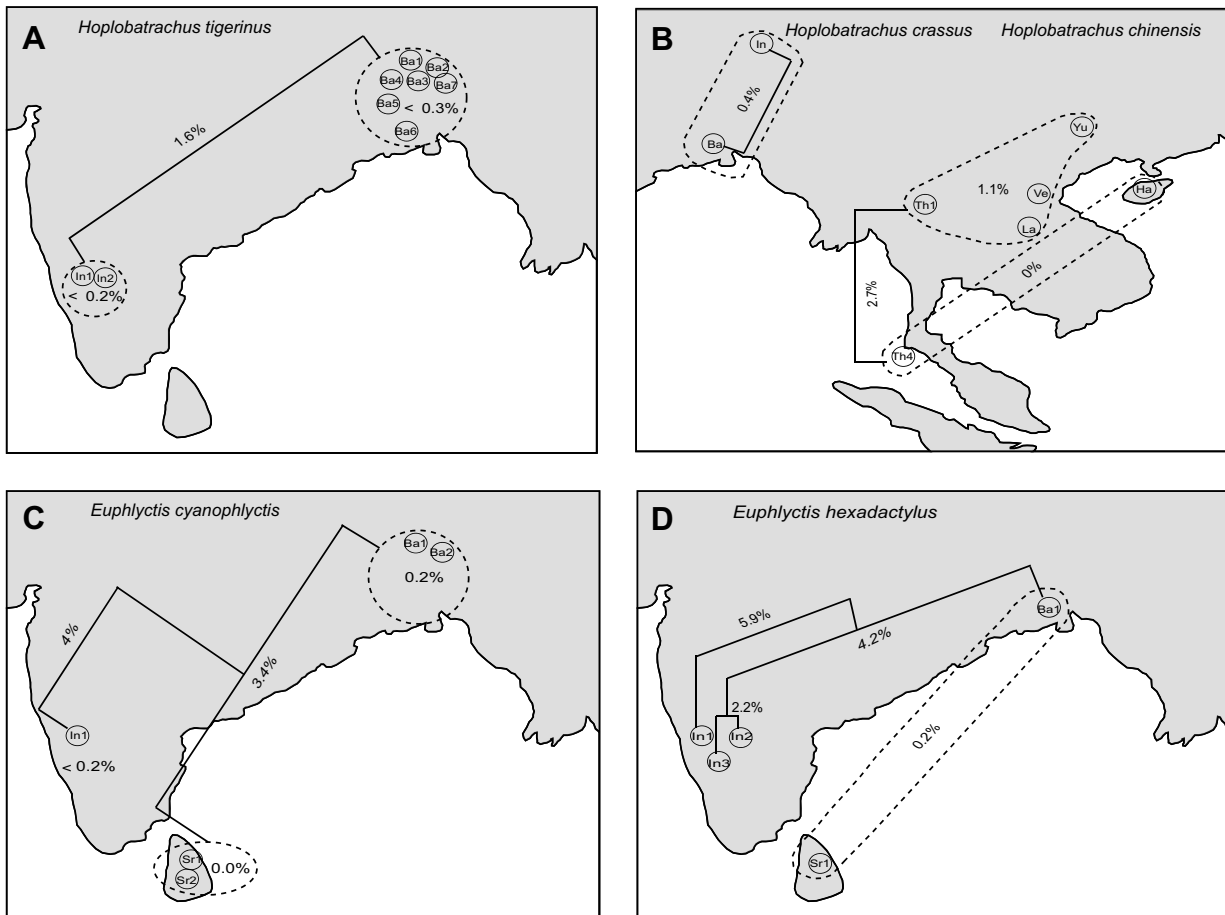


Fig. 2. Distribution of haplotypes for each *Hoplobatrachus* and *Euphlyctis* species. The average nucleotide divergence of 16S rRNA gene between haplotypes is denoted. Dotted circles unite similar haplotypes (<1.1% nucleotide difference), and solid lines show the phylogenetic relationships among haplotypes (as in Fig. 3). (A) *H. tigerinus*, (B) *H. crassus*, and *H. chinensis*, (C) *E. cyanophlyctis*, (D) *E. hexadactylus*. Abbreviations for haplotype are followings: Ba from Bangladesh, In from India, Sr from Sri Lanka, Th from Thailand, Ve from Vietnam, La from Laos. Yu from Yunan, and Ha from Hainan. Yunan and Hainan haplotypes (Yu and Ha) of *H. chinensis* and Sri Lanka haplotype (Sr) of *E. hexadactylus* are from DDBJ database (Accession Nos. DQ458251, DQ458250, and AF215389, respectively).

1.8%, and 1.6%) (Table 2); consequently, in *H. tigerinus*, two major haplotypes could be recognized corresponding to two geographic regions (named Htig-Ba and Htig-In) (Fig. 2A). In the case of *H. chinensis* ($N = 14$), we found 13, 9, and 7 haplotypes for Cyt *b*, 12S, and 16S rRNA genes, respectively (Table 1). Almost all haplotypes (i.e., Hchi-Th1, -Th2, -Th3, -La, and -Ve; see Table 1) showed low nucleotide divergence (Table 2); however, the haplotype found from Phang Nga, Thailand (Hchi-Th4) showed high nucleotide divergence from other Thailand populations (13.4%, 5.5%, and 2.7%; Table 2) (Fig. 2B). In the *H. crassus* taxa ($N = 2$), two haplotypes were found from Khulna, Sundarban (Bangladesh) and Assam (India) (Table 1), and very low nucleotide divergence was observed between these populations (0.9%, 0.2%, and 0.4%; Table 2) (Fig. 2B). In African *H. occipitalis* ($N = 4$), we found 4, 3, and 2 haplotypes with low nucleotide divergence in Cyt *b*, 12S, and 16S rRNA genes, respectively (1.1%, 0.2%, and 0.4%; Tables 1 and 2). In the *E. cyanophlyctis* taxa ($N = 24$), there were 16, 8, and 7 haplotypes in Cyt *b*, 12S, and 16S rRNA genes, respectively (Table 1). These haplotypes could be categorized into three major groups corresponding to the Bangladesh, India, and Sri Lanka populations (Ecy-Ba, -In, and -Sr). Although nucleotide divergence was very low within each group (<1% for all mitochondrial genes; Table 2), interpopulation divergence was very high (e.g., 13.6%, 5.1%, and 4.0% between Bangladesh and India; Table 2) (Fig. 2C). In the *E. hexadactylus* taxa ($N = 12$), four major haplotypes could be recognized: one from Khulna, Sundarban, Bangladesh (Ehex-Ba) and the remaining three

from the Western Ghats, India (Table 1). Two Indian haplotypes were found from only a single locality (Adyar, Western Ghats) (Ehex-In1 and -In2) and the other was observed from Mudigere (Ehex-In3). Among these haplotypes, nucleotide divergence between Ehex-In2 and -In3 was moderate (10.0%, 4.4%, and 2.2% for Cyt *b*, 12S, and 16S rRNA genes, respectively), and other interpopulation comparisons showed very high nucleotide divergence (16.8–20.1%, 5.4–13.0%, and 3.7–6.3%) (Table 2 and Fig. 2D). This nucleotide divergence matched the interspecies-level divergence found in the present study (16.8–23.0%, 4.1–12.8%, and 3.2–9.1%; Table 2).

3.2. Phylogenetic analyses

To understand the interspecies and interpopulation relationships of *Hoplobatrachus* and *Euphlyctis* taxa, we performed phylogenetic analyses. The partition homogeneity test (Farris et al., 1995) revealed that the three mitochondrial genes analyzed here were suitable for combination (homogeneity not rejected, $P = 0.543$ for Cyt *b* vs. 12S rRNA, $P = 0.993$ for Cyt *b* vs. 16S rRNA, and $P = 0.704$ for 12S rRNA vs. 16S rRNA); thus, we used the combined data (1544 bp) of these genes, which contained 493 parsimoniously informative sites.

