

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

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1 **Abstract**

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

1 **Key words:** Genetic divergence; Molecular phylogeny; Mitochondrial genes;

2 Divergence time; Amphibia; *Hoplobatrachus*; *Euphlyctis*; Cryptic species;

3 Bangladesh

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1 **1. Introduction**

2 Bangladesh, located in the tropical climatic zone, features one of the world's
3 largest deltas (Ganges–Brahmaputra river delta) formed by Miocene sedimentation
4 and subsidence during continent-continent collision (Uddin and Lundberg, 2004) and
5 is endowed with a rich diversity of unique flora and fauna. Biogeographically, this
6 country is part of the Oriental region, nestled between the Indo-Himalayan and Indo-
7 Chinese subregions of the Orient (Nishat et al., 2002). Although the amphibian fauna
8 of the Western Ghats, India includes a large number of endemic taxa (Inger and Dutta,
9 1986), the available information on Bangladesh amphibian fauna lists only 22 frog
10 species (Islam et al., 2000). A recent herpetofaunal survey reported the occurrence of
11 some interesting species in Bangladesh for the first time (Reza et al., 2007), but the
12 genetic divergence and evolutionary aspects of the herpetofauna of Bangladesh have
13 basically been neglected.

14 Among the amphibian fauna reported from Bangladesh, *Hoplobatrachus* and
15 *Euphlyctis* frogs were the most common species, and during the 1980s Bangladesh
16 was a major world supplier of frogs. The Bangladesh Government eventually banned
17 the exporting of frogs in order to maintain the country's natural resources and
18 ecological balance. As for the genus *Hoplobatrachus*, *H. tigerinus* (Indian bullfrog) is
19 one of the most widely distributed species in Bangladesh, whereas the distribution of
20 *H. crassus* (Jerdon's bullfrog) is not clear due to insufficient data (Islam et al., 2000).
21 These two species are also distributed in other Asian countries such as India, Nepal,

1 Bhutan, and Sri Lanka (Frost, 2007). Two more species belonging to the genus
2 *Hoplobatrachus* are distributed in other countries: *H. chinensis* in Myanmar, China,
3 Thailand, and Malaysia, and *H. occipitalis* in several African countries (Frost, 2007).
4 As for the genus *Euphlyctis*, *E. cyanophlyctis* (Indian skipper frog) and *E.*
5 *hexadactylus* (Indian green frog) are known from Bangladesh (Islam et al., 2000). The
6 type localities of these two species are not clear, but Frost (2007) and Bauer (1998)
7 suggested that they might be in Tranquebar and Pondichéry located in Southeast
8 India near Sri Lanka. They also show wide distribution in other Asian countries: *E.*
9 *cyanophlyctis* in India, Pakistan, Afghanistan, Nepal, Sri Lanka, Myanmar, and
10 Vietnam, and *E. hexadactylus* in India, Pakistan, and Sri Lanka (Frost, 2007). Among
11 them, *E. cyanophlyctis* from the northwestern highlands of Pakistan was recognized as
12 a subspecies, *E. cyanophlyctis microspinulata* (Khan, 1997). Two more species
13 belonging to the genus *Euphlyctis* are distributed in other Asian countries: *E. ghoshi*,
14 known only from its type locality (Manipur, India), and *E. ehrenbergii*, inhabiting the
15 southwestern Arabian Peninsula (Saudi Arabia and Yemen) (Frost, 2007).

16 It is well known that the genus *Hoplobatrachus* is the sister taxon to the genus
17 *Euphlyctis* (Kosuch et al., 2001; Grosjean et al., 2004; Kurabayashi et al., 2005; Frost
18 et al., 2006). The species of these two genera were formerly regarded as members of
19 the genus *Rana*. However, Dubois (1987, 1992) suggested that the genus *Rana* was a
20 phylogenetically heterogeneous group, and transferred many species from *Rana* to
21 other genera including *Hoplobatrachus* and *Euphlyctis*. Although several studies have

1 been performed for phylogenetic analyses of higher taxa including these genera
2 (Bossuyt et al., 2006; Kosuch et al., 2001; Roelants et al., 2004; Vences et al., 2003),
3 there has been no investigation regarding detailed species composition, genetic
4 relationships and phylogeographic patterns among *Hoplobatrachus* and *Euphlyctis*
5 groups in Bangladesh and neighboring countries.