Fig. 3 shows the resultant ML tree ($-lnL = 10414.34$), and BI analysis showed the same topology. MP analysis also reconstructed a similar topology. However, in the MP tree, monophyly of *H. occi-*

Table 2
Percent nucleotide sequence divergence within and among species at three mitochondrial genes

Species	Level	Combination	Percent sequence divergence		
			Average (Minimum–Maximum)	Cyt <i>b</i>	12S rRNA
<i>H. tigerinus</i>	Intrapopulation	Htig-Ba	0.6 (0.2–0.9)	0.2 (0–0.4)	0.3 (0–0.4)
	Interpopulation	Htig-In	0.4	0.0	0.2
<i>H. chinensis</i>	Intrapopulation	Hchi-Th1 to 4	6.8 (0.2–13.5)	2.9 (0.2–5.5)	1.5 (0.2–2.8)
		Hchi-Th1 to 3	0.3 (0.2–0.4)	0.3 (0.2–0.4)	0.3 (0.2–0.4)
	Interpopulation	Hchi-Th1-3 vs. Hchi-Th4	13.4	5.5	2.7
		Hchi-Th vs. Hchi-La	6.6 (3.9–14.3)	1.8 (0.6–5.5)	1.6 (1.1–2.8)
		Hchi-Th vs. Hchi-Ve	5.9 (3.2–13.7)	1.5 (0–5.5)	1.2 (0.7–2.4)
		Hchi-La vs. Hchi-Ve	1.6	0.4	0.4
<i>H. crassus</i>	Intrapopulation	Hcra-Ba vs. Hcra-In	0.9	0.2	0.4
<i>H. occipitalis</i>	Interpopulation	Hocc-Ta	1.1	0.2	0.2
<i>E. cyanophlyctis</i>	Intrapopulation	Ecya-Ba	0.2	0.2	0.2
		Ecya-Sr	0.5	0.6	0
	Interpopulation	Ecya-Ba vs. Ecya-In	13.6 (13.5–13.7)	5.1 (5.0–5.2)	4.0 (3.9–4.1)
		Ecya-Ba vs. Ecya-Sr	14.5 (14.1–14.8)	3.3 (2.9–3.6)	3.4 (3.3–3.5)
<i>E. hexadactylus</i>	Interpopulation	Ecya-In vs. Ecya-Sr	15.1 (15.0–15.2)	3.9 (3.8–4.0)	2.0
		Ehex-Ba vs. Ehex-In	16.9 (10.0–20.1)	8.6 (4.4–13.0)	4.8 (2.2–6.3)
<i>E. hexadactylus</i>	Interpopulation	Ehex-In1 vs. 2	20.1	11.9	6.3
		Ehex-In1 vs. 3	19.4	13.0	6.3
		Ehex-In 2 vs. 3	10.0	4.4	2.2
		Ehex-In1 vs. Ehex-Ba	17.8	10.7	5.9
		Ehex-In2 vs. Ehex-Ba	16.8	5.4	4.6
		Ehex-In3 vs. Ehex-Ba	17.5	5.9	3.7
		Intraspecies	<i>H. tigerinus</i>	4.0 (0.2–9.8)	0.8 (0–1.9)
	<i>H. chinensis</i>		6.2 (0.2–13.5)	2.1 (0.2–5.5)	1.4 (0.2–2.8)
	<i>H. crassus</i>		0.9	0.2	0.4
	<i>H. occipitalis</i>		1.1	0.2	0.2
	<i>E. cyanophlyctis</i>		11.6 (0.2–15.2)	3.2 (0.2–5.2)	2.6 (0–4.1)
	<i>E. hexadactylus</i>		16.9 (10.0–20.1)	8.6 (4.4–13.0)	4.8 (2.2–6.3)
	Interspecies	<i>H. tig.</i> vs. <i>H. chi.</i>	16.8 (15.0–18.2)	4.1 (3.4–6.3)	3.2 (2.6–3.9)
		<i>H. tig.</i> vs. <i>H. cra.</i>	19.6 (19.0–20.5)	6.1 (5.2–6.5)	4.7 (4.1–5.0)
		<i>H. tig.</i> vs. <i>H. occ.</i>	20.5 (20.0–21.0)	7.7 (6.9–8.2)	8.1 (7.8–8.3)
		<i>H. chi.</i> vs. <i>H. cra.</i>	19.5 (18.0–20.5)	6.2 (5.7–6.9)	4.7 (4.4–5.0)
		<i>H. chi.</i> vs. <i>H. occ.</i>	20.9 (20.0–21.7)	8.2 (7.8–8.6)	7.6 (7.2–8.0)
		<i>H. cra.</i> vs. <i>H. occ.</i>	23.0 (23.0–23.2)	8.6 (8.4–8.8)	9.1 (8.9–9.4)
<i>E. cya.</i> vs. <i>E. hex.</i>		21.5 (19–24.6)	12.8 (12.0–14.0)	8.8 (7.8–11.1)	

pitalis and other *Hoplobatrachus* supported by ML and BI analyses was not recovered, whereas the basal split of *H. occipitalis* at the root of all other *Hoplobatrachus* and *Euphlyctis* was supported by moderate BP (60%). Furthermore, in the MP tree, the relationship between *H. chinensis* and *H. tigerinus* could not be clarified (i.e., *H. chinensis* from Phang Nga, Thailand became the sister taxon with respect to the clade of other *H. tigerinus* and *H. chinensis*).

The ML tree showed that six major clades corresponding to six species used here could be recognized. These clades were basically supported by high BP and BPP values (excluding *H. chinensis* from Phang Nga; see below), but the basal split of *H. occipitalis* from other *Hoplobatrachus* was not supported (Fig. 3). In the *E. hexadactylus* clade, four distinct subgroups could be found. Interestingly, among these subgroups, the specimen from Adyar (Ehex-In1) formed the sister taxon to a clade containing all other specimens, and Bangladesh (Ehex-Ba) and two other Indian taxa (Ehex-In2 and -In3) became monophyletic (Fig. 3). The *E. cyanophlyctis* clade consisted of three major geographic subgroups that clearly corresponded to the India (Ecya-In), Bangladesh (Ecya-Ba1 and -Ba2), and Sri Lanka (Ecya-Sr1 and -Sr2) groups. Among them, Bangladesh and Sri Lanka subgroups became monophyletic, but with low statistical support (67% and 70%; Fig. 3). Within the *H. chinensis* clade, Thailand, Vietnam, and Laos populations formed an obvious clade, but the specimen from Phang Nga (Hchi-Th4) showed a degree of divergence from the other *H. chinensis* taxa and monophyly with

other *H. chinensis* taxa was only moderately supported (Fig. 3). In the *H. tigerinus* clade, two major subgroups were recognized. These *H. tigerinus* subgroups clearly corresponded to the sampling localities: one subgroup consisted of two haplotypes from the Indian population and the other consisted of seven haplotypes from the Bangladesh population (Fig. 3).

Consequently, the following groups were not supported by high BP and BPP values in our analyses: (1) *H. tigerinus* and *H. chinensis*, (2) Phang Nga *H. chinensis* (Hchi-Th4) grouped with other *H. chinensis*, (3) sister-group relationship of African *H. occipitalis* with respect to the Asian *Hoplobatrachus* species, and (4) sister-group relationship of Bangladesh and Sri Lanka *E. cyanophlyctis*. Thus, we investigated alternative phylogenetic hypotheses for these phylogenetic relationships by conducting AU, KH, and SH tests. These tests could not reject other hypothetical topologies for these problematic relationships. The results are shown in Electronic Supplement 3.

3.3. Estimation of divergence time

We estimated divergence times among *Hoplobatrachus* and *Euphlyctis* taxa by Bayesian molecular dating based on the ML and BI tree topology (Fig. 4). As for the problematic *H. occipitalis* position, we tried three alternative tree topologies (i.e., *H. occipitalis* + other *Hoplobatrachus*, *H. occipitalis* + all *Euphlyctis*, and polytomy of *H. occipitalis*, *Hoplobatrachus*, and *Euphlyctis*). These different

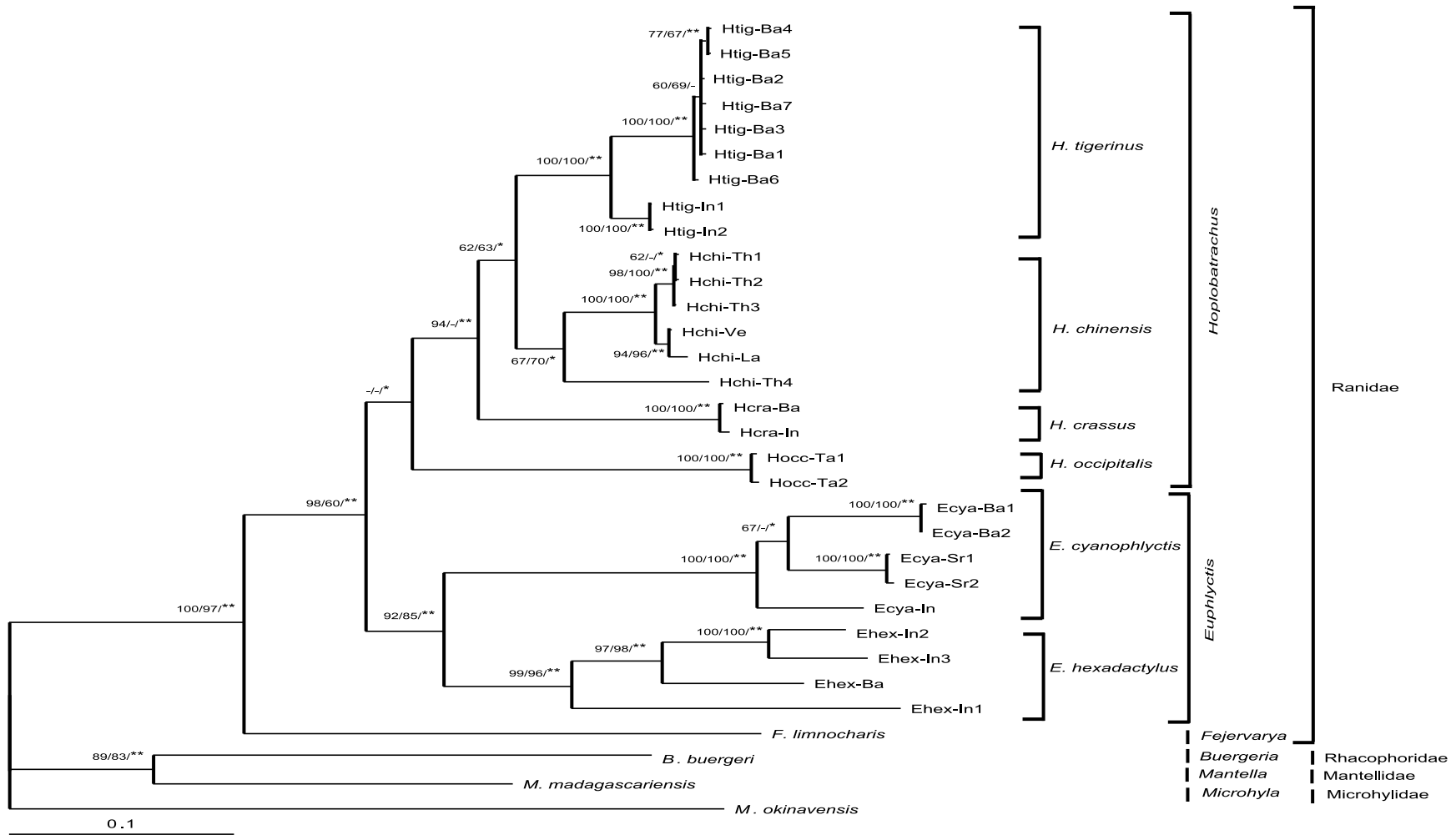


Fig. 3. Maximum likelihood (ML) tree ($-\ln L = 10414.34$) based on the nucleotide sequence of 1544 bp of mitochondrial (Cyt *b* + 12S rRNA + 16S rRNA) genes with GTR + I + G substitution model from 28 haplotypes (Table 1) of *Hoplobatrachus* and *Euphylyctis* species with *M. okinavensis* as an outgroup. The Bootstrap support (above 50%) is given in order for ML/MP (100/100). Asterisks represent Bayesian posterior probability (BPP; * >95% and ** >99%). The scale bar represents branches in terms of nucleotide substitutions per site for the ML tree.

topologies did not significantly affect the time estimation (Table 3); thus, we used only the result from the *Hoplobatrachus* monophyly constraint (Fig. 4).

If we accepted the monophyly of all *Hoplobatrachus*, the African *H. occipitalis* first branched from Asian *Hoplobatrachus* lineage at 25.6 Ma (E in Fig. 4). Within Asian *Hoplobatrachus*, *H. crassus* was the first to split from the others and the timing was estimated as 19.5 Ma (G in Fig. 4). The branching time between *H. chinensis* and *H. tigerinus* was estimated as 15.9 Ma (I in Fig. 4). Within *H. chinensis*, the Phang Nga haplotype (Hchi-Th4) separated from a lineage ancestral to all others at 12.0 Ma (J in Fig. 4); other Thailand and Vietnam haplotypes split at 2.3 Ma (P in Fig. 4). Within the *Euphlyctis* clade, the split of *E. cyanophlyctis* and *E. hexadactylus* was estimated as 23.4 Ma (F in Fig. 4). Within the *E. hexadactylus* taxa, an Indian haplotype (Ehex-In1) was the first to branch at 16.3 Ma; then, Bangladesh *E. hexadactylus* (Ehex-Ba) split from the other Indian lineage at 10.7 Ma (K in Fig. 4), and two Indian haplotypes (Ehex-In2 and -In3) separated at 5.2 Ma (N in Fig. 4). In the case of the *E. cyanophlyctis* clade, the Indian haplotype was the first to branch at 7.1 Ma (L in Fig. 4) and the split of Sri Lankan and Bangladesh haplotypes was estimated at 6.0 Ma (O in Fig. 4).

4. Discussion

4.1. Intraspecific differentiation and possible cryptic species

In the intraspecies comparisons, we found several haplotypes having a degree of sequence divergence more typical of interspecies comparisons (Table 2 and Fig. 2). First, Bangladesh and Indian populations of *H. tigerinus* possessed clearly distinct haplotypes. The average sequence divergence between Bangladesh (Htig-Ba) and Indian (Htig-In) haplotypes was high (9.3%, 1.8%, and 1.6% in Cyt *b* and 12S and 16S rRNA genes, respectively) compared with the values of 0.6%, 0.2%, and 0.3% within Bangladesh populations and 0.4%, 0%, and 0.2% within Indian populations (Table 2). Similarly, the haplotype of *H. chinensis* from Phang Nga, Thailand (Hchi-Th4) showed high nucleotide divergence compared with other Thailand populations (13.4%, 5.5%, and 2.7%; Table 2) (Fig. 2B). The haplotype of Bangladesh *E. cyanophlyctis* (Ecy-Ba) also showed high nucleotide divergence with respect to the Indian and Sri Lankan haplotypes (13.6% and 14.5% for Cyt *b*, 5.1% and 3.3% for 12S rRNA, and 4.0% and 3.4% for 16S rRNA; Table 2) (Fig. 2C). These obviously distinguishable haplotype groups occurred in separate geographic areas, suggesting that these haplotypes were maintained by allopatric separation and lack of