6 The increasing utilization of molecular data has led to the reorganization of
7 amphibian taxonomy (Biju and Bossuyt, 2003; Borkin et al., 2004; Bossuyt et al.,
8 2006; De la Riva et al., 2000; Frost et al., 2006; Meegaskumbura et al., 2002) and the
9 discovery of many cryptic species (Bickford et al., 2006; Fouquet et al., 2007a, b;
10 Köhler et al., 2005; Stuart et al., 2006). Recent analyses of molecular and allozyme
11 data on samples from Asian countries suggested the underestimation of diversity of
12 amphibian fauna in this region as well as among these groups (Kurabayashi et al.,
13 2005; Djong et al., 2007a, b; Kuramoto et al., 2007; Sumida et al., 2007; Islam et al.,
14 2008). Inger (1999) suggested that additional samplings in South Asia would
15 undoubtedly increase the number of species known from each area and illuminate
16 detailed information on the distribution of species.

17 In order to elucidate the genetic diversity and phylogenetic relationships among
18 *Hoplobatrachus* and *Euphlyctis* groups from Bangladesh and neighboring countries,
19 we performed molecular phylogenetic analyses using mitochondrial *Cyt b* and 12S
20 and 16S rRNA gene data from 252 frog specimens. Based on the results, we showed
21 the possible existence of several cryptic species in these frog groups. We also

1 estimated the divergence times among these taxa to determine the paleogeological
2 events that had caused these divergences.

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4 **2. Materials and Methods**

5 2.1. Specimens

6 A total of 252 individuals consisting of four species of the genus
7 *Hoplobatrachus* (*H. tigerinus*, *H. crassus*, *H. chinensis*, and *H. occipitalis*) and two
8 species of the genus *Euphlyctis* (*E. cyanophlyctis* and *E. hexadactylus*) were used in
9 the present study (Table 1, Fig. 1). Among them, 201 individuals were collected from
10 17 localities in Bangladesh, 46 individuals from 20 localities in India, Nepal, Sri
11 Lanka, Thailand, Laos, and Vietnam, and three individuals of *H. occipitalis* were
12 commercially obtained from Tanzania. Species identification was based on Dubois
13 (1992) and Frost (2007) classifications. Details of specimens are shown in electric
14 supplement 1.

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16 2.2. DNA extraction

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

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1 2.3. PCR and sequencing

2 PCR amplification was performed on partial sequences of Cyt *b* (564 bp), 12S
3 rRNA (689 bp), and 16S rRNA (517 bp) genes. These segments corresponded to the
4 sites 16785–17348, 4474–5163, and 6251–6765, respectively, in the *Fejervarya*
5 *limnocharis* complete mtDNA sequence (Accession No. AY158705, Liu et al., 2005).
6 The following sets of primers were used for PCR amplification: Cytb Fow-1-1 (Sano
7 et al., 2005) and Cytb Rev-1 (Kurabayashi, unpublished) for Cyt *b* gene, FS01 and
8 RFR60 for 12S rRNA gene (Sumida et al., 1998), and F51 and R51 for 16S rRNA
9 gene (Sumida et al., 2002). The sequences of the primers are available from electric
10 supplement 2. PCR mixtures were prepared with the TaKaRa Ex TaqTM Kit (TaKaRa
11 Bio Inc.) as recommended by the manufacturer's protocol. Cyt *b* and 12S and 16S
12 rRNA segments were amplified by 35 cycles, each cycle consisting of denaturation for
13 10 sec at 98°C, annealing for 30 sec at 47.5°C (10 cycles), 45.0°C (10 cycles) and
14 42.5°C (15 cycles), and extension for 1 min 20 sec at 72°C. The PCR products were
15 purified by ethanol precipitation. The amplified Cyt *b* and 12S and 16S rRNA gene
16 segments were directly sequenced for both strands using the BigDye Terminator
17 Cycle Sequencing Kit (ABI) with automated DNA Sequencer (3100-Avant, ABI). The
18 resultant sequences were deposited in the DDBJ database under Accession Nos.
19 AB274044–AB274170, AB273137–AB273176, AB272583–AB272608, AB290594–
20 AB290612, and AB290412–AB290434 (Table 1).

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1 2.4. Selection of haplotypes

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

10

11 2.5. Phylogenetic analyses

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

20 Phylogenetic analysis based on the combined data was performed by maximum
21 likelihood (ML), maximum parsimony (MP), and Bayesian inference (BI) methods. In

1 all analyses, *Microhyla okinavensis* was used as the outgroup; the sister-taxon
2 relationship of Microhylidae (+ Afrobatrachia) and ranids (= Natanaura sensu Frost et
3 al., 2006) was well corroborated (e.g., van der Meijden et al., 2005; Van Bocxlaer et
4 al., 2006; Igawa et al., 2008). MP analysis was performed using PAUP*4.0b10
5 (Swofford, 2003). A heuristic search with 100 replicates of random sequence addition
6 and TBR branch swapping was used, and all sites were of equal weighting. Clade
7 support under MP was evaluated using 2000 replicates of nonparametric bootstrapping
8 (nBP). For BI and ML analysis, appropriate substitution models (GTR+G+I) were
9 chosen using the Akaike information criterion (AIC) as implemented in Modeltest 3.7
10 (Posada and Crandall, 1998). ML analysis based on the combined data was performed
11 using PAUP* with heuristic search and TBR swapping. Nonparametric BP under ML
12 was calculated using PHYML 2.4.4 (Guindon and Gascuel, 2003) with 1000 replicates.
13 BI analysis was performed using MrBayes Ver. 3.1.2 (Ronquist and Huelsenbeck,
14 2003). The following settings were used for BI analysis: Number of Markov chain
15 Monte Carlo (MCMC) generations = 15×10^5 , sampling frequency = 10. The burn-in
16 size was determined by checking the convergence of $-\log$ likelihood ($-\ln L$) values and
17 the first 1×10^5 generations were discarded. The statistical support of the BI tree was
18 evaluated by Bayesian posterior probability (BPP). The sequence divergence was
19 computed with MEGA Ver. 4.0 (Tamura et al., 2007).

20 Alternative phylogenetic hypotheses among *Hoplobatrachus* and *Euphlyctis*
21 were compared using the approximately unbiased (AU), Kishino-Hasegawa (KH) and

1 Shimodaira-Hasegawa (SH) tests as implemented in CONSEL Ver. 0.1i (Shimodaira
2 and Hasegawa, 2001). Site-wise $\ln L$ values were calculated using PAML (Yang,
3 1997) and used as input for the program.

4

5 2.6. Divergence-time estimation

6 For divergence-time estimation, we used the MultiDivtime software package
7 (Thorne and Kishino, 2002). To focus on species-level divergence in the analysis, we
8 decreased the number of OTUs based on the results from the previous ML and BI
9 analyses. Because of ambiguous phylogenetic positions of *H. occipitalis*, we
10 separately conducted divergence-time estimation based on three alternative tree
11 topologies; i.e., *H. occipitalis* + *Hoplobatrachus*, *H. occipitalis* + *Euphlyctis*, and
12 polytomy of *H. occipitalis*, *Hoplobatrachus*, and *Euphlyctis*. In all estimations, we
13 optimized the parameters for estimation using ‘baseml’ in the PAML package. Then,
14 the branch lengths of the initial trees and the divergence times were estimated using
15 the ‘estbranches’ and ‘multidivtime’ programs in the Multidivtime package. In the
16 analyses, as a reference point for divergence estimation, we applied the divergence
17 between Mantellidae and Rhacophoridae 92.6–53.6 million years ago (Ma) (Bossuyt
18 and Milinkovitch, 2001). We also applied the divergence between *Hoplobatrachus*
19 and *Euphlyctis* 30–25 Ma, estimated by two recent studies (Bossuyt et al., 2006;
20 Roelants et al., 2004).