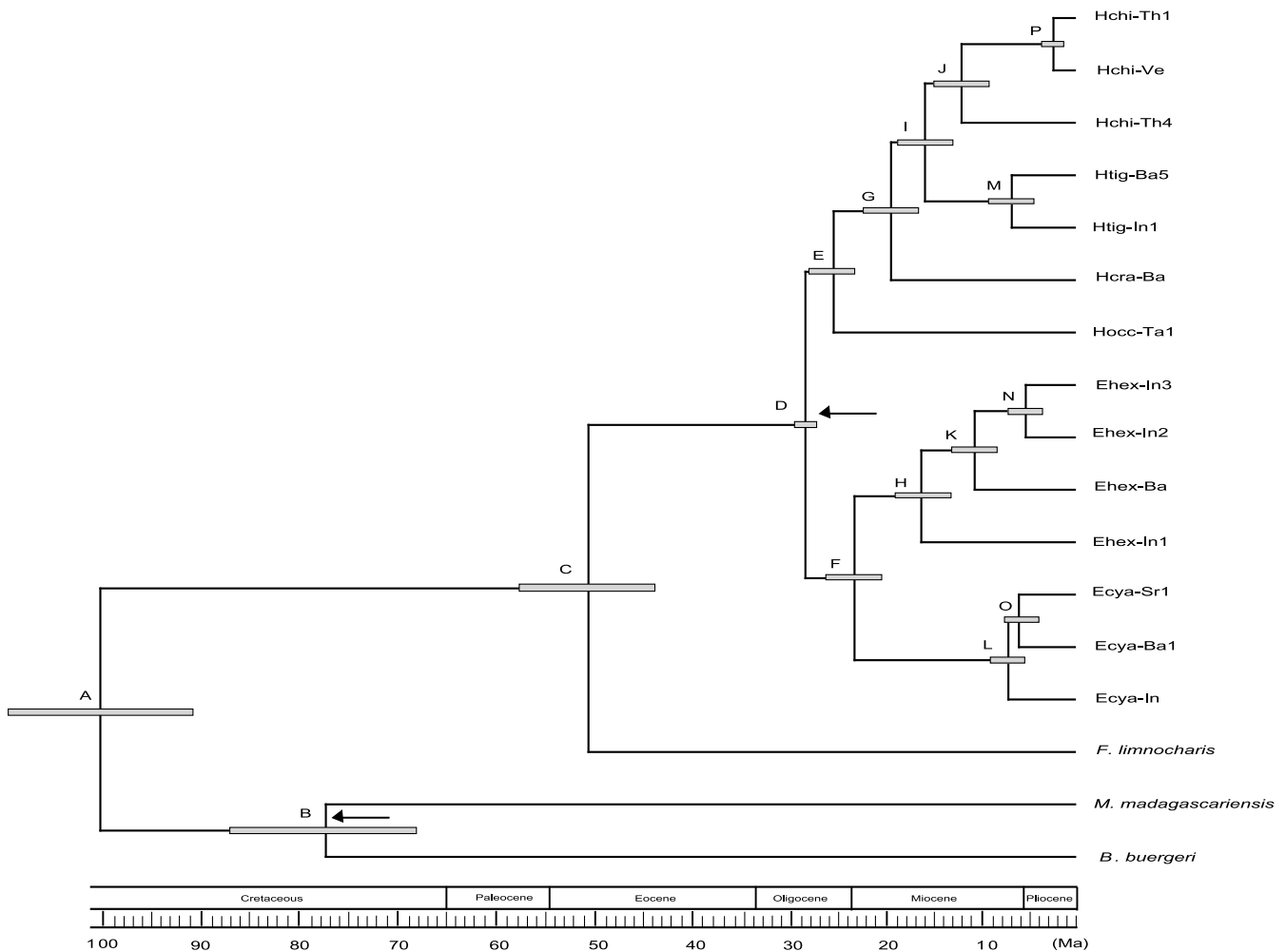


Fig. 4. Estimated divergence time. The range of 95% credibility interval is indicated by gray rectangles. The phylogenetic relationships were assumed on the ML and BI results (as in Fig. 3). Arrows show the fixed reference points used here. The divergence time between Mantellidae and Rhacophoridae of 92.6–53.6 million years ago (Ma) was estimated by Bossuyt and Milinkovitch (2001), and the divergence between *Hoplobatrachus* and *Euphlyctis* of 30–25 Ma was by two recent studies (Bossuyt et al., 2006; Roelants et al., 2004).

Table 3
Divergence time estimates (mean \pm SD and 95% confidence interval) for different nodes based on tree topologies

Branching node	Comparative tree topologies			Accepted interval	
	<i>H. occipitalis</i> + <i>Hoplobatrachus</i>		Polytomy (<i>H. occipitalis</i> , <i>Hoplobatrachus</i> , and <i>Euphyctis</i>)		
	Estimated time \pm SD	95% Interval			
Mantellidae and Rhacophoridae (B)	79.3 \pm 8.3	61.5–91.8	80.1 \pm 8.1	62.0–92.0	61.5–92.0
Between Indian populations of <i>E. hexadactylus</i> (N)	5.2 \pm 1.4	2.9–8.5	4.5 \pm 1.3	2.4–7.4	2.7–8.1
Between Indian and Bangladesh populations of <i>E. hexadactylus</i> (K)	10.7 \pm 2.1	7.0–15.3	9.1 \pm 1.9	5.8–13.3	6.5–14.5
Between another Indian populations and the other populations of <i>E. hexadactylus</i> (Basal <i>E. hexadactylus</i>) (H)	16.3 \pm 2.6	11.6–21.8	14 \pm 2.3	9.8–19.0	11.0–20.5
Between Sri Lankan and Bangladesh populations of <i>E. cyanophlyctis</i> (O)	6 \pm 1.6	3.5–9.5	5 \pm 1.3	3.0–8.1	3.2–8.8
Between Indian and other populations of <i>E. cyanophlyctis</i> (Basal <i>E. cyanophlyctis</i>) (L)	7.1 \pm 1.7	4.3–11.1	6 \pm 1.5	3.6–9.4	4.1–10.3
Between <i>E. hexadactylus</i> and <i>E. cyanophlyctis</i> (Basal <i>Euphyctis</i>) (F)	23.4 \pm 2.5	18.5–28.2	20.1 \pm 2.5	15.3–25.1	17.3–27.3
Between <i>H. occipitalis</i> and the other <i>Euphyctis</i> (Basal <i>Euphyctis</i>)	—	—	24.8 \pm 2.2	20.3–28.8	20.3–28.8
Between Indian and Bangladesh populations of <i>H. tigerinus</i> (M)	6.7 \pm 1.8	3.8–10.7	8.4 \pm 2.2	4.8–13.4	3.8–13.4
Between Thailand and Vietnam populations of <i>H. chinensis</i> (P)	2.3 \pm 1.0	1.0–4.4	2.9 \pm 1.2	1.2–5.7	1.1–5.0
Between another Thailand and other populations of <i>H. chinensis</i> (I)	12 \pm 2.4	7.8–17.1	14.3 \pm 2.7	9.4–20.1	8.7–18.6
Between <i>H. tigerinus</i> and <i>H. chinensis</i> (I)	15.9 \pm 2.5	11.3–21.1	19 \pm 2.7	13.8–24.5	12.6–22.8
Between <i>H. crassus</i> and <i>H. tigerinus</i> + <i>H. chinensis</i> (G)	19.5 \pm 2.6	14.6–24.7	23 \pm 2.6	17.9–27.9	16.4–26.3
Between <i>H. occipitalis</i> and the other <i>Hoplobatrachus</i> (Basal <i>Hoplobatrachus</i>) (E)	25.6 \pm 2.0	21.4–29.1	—	—	21.4–29.1
<i>Hoplobatrachus</i> + <i>Euphyctis</i> + <i>H. occipitalis</i>	—	—	—	—	25.5–30.0
Between <i>Hoplobatrachus</i> and <i>Euphyctis</i> (D)	28.6 \pm 1.2	25.7–30.0	28.5 \pm 1.2	25.5–30.0	25.7–30.0
Between <i>F. limnocharis</i> and <i>Hoplobatrachus</i> + <i>Euphyctis</i> (C)	51.7 \pm 6.1	40.3–64.6	53.5 \pm 6.8	41.2–67.8	40.3–67.8
Between Dicoglossidae and Mantellidae + Rhacophoridae (A)	103.3 \pm 8.2	87.6–119.5	104.9 \pm 8.0	89.5–120.7	87.6–120.7

constant gene flow. Remarkably, the four major haplotypes of *E. hexadactylus* show high nucleotide divergence from each other (Table 2). Even though three of these haplotypes were also found in separate areas [Khulna (Sundarban, Bangladesh), Mudigere and Adyar (Western Ghats, India)], Ehex-In1 and Ehex-In2 haplotype groups occurred in the same locality, Adyar (Western Ghats, India) (Fig. 2D).