21

1 **3. Results**

2 3.1. Haplotypes and sequence divergence

3 A total of 102 (93 from Bangladesh and 9 from India) haplotypes were found
4 in *H. tigerinus* taxa (N = 182 from Bangladesh and N = 11 from India) in Cyt *b* genes.
5 We found 25 and 13 haplotypes in 12S and 16S rRNA genes, respectively, in *H.*
6 *tigerinus* (Table 1). The high number of Cyt *b* haplotypes was due to the huge number
7 of silent mutations at the third codon position of this gene, and the same situation was
8 observed in other taxa. In the *H. tigerinus* Bangladesh populations, we found seven
9 haplotypes (Htig-Ba1 ~ -Ba7) (Fig. 2A). A very low level of nucleotide divergence
10 was observed for each gene among these haplotypes (average divergence is 0.6%,
11 0.2%, and 0.3% for Cyt *b*, 12S and 16S rRNA genes, respectively) (Table 2).
12 However, between Bangladesh and Indian haplotypes, there was a degree of
13 nucleotide divergence (9.3%, 1.8%, and 1.6%) (Table 2); consequently, in *H. tigerinus*,
14 two major haplotypes could be recognized corresponding to two geographic regions
15 (named Htig-Ba and Htig-In) (Fig. 2A). In the case of *H. chinensis* (N = 14), we found
16 13, 9 and 7 haplotypes for Cyt *b*, 12S and 16S rRNA genes, respectively (Table 1).
17 Almost all haplotypes (i.e., Hchi-Th1, -Th2, -Th3, -La, and -Ve; see Table 1) showed
18 low nucleotide divergence (Table 2); however, the haplotype found from Phang Nga,
19 Thailand (Hchi-Th4) showed high nucleotide divergence from other Thailand
20 populations (13.4%, 5.5%, and 2.7%; Table 2) (Fig. 2B). In the *H. crassus* taxa (N =
21 2), two haplotypes were found from Khulna, Sundarban (Bangladesh) and Assam

1 (India) (Table 1), and very low nucleotide divergence was observed between these
2 populations (0.9%, 0.2%, and 0.4%; Table 2) (Fig. 2B). In African *H. occipitalis* (N =
3 4), we found 4, 3 and 2 haplotypes with low nucleotide divergence in Cyt *b*, 12S and
4 16S rRNA genes, respectively (1.1%, 0.2%, and 0.4%; Tables 1, 2). In the *E.*
5 *cyanophlyctis* taxa (N = 24), there were 16, 8 and 7 haplotypes in Cyt *b*, 12S and 16S
6 rRNA genes, respectively (Table 1). These haplotypes could be categorized into three
7 major groups corresponding to the Bangladesh, India and Sri Lanka populations
8 (Ecya-Ba, -In, and -Sr). Although nucleotide divergence was very low within each
9 group (< 1% for all mitochondrial genes; Table 2), interpopulation divergence was
10 very high (e.g., 13.6%, 5.1%, and 4.0% between Bangladesh and India; Table 2) (Fig.
11 2C). In the *E. hexadactylus* taxa (N = 12), four major haplotypes could be recognized:
12 one from Khulna, Sundarban, Bangladesh (Ehex-Ba) and the remaining three from the
13 Western Ghats, India (Table 1). Two Indian haplotypes were found from only a single
14 locality (Adyar, Western Ghats) (Ehex-In1 and -In2) and the other was observed from
15 Mudigere (Ehex-In3). Among these haplotypes, nucleotide divergence between Ehex-
16 In2 and -In3 was moderate (10.0%, 4.4%, and 2.2% for Cyt *b*, 12S and 16S rRNA
17 genes, respectively), and other interpopulation comparisons showed very high
18 nucleotide divergence (16.8–20.1%, 5.4–13.0%, and 3.7–6.3%) (Table 2, Fig. 2D).
19 This nucleotide divergence matched the interspecies-level divergence found in the
20 present study (16.8–23.0%, 4.1–12.8%, and 3.2–9.1%; Table 2).

21

1 3.2. Phylogenetic analyses

2 To understand the interspecies and interpopulation relationships of
3 *Hoplobatrachus* and *Euphlyctis* taxa, we performed phylogenetic analyses. The
4 partition homogeneity test (Farris et al., 1995) revealed that the three mitochondrial
5 genes analyzed here were suitable for combination (homogeneity not rejected, $P =$
6 0.543 for Cyt *b* vs. 12S rRNA, $P = 0.993$ for Cyt *b* vs. 16S rRNA, and $P = 0.704$ for
7 12S rRNA vs. 16S rRNA); thus, we used the combined data (1,544 bp) of these genes,
8 which contained 493 parsimoniously informative sites.

9 Figure 3 shows the resultant ML tree ($-\ln L = 10414.34$), and BI analysis
10 showed the same topology. MP analysis also reconstructed a similar topology.
11 However, in the MP tree, monophyly of *H. occipitalis* and other *Hoplobatrachus*
12 supported by ML and BI analyses was not recovered, whereas the basal split of *H.*
13 *occipitalis* at the root of all other *Hoplobatrachus* and *Euphlyctis* was supported by
14 moderate BP (60%). Furthermore, in the MP tree, the relationship between *H.*
15 *chinensis* and *H. tigerinus* could not be clarified (i.e., *H. chinensis* from Phang Nga,
16 Thailand became the sister taxon with respect to the clade of other *H. tigerinus* and *H.*
17 *chinensis*).

18 The ML tree showed that six major clades corresponding to six species used
19 here could be recognized. These clades were basically supported by high BP and BPP
20 values (excluding *H. chinensis* from Phang Nga; see below), but the basal split of *H.*
21 *occipitalis* from other *Hoplobatrachus* was not supported (Fig. 3). In the *E.*

1 *hexadactylus* clade, four distinct subgroups could be found. Interestingly, among these
2 subgroups, the specimen from Adyar (Ehex-In1) formed the sister taxon to a clade
3 containing all other specimens, and Bangladesh (Ehex-Ba) and two other Indian taxa
4 (Ehex-In2 and -In3) became monophyletic (Fig. 3). The *E. cyanophlyctis* clade
5 consisted of three major geographic subgroups that clearly corresponded to the India
6 (Ecya-In), Bangladesh (Ecya-Ba1 and –Ba2), and Sri Lanka (Ecya-Sr1 and –Sr2)
7 groups. Among them, Bangladesh and Sri Lanka subgroups became monophyletic, but
8 with low statistical support (67% and 70%; Fig. 3). Within the *H. chinensis* clade,
9 Thailand, Vietnam, and Laos populations formed an obvious clade, but the specimen
10 from Phang Nga (Hchi-Th4) showed a degree of divergence from the other *H.*
11 *chinensis* taxa and monophyly with other *H. chinensis* taxa was only moderately
12 supported (Fig. 3). In the *H. tigrinus* clade, two major subgroups were recognized.
13 These *H. tigrinus* subgroups clearly corresponded to the sampling localities: one
14 subgroup consisted of two haplotypes from the Indian population and the other
15 consisted of seven haplotypes from the Bangladesh population (Fig. 3).