Recent molecular works suggested that the values of intra- and interspecific sequence divergence can help to identify cryptic species. Vences et al. (2005) reported on conspecific 16S rRNA haplotypes of up to 6% pairwise distance in mantellid frogs. Fouquet et al. (2007a) provided evidence that reproductively isolated cryptic species can be separated by 3.8% (*Rhinella*) and 4.3% (*Scinax*) based on 16S rRNA gene sequences. However, Fouquet et al. (2007b) suggested that a 3% threshold may prove to be a useful tool to document tropical frog biodiversity. According to these studies, the present nucleotide divergence found in *H. chinensis* (Phang Nga, Thailand vs. all others), *E. cyanophlyctis*, and *E. hexadactylus* suggested the presence of cryptic species within currently recognized species. The sympatric distribution of Ehex-In1 and Ehex-In2 haplotypes (nucleotide divergence is 20.1%, 11.9%, and 6.3% for Cyt b, 12S, and 16S RNA genes, respectively) clearly indicates the occurrence of different *E. hexadactylus* species in Adyar (Western Ghats, India).

As described above, we found three and four distinct haplotype groups having species-level nucleotide divergence in *E. cyanophlyctis* (Ecy-Ba, -Sr, and -In; Fig. 2C) and *E. hexadactylus* (Ehex-Ba, -In1, -In2, and -In3; Fig. 2D), respectively. The type localities of these two species were suggested as Tranquebar (Bauer, 1998) and Pondichéry (Frost, 2007), respectively (both located in Southeast India near Sri Lanka). In the present study, specimens from the type localities were not available, so it is difficult to specify which haplotype group corresponds to the nominal species. However, it is possible that the Sri Lanka *E. cyanophlyctis* haplotype (Ecy-Sr) group corresponds to the “real” *E. cyanophlyctis*, because Sri Lanka is very close to the type locality and was connected to Southeast India during the Pleistocene period (>1.0 Ma; Bossuyt et al., 2004). Furthermore, *Rana bengalensis* named after ‘Bengal’ (presently Bangladesh and West Bengal of India) is currently considered a synonym of *E. cyanophlyctis* (Frost, 2007). Thus, the Bangladesh *E. cyanophlyctis* haplotype (Ecy-Ba) group might correspond to this species. Furthermore, in the case of *E. hexadactylus*, the 16S rRNA gene sequence of the Sri Lankan specimen (Kosuch et al., 2001, Accession No. AF215389) is very similar to that of the Bangladesh haplotype (0.2%) (Fig. 2D). If the specimen from Sri Lanka corresponds to the nominal species, the haplotype group from Bangladesh may be the “real” *E. hexadactylus*, in which case other haplotypes from the Western Ghats are considered distinct species. As for the genus *Euphyctis*, another species, *E. ghoshi*, has been identified only from Manipur, India (Chanda, 1990). However, as genetic analysis has never been performed for this species, one of the Indian *Euphyctis* haplotypes found here may correspond to that of *E. ghoshi*. As for *H. chinensis*, we did not use specimens from China. However, Che et al. (2007) also showed two distinguishable *H. chinensis* haplotypes (with 9.3% and 3.0% sequence divergence for 12S and 16S rRNA genes, respectively) from Hainan and Yunan, China, and the haplotypes matched our Hchi-Th4 haplotype (0% sequence divergence in 16S rRNA gene; Fig. 2B) and other *H. chinensis* haplotypes (1.1%; Fig. 2B), respectively. The type locality of this species is unclear, but is possibly in the vicinity of Canton, China (Frost, 2007), and Hainan is very close to Canton. Thus, our results imply that *H. chinensis* as currently recognized contains two distinct species; one species (Hchi-Th4) (the possible nominal species) might occupy the wide coastal region of Southeast Asia, and the other seems to inhabit southeastern China and neighboring countries.

Although the distribution of *H. crassus* in Bangladesh was unclear (Islam et al., 2000), we could find *H. crassus* in the Sundarban mangrove forest of Khulna, Bangladesh. It is also noteworthy that the physical distance between Sundarban, Khulna (Bangladesh) and Assam (India) is large (about 1100 km) (Fig. 2B), but the haplotypes of *H. crassus* from these two populations (Hcra-Ba and -In) have almost the same nucleotide sequence. This low divergence might represent recent population expansion through the Ganges–Brahmaputra delta (Table 2).

In the present study, we could not perform detailed morphological comparisons, and we lacked the specimens from type localities for some species. Thus, at present, we avoid further taxonomic discussion. However, our results clarified the underestimation of the richness of amphibian fauna in this region, indicating the possible occurrence of many cryptic species among these groups and strongly suggest that taxonomic revisions are needed for *Hoplobatrachus* and *Euphlyctis* taxa.

4.2. Divergence times and possible events causing *Hoplobatrachus* and *Euphlyctis* divergence

It is generally proposed that several Asian ranid (= Natatanuran sensu Frost et al., 2006) lineages occurred in the Indian subcontinent after the split from Gondwanaland (starting around 150 Ma) and migrated to Asia via subcontinental drift and collision with Eurasia (e.g., Roelants et al., 2004; van der Meijden et al., 2005; Bossuyt et al., 2006). The Dicroglossini group (including *Hoplobatrachus* and *Euphlyctis*) is included in this explanation (e.g., Bossuyt and Milinkovitch, 2001). In this study, we could not clarify the phylogenetic position of African *H. occipitalis*; however, the separation

of this species from other Asian *Hoplobatrachus* and *Euphlyctis* taxa was estimated at around 25 Ma (Table 3 and Fig. 4). Similar separation times for this African taxon have been estimated from several studies (25–8 Ma, Kosuch et al., 2001; approx. 10 Ma, Vences et al., 2003), and Kosuch et al. (2001) suggested that the split of African *H. occipitalis* and Asian taxa was not correlated with Gondwanan vicariance (i.e., “Out of Africa” hypothesis), but rather *H. occipitalis* returned from Asia to Africa after the India–Eurasia collision (out of Asia). Furthermore, the separation between the genera *Hoplobatrachus* and *Euphlyctis* has been estimated as 30–25 Ma in at least two independent studies (Roelants et al., 2004; Bossuyt et al., 2006). Thus, the ancestors of *Hoplobatrachus* and *Euphlyctis* would have occurred in the Indian subcontinent before the India–Eurasia collision (23–20 Ma; Alam et al., 2003; Uddin and Lundberg, 2004).

In our estimation (Fig. 4), the first splits occurred in both the *Hoplobatrachus* and *Euphlyctis* lineages at around 22 Ma (split of *H. occipitalis* from others and split between *E. hexadactylus* and *E. cyanophlyctis*). This age seems to correlate with the timing of the India–Eurasia collision (23–20 Ma; Alam et al., 2003; Uddin and Lundberg, 2004) (Fig. 5A), suggesting that the collision and the following climate change and/or the expansion of inhabitable areas might have led to the initial adaptive radiation of these frog lineages. Then, in the *Hoplobatrachus* lineage, *H. crassus* separated from other lineages at around 19.5 Ma, and the split of *H. chinensis* and *H. tigerinus* was estimated as 15.9 Ma. In the *E. hexadactylus* lineage, the Ehex-In1 haplotype was the first to split at 16.3 Ma. We could not identify specific geographic events for the above split ages. However, at 20–14 Ma, the uplift of the Himalayas through the North and Indo-Burman ranges (Uddin and Lundberg, 2004;

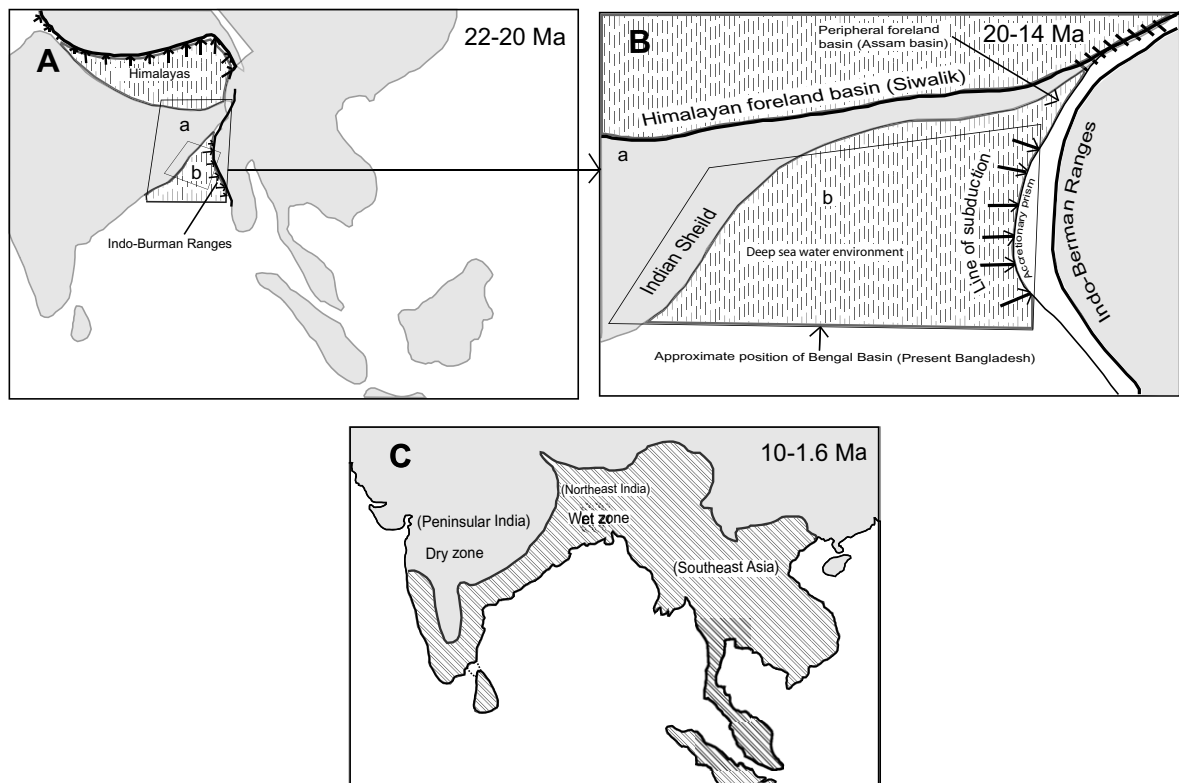


Fig. 5. Summary of paleogeography in the Indian subcontinent. Collision of Indian tectonic plate with Eurasian plate and subsequent geographic events are shown. (A) Subduction and formation of Himalayas and Indo-Burman ranges during the early Miocene (22–20 Ma). (B) Formation of Bengal basin and filled by sedimentation during the middle Miocene (20–14 Ma). (C) Map of Asia showing dry and wet zones (10–1.6 Ma). Hatched and gray areas represent the wet zone (over 250 cm of rainfall) and dry zone (rainfall between 50 and 100 cm), respectively. Here, Sri Lanka is shown as being connected to South India, because it was geologically part of the Deccan plate and was separated from India by a shallow strait that might have served as a land bridge during times of lowered sea level. This land bridge might have facilitated the movement of flora and fauna between peninsular India and Sri Lanka. (A) and (B) modified after Uddin and Lundberg (2004) and Alam et al. (2003), and (C) modified after Karanth (2003).

Alam et al., 2003) was caused by the continental collision, and the formation of the present Bangladesh land by sedimentation was not completed (i.e., Bengal basin; Alam et al., 2003; Uddin and Lundberg, 2004) (Fig. 5B), suggesting that the ancestors of *H. crassus*, *H. tigerinus*, and *E. hexadactylus* could not have immediately spread to North and Southeast Asian areas at the time of their split. Although *H. crassus*, *H. tigerinus*, and *E. hexadactylus* currently show a wide distribution, major speciation events in *Hoplobatrachus* and *Euphlyctis* might have occurred in the Indian subcontinent.

In the *H. chinensis* taxa, the first split separated the Hchi-Th4 haplotype from others at around 12 Ma. In this period, the present Bangladesh land seems to have been formed (Alam et al., 2003; Uddin and Lundberg, 2004) and frog taxa could have expanded their habitat to Southeast Asia through this area. Considering the present distribution of *H. chinensis* (East and Southeast Asia, but not India), its immediate ancestors likely occurred and diverged in East and Southeast Asia rather than in India.

South Asian biogeography is marked by a disjunct distribution pattern of closely related organisms. Such a pattern has been reported for many animals (mammals, birds, freshwater fish, amphibians, reptiles, and insects) and plants (Karanth, 2003; Gaston and Zacharias, 1996; Das, 1996, 2002; Daniel, 2002) in this area. The formation of this unique distribution pattern is believed to have begun in the Middle Miocene (18–11 Ma) (Ashton and Gunatilleke, 1987). Before this period, humid forest extended continuously from Northeast to Southern India as well as to Bangladesh (Poole and Davies, 2001). However, by Upper Siwalik times (before 5.1–1.6 Ma, Fig. 5C), aridification occurred and the tropical forest was largely replaced by savanna in central India; the dried zone was presumed to be a barrier for many organisms (Karanth, 2003). Interestingly, the estimated branching ages of the Western Ghats, Indian and Southeast Asian haplotypes of *H. tigerinus* (6.7 ± 1.8 Ma), and *E. cyanophlyctis* (7.1 ± 1.7 Ma) seem to match the period of dry-zone formation. This might suggest that before this period the ancestors of these taxa were widely distributed in South and Southeast Asia; however, aridification of central India blocked the gene flow between the West India and Southeast Asian areas. In *E. cyanophlyctis* taxa, the Western Ghats haplotypes (Ecy-In) split at 7.1 Ma from the Sri Lanka and Bangladesh haplotypes and the latter split at 6.0 Ma. Although central India had dried up, the eastern coast remained wet during this period [and Sri Lanka was intermittently connected to the Indian mainland during the Pleistocene (>1.0 Ma; Bossuyt et al., 2004)] (Fig. 5C). The split ages of *E. cyanophlyctis* taxa may suggest that, unlike central India, the eastern side of India might have been a corridor for amphibian migration during the late Miocene. The presence of very similar *E. hexadactylus* haplotypes in both Sri Lanka and Bangladesh (see above) might support this idea. Two *E. hexadactylus* haplotypes from the Western Ghats (Ehex-In2 from Adyar and Ehex-In3 from Mudigere) split around 5.2 Ma, and this period is also consistent with the drying age of central India. However, as in eastern India, it is considered that tropical forests expanded in the Western Ghats region during this period (Karanth, 2003). Thus, the divergence between Ehex-In2 (Adyar) and Ehex-In3 (Mudigere) haplotypes does not appear to have been caused by a vicariance geographic event or environmental change (i.e., vicariant divergence) but by range expansion.

In this study, we investigated the divergence patterns of Asian *Hoplobatrachus* and *Euphlyctis* taxa based on estimated divergence times and paleogeological events, and proposed that (1) major speciation events of these anuran taxa might have occurred in South Asian areas, (2) the formation of Bangladesh land may have allowed the spread of frog taxa to Southeast Asian areas, and (3) the aridification of central India might have affected the gene flow of widely distributed species. These results might be useful as a guideline for biogeographical studies in this region. At the same

time, we could not specify the causes of some speciation events (e.g., *H. crassus*, *H. tigerinus*, and *E. hexadactylus*) due to lack of detailed investigation in East India, a possible corridor connecting South and Southeast Asian anuran fauna. Further extensive sampling at this area is needed to clarify the evolutionary process of these frog taxa.