16 Consequently, the following groups were not supported by high BP and BPP
17 values in our analyses: (1) *H. tigrinus* and *H. chinensis*, (2) Phang Nga *H. chinensis*
18 (Hchi-Th4) grouped with other *H. chinensis*, (3) sister-group relationship of African *H.*
19 *occipitalis* with respect to the Asian *Hoplobatrachus* species, and (4) sister-group
20 relationship of Bangladesh and Sri Lanka *E. cyanophlyctis*. Thus, we investigated
21 alternative phylogenetic hypotheses for these phylogenetic relationships by

1 conducting AU, KH, and SH tests. These tests could not reject other hypothetical
2 topologies for these problematic relationships. The results are shown in electric
3 supplement 3.

4

5 3.3 Estimation of divergence time

6 We estimated divergence times among *Hoplobatrachus* and *Euphlyctis* taxa by
7 Bayesian molecular dating based on the ML and BI tree topology (Fig. 4). As for the
8 problematic *H. occipitalis* position, we tried three alternative tree topologies (i.e., *H.*
9 *occipitalis* + other *Hoplobatrachus*, *H. occipitalis* + all *Euphlyctis*, and polytomy of *H.*
10 *occipitalis*, *Hoplobatrachus*, and *Euphlyctis*). These different topologies did not
11 significantly affect the time estimation (Table 3); thus, we used only the result from
12 the *Hoplobatrachus* monophyly constraint (Fig. 4).

13 If we accepted the monophyly of all *Hoplobatrachus*, the African *H. occipitalis*
14 first branched from Asian *Hoplobatrachus* lineage at 25.6 Ma (E in Fig. 4). Within
15 Asian *Hoplobatrachus*, *H. crassus* was the first to split from the others and the timing
16 was estimated as 19.5 Ma (G in Fig. 4). The branching time between *H. chinensis* and
17 *H. tigerinus* was estimated as 15.9 Ma (I in Fig. 4). Within *H. chinensis*, the Phang
18 Nga haplotype (Hchi-Th4) separated from a lineage ancestral to all others at 12.0 Ma
19 (J in Fig. 4); other Thailand and Vietnam haplotypes split at 2.3 Ma (P in Fig. 4).
20 Within the *Euphlyctis* clade, the split of *E. cyanophlyctis* and *E. hexadactylus* was
21 estimated as 23.4 Ma (F in Fig. 4). Within the *E. hexadactylus* taxa, an Indian

1 haplotype (Ehex-In1) was the first to branch at 16.3 Ma; then, Bangladesh *E.*
2 *hexadactylus* (Ehex-Ba) split from the other Indian lineage at 10.7 Ma (K in Fig. 4),
3 and two Indian haplotypes (Ehex-In2 and -In3) separated at 5.2 Ma (N in Fig. 4). In
4 the case of the *E. cyanophlyctis* clade, the Indian haplotype was the first to branch at
5 7.1 Ma (L in Fig. 4) and the split of Sri Lankan and Bangladesh haplotypes was
6 estimated at 6.0 Ma (O in Fig. 4).

7

8 **4. Discussion**

9 4.1. Intraspecific differentiation and possible cryptic species

10 In the intraspecies comparisons, we found several haplotypes having a degree
11 of sequence divergence more typical of interspecies comparisons (Table 2, Fig. 2).
12 First, Bangladesh and Indian populations of *H. tigerinus* possessed clearly distinct
13 haplotypes. The average sequence divergence between Bangladesh (Htig-Ba) and
14 Indian (Htig-In) haplotypes was high (9.3%, 1.8%, and 1.6% in *Cyt b* and 12S and
15 16S rRNA genes, respectively) compared with the values of 0.6%, 0.2%, and 0.3%
16 within Bangladesh populations and 0.4%, 0% and 0.2% within Indian populations
17 (Table 2). Similarly, the haplotype of *H. chinensis* from Phang Nga, Thailand (Hchi-
18 Th4) showed high nucleotide divergence compared with other Thailand populations
19 (13.4%, 5.5%, and 2.7%; Table 2) (Fig. 2B). The haplotype of Bangladesh *E.*
20 *cyanophlyctis* (Ecy-Ba) also showed high nucleotide divergence with respect to the
21 Indian and Sri Lankan haplotypes (13.6% and 14.5% for *Cyt b*, 5.1% and 3.3% for