Acknowledgments

We acknowledge to Dr. A. Dubois and Dr. A. Ohler, Muséum National d' Histoire Naturelle, France, for their kindness in collecting and supplying samples, and to Dr. S.H. Joshy of St. Aloysius College, India, for his valuable help in collecting specimens. We are also grateful to Dr. A. Larson and two anonymous reviewers for improving an early revision of the manuscript. This work was supported by a Grant-in-Aid for Scientific Research (C) (No. 17570082) to M. Sumida from the Ministry of Education, Culture, Sports, Science and Technology, Japan. The present molecular work was performed with the gracious cooperation of the Analysis Center of Life Science and Natural Science Center for Basic Research and Development, Hiroshima University.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2008.04.020.

References

- Alam, M., Alam, M.M., Curray, J.R., Chowdhury, M.L.R., Gani, R.M., 2003. An overview of the sedimentary geology of the Bengal Basin in relation to the regional tectonic framework and basin-fill history. *Sediment. Geol.* 155, 179–208.
- Ashton, P.S., Gunatilleke, C.V.S., 1987. New light on the plant geography of Ceylon. I. Historical plant geography. *J. Biogeogr.* 14, 249–285.
- Bauer, A.M., 1998. South Asian herpetological specimens of historical note in the zoological museum, Berlin. *Hymadryad* 23, 133–149.
- Bickford, D., Lohman, D.J., Sodhi, N.S., Ng, P.K.L., Meier, R., Winker, K., Ingram, K.I., Das, I., 2006. Cryptic species as a window on diversity and conservation. *Trends Ecol. Evol.* 22, 148–155.
- Biju, S.D., Bossuyt, F., 2003. New frog family from India reveals an ancient biogeographical link with the Seychelles. *Nature* 425, 711–713.
- Borkin, L.Y., Litvinchuk, S.N., Rozanov, Y.M., Skorinov, D.V., 2004. On cryptic species (from the example of amphibians). *Zool. Zh.* 83, 936–960.
- Bossuyt, F., Milinkovitch, M.C., 2001. Convergent adaptive radiations in Madagascar and Asian ranid frogs reveal covariation between larval and adult traits. *Proc. Natl. Acad. Sci. USA* 97, 6585–6590.
- Bossuyt, F., Meegaskumbura, M., Beenaerts, N., Gower, D.J., Pethiyagoda, R., Roelants, K., Mannaert, A., Wilkison, M., Bahir, M.M., Manamendra-Arachchi, K., Ng, P.K.L., Schneider, C.J., Oommen, O.V., Milinkovitch, M.C., 2004. Local endemism within the Western Ghats–Sri Lanka biodiversity hotspots. *Science* 306, 479–481.
- Bossuyt, F., Brown, R.M., Hillis, D.M., Cannatella, D.C., Milinkovitch, M.C., 2006. Phylogeny and biogeography of a cosmopolitan frog radiation: late cretaceous diversification resulted in continent-scale endemism in the family Ranidae. *Syst. Biol.* 55, 579–594.
- Castresana, J., 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol. Biol. Evol.* 17, 540–552.
- Chanda, S.K., 1990. A new frog of the genus *Rana* (Ranidae: Anura) from Manipur, Northeastern India. *Hamadryad* 15, 16–17.
- Che, J., Pang, J., Zhao, H., Wu, G., Zhao, E., Zhang, Y., 2007. Molecular phylogeny of the Chinese ranids inferred from nuclear and mitochondrial DNA sequences. *Biochem. Syst. Ecol.* 35, 29–39.
- Daniel, J.C., 2002. *The Book of Indian Reptiles and Amphibians*. Oxford University Press, Mumbai.
- Das, I., 1996. *Biogeography of the Reptiles of South Asia*. Kreiger Publishing Company, Malabar, FL.
- Das, I., 2002. *An Introduction to the Amphibians and Reptiles of Tropical Asia*. Natural History Publications (Borneo), Kota Kinabalu.
- De la Riva, I., Köhler, J., Lötters, S., Reichle, S., 2000. Ten years of research on Bolivian amphibians: updated checklist, distribution, taxonomic problems, literature and iconography. *Rev. Esp. Herpetol.* 14.
- Djong, T.H., Islam, M.M., Nishioka, M., Matsui, M., Ota, H., Kuramoto, M., Khan, M.M.R., Alam, M.S., De Silva, A., Khonsue, W., Sumida, M., 2007a. Genetic relationships and reproductive isolation mechanisms among the *Fejervarya limnocharis* complex from Indonesia (Java) and other countries. *Zool. Sci.* 24, 360–375.

- Djong, T.H., Matsui, M., Kuramoto, M., Belabut, D.M., Yong, H.S., Nishioka, M., Sumida, M., 2007b. Morphological divergence, reproductive isolating mechanism, and molecular phylogenetic relationships among Indonesia, Malaysia, and Japan populations of the *Fejervarya limnocharis* complex (Anura, Ranidae). *Zool. Sci.* 24, 1197–1212.
- Dubois, A., 1987. *Miscellanea taxinomica batrachologica* (1). *Alytes* 5, 7–95.
- Dubois, A., 1992. Notes sur la classification des Ranidae (Amphibiens Anoures). *Bull. Mens. Soc. Linn.* 61, 305–352.
- Farris, J.S., Källersjö, M., Kluge, A.G., Bult, C., 1995. Constructing a significance test for incongruence. *Syst. Biol.* 44, 570–672.
- Fouquet, A., Vences, M., Salducci, M.D., Meyer, A., Marty, C., Blanc, M., Gilles, A., 2007a. Revealing cryptic diversity using molecular phylogenetics and phylogeography in frogs of the *Sinxin rubber* and *Rhinella margaritifera* species groups. *Mol. Phylogenet. Evol.* 43, 567–582.
- Fouquet, A., Gilles, A., Vences, M., Marty, C., Blanc, M., Gemmill, N.J., 2007b. Underestimation of species richness in neotropical frogs revealed by mtDNA analysis. *PLoS One* 10, 1–10.
- Frost, D.R., 2007. Amphibian Species of the World: An Online Reference 5.0. Electronic database available at <<http://research.amnh.org/herpetology/amphibia/index.php>>.
- Frost, D.R., Grant, T., Faivovich, J., Bain, R.H., Haas, A., Haddad, C.F.B., de Sa, R.O., Channing, A., Wilkinson, M., Donnellan, S.C., Raxworthy, C., Campbell, J.A., Blotto, B.L., Moler, P., Drewes, R.C., Nussbaum, R.A., Lynch, J.D., Green, D.M., Wheeler, W.C., 2006. The amphibian tree of life. *Bull. Amer. Mus. Nat. Hist.* 297, 1–370.
- Gaston, A.J., Zacharias, V.J., 1996. The recent distribution of endemic and disjunct birds in Kerala State: preliminary results of an ongoing survey. *J. Bombay Nat. Hist. Soc.* 93, 389–400.
- Grosjean, S., Vences, M., Dubois, A., 2004. Evolutionary significance of oral morphology in the carnivorous tadpoles of tiger frogs, genus *Hoplobatrachus* (Ranidae). *Biol. J. Linn. Soc.* 81, 171–181.
- Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.* 52, 696–704.
- Igawa, T., Kurabayashi, A., Usuki, C., Fujii, T., Sumida, M., 2008. Complete mitochondrial genomes of three neobatrachian anurans: a case study of divergence time estimation using different data and calibration settings. *Gene* 407, 116–129.
- Inger, R.F., 1999. Distribution of amphibians in Southern Asia and adjacent islands. In: Duellman, W.E. (Ed.), *Patterns of Distribution of Amphibians: A Global Perspective*. Johns Hopkins University Press, Baltimore, MD, USA, pp. 445–482.
- Inger, R.F., Dutta, S.K., 1986. An overview of the amphibian fauna of India. *J. Bombay Nat. Hist. Soc.* 83, 135–146.
- Islam, M.A., Ameen, M., Nishat, A., 2000. Red Book of Threatened Amphibians and Reptiles of Bangladesh. IUCN, The World Conservation Union, Dhaka, Bangladesh.
- Islam, M.M., Khan, M.M.R., Djong, H.T., Alam, M.S., Sumida, M., 2008. Genetic differentiation of *Fejervarya limnocharis* complex from Bangladesh and other Asian countries elucidated by allozyme analyses. *Zool. Sci.* 25, 261–272.
- Karanth, K.P., 2003. Evaluation of disjunct distributions among wet-zone species of the Indian subcontinent: testing various hypotheses using a phylogenetic approach. *Curr. Sci.* 85, 10.
- Khan, M.S., 1997. A new subspecies of common skittering frog *Euphylyctis cyanophlyctis* (Schneider, 1977) from Balochistan, Pakistan. *Pak. J. Zool.* 29, 107–112.
- Köhler, J., Vietes, R., Bonett, R.M., Garcia, B.F.H., Glaw, F., Steinke, F., Vences, M., 2005. New amphibians and global conservation: a boost in species discoveries in a highly endangered vertebrate group. *BioScience* 55, 693–696.
- Kosuch, J., Vences, M., Dubois, A., Ohler, A., Bohme, W., 2001. Out of Asia: mitochondrial DNA evidence for an oriental origin of tiger frogs, genus *Hoplobatrachus*. *Mol. Phylogenet. Evol.* 21, 398–407.
- Kurabayashi, A., Kuramoto, M., Joshy, H., Sumida, M., 2005. Molecular phylogeny of the ranid frogs from Southwest India based on the mitochondrial ribosomal RNA gene sequences. *Zool. Sci.* 22, 525–534.
- Kurabayashi, A., Usuki, C., Mikami, N., Fujii, T., Yonekawa, H., Sumida, M., Hasegawa, M., 2006. Complete nucleotide sequence of the mitochondrial genome of a Malagasy poison frog *Mantella madagascariensis*: evolutionary implications on mitochondrial genomes of higher anuran groups. *Mol. Phylogenet. Evol.* 39, 223–236.
- Kuramoto, M., Joshy, S.H., Kurabayashi, A., Sumida, M., 2007. The genus *Fejervarya* (Anura: Ranidae) in central Western Ghats, India, with description of four new cryptic species. *Cur. Herpetol.* 26, 81–105.
- Liu, Z.-Q., Wang, Y.Q., Su, B., 2005. The mitochondrial genome organization of the rice frog, *Fejervarya limnocharis* (Amphibia: Anura): a new gene order in the vertebrate mtDNA. *Gene* 346, 145–151.
- Meegaskumbura, M., Bossuyt, F., Pethiyagoda, R., Manamendra-Arachchi, K., Bahir, M., Milinkovitch, M.C., Schneider, C.J., 2002. Sri Lanka: an amphibian hotspot. *Science* 298, 379.
- Nishat, A., Huq, S.M.I., Barua, S.P., Reza, A.H.M.A., Khan, A.S.M., 2002. Bio-ecological Zones of Bangladesh. IUCN, The World Conservation Union, Bangladesh Country Office. XII+ 141 pp.
- Poole, I., Davies, C., 2001. *Glutoxylon* Chowdhury (Anacardiaceae): the first record of fossil wood from Bangladesh. *Rev. Palaeobot. Palynol.* 113, 261–272.
- Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14, 817–818.
- Reza, A.H.M., 2007. Destination Bangladesh: from the Himalayas to the Bay of Bengal. *Iguana* 14 (2), 106–114.
- Roelants, K., Jiang, J., Bossuyt, F., 2004. Endemic ranid (Amphibia: Anura) genera in southern mountain ranges of the Indian subcontinent represent ancient frog lineages: evidence from molecular data. *Mol. Phylogenet. Evol.* 31, 730–740.
- Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19, 1572–1574.
- Sano, N., Kurabayashi, A., Fujii, T., Yonekawa, H., Sumida, M., 2004. Complete nucleotide sequence and gene rearrangement of the mitochondrial genome of the bell-ring frog, *Buergeria buergeri* (family Rhacophoridae). *Genes Genet. Syst.* 79, 151–163.
- Sano, N., Kurabayashi, A., Fujii, T., Yonekawa, H., Sumida, M., 2005. Complete nucleotide sequence of the mitochondrial genome of Schlegel's tree frog *Rhacophorus schlegelii* (family Rhacophoridae): duplicated control regions and gene rearrangements. *Genes Genet. Syst.* 80, 213–224.
- Shimodaira, H., Hasegawa, M., 2001. CONSEL: a program for assessing the confidence of phylogenetic tree selection. *Bioinformatics* 17, 1246–1247.
- Stuart, B.L., Inger, R.F., Voris, H.K., 2006. High level of cryptic species diversity revealed by sympatric lineages of Southeast Asian forest frogs. *Biol. Lett.* 2, 470–474.
- Sumida, M., Ogata, M., Kaneda, H., Yonekawa, H., 1998. Evolutionary relationships among Japanese pond frogs inferred from mitochondrial DNA sequences of cytochrome *b* and 12S ribosomal RNA genes. *Genes Genet. Syst.* 73, 121–133.
- Sumida, M., Kondo, Y., Kanamori, Y., Nishioka, M., 2002. Inter- and intraspecific evolutionary relationships of the rice frog *Rana limnocharis* and the allied species *R. cancrivora* inferred from crossing experiments and mitochondrial DNA sequences of the 12S and 16S rRNA genes. *Mol. Phylogenet. Evol.* 25, 293–305.
- Sumida, M., Kotaki, M., Islam, M.M., Djong, T.H., Igawa, T., Kondo, Y., Matsui, M., De Silva, A., Khonsue, W., Nishioka, M., 2007. Evolutionary relationships and reproductive isolating mechanisms in the rice frog (*Fejervarya limnocharis*) species complex from Sri Lanka, Thailand, Taiwan and Japan, inferred from mtDNA gene sequences, allozymes, and crossing experiments. *Zool. Sci.* 24, 547–562.
- Swofford, D.L., 2003. PAUP^{*}. *Phylogenetic Analysis Using Parsimony* (and Other Methods). Version 4. Sinauer Associates, Sunderland, MA.
- Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24, 1596–1599.
- Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680.
- Thorne, J.L., Kishino, H., 2002. Divergence time and evolutionary rate estimation with multilocus data. *Syst. Biol.* 51, 689–702.
- Uddin, A., Lundberg, N., 2004. Miocene sedimentation and subsidence during continent–continent collision, Bengal basin, Bangladesh. *Sediment. Geol.* 164, 131–146.
- Van Bocxlaer, I., Roelants, K., Biju, S.D., Nagaraju, J., Bossuyt, F., 2006. Late Cretaceous vicariance in Gondwanan amphibians. *PLoS One* 1, 1–6.
- Van der Meijden, A., Vences, M., Hoegg, S., Meyer, A., 2005. A previously unrecognized radiation of ranid frogs in Southern Africa revealed by nuclear and mitochondrial DNA sequences. *Mol. Phylogenet. Evol.* 37, 674–685.
- Vences, M., Vieites, D.R., Glaw, F., Brinkmann, H., Kosuch, J., Veith, M., Meyer, A., 2003. Multiple overseas dispersal in amphibians. *Proc. R. Soc. Lond. B Biol. Sci.* 270, 2435–2442.
- Vences, M., Thomas, M., van der Meijden, A., Chiari, Y., Vietes, D.R., 2005. Comparative performance of the 16S rRNA gene in DNA barcoding of amphibians. *Front. Zool.* 2, 5.
- Yang, Z., 1997. PAML: a program package for phylogenetic analysis by maximum likelihood. *Comput. Appl. Biosci.* 13, 555–556.