1 12S rRNA, and 4.0% and 3.4% for 16S rRNA ; Table 2) (Fig. 2C). These obviously
2 distinguishable haplotype groups occurred in separate geographic areas, suggesting
3 that these haplotypes were maintained by allopatric separation and lack of constant
4 gene flow. Remarkably, the four major haplotypes of *E. hexadactylus* show high
5 nucleotide divergence from each other (Table 2). Even though three of these
6 haplotypes were also found in separate areas [Khulna (Sundarban, Bangladesh),
7 Mudigere and Adyar (Western Ghats, India)], Ehex-In1 and Ehex-In2 haplotype
8 groups occurred in the same locality, Adyar (Western Ghats, India) (Fig. 2D).

9 Recent molecular works suggested that the values of intra- and interspecific
10 sequence divergence can help to identify cryptic species. Vences et al. (2005)
11 reported on conspecific 16S rRNA haplotypes of up to 6% pairwise distance in
12 mantellid frogs. Fouquet et al. (2007a) provided evidence that reproductively
13 isolated cryptic species can be separated by 3.8% (*Rhinella*) and 4.3% (*Scinax*)
14 based on 16S rRNA gene sequences. However, Fouquet et al. (2007b) suggested
15 that a 3% threshold may prove to be a useful tool to document tropical frog
16 biodiversity. According to these studies, the present nucleotide divergence found in *H.*
17 *chinensis* (Phang Nga, Thailand vs. all others), *E. cyanophlyctis*, and *E. hexadactylus*
18 suggested the presence of cryptic species within currently recognized species. The
19 sympatric distribution of Ehex-In1 and Ehex-In2 haplotypes (nucleotide divergence is
20 20.1%, 11.9%, and 6.3% for Cyt *b*, 12S, and 16S RNA genes, respectively) clearly

1 indicates the occurrence of different *E. hexadactylus* species in Adyar (Western Ghats,
2 India).

3 As described above, we found three and four distinct haplotype groups having
4 species-level nucleotide divergence in *E. cyanophlyctis* (Ecya-Ba, -Sr, and -In; Fig
5 2C) and *E. hexadactylus* (Ehex-Ba, -In1, -In2, and -In3; Fig 2D), respectively. The
6 type localities of these two species were suggested as Tranquebar (Bauer, 1998) and
7 Pondichéry (Frost, 2007), respectively (both located in Southeast India near Sri
8 Lanka). In the present study, specimens from the type localities were not available, so
9 it is difficult to specify which haplotype group corresponds to the nominal species.
10 However, it is possible that the Sri Lanka *E. cyanophlyctis* haplotype (Ecya-Sr) group
11 corresponds to the “real” *E. cyanophlyctis*, because Sri Lanka is very close to the type
12 locality and was connected to Southeast India during the Pleistocene period (> 1.0 Ma;
13 Bossuyt et al., 2004). Furthermore, *Rana bengalensis* named after ‘Bengal’ (presently
14 Bangladesh and West Bengal of India) is currently considered a synonym of *E.*
15 *cyanophlyctis* (Frost, 2007). Thus, the Bangladesh *E. cyanophlyctis* haplotype (Ecya-
16 Ba) group might correspond to this species. Furthermore, in the case of *E.*
17 *hexadactylus*, the 16S rRNA gene sequence of the Sri Lankan specimen (Kousch et al.,
18 2001, Accession No. AF215389) is very similar to that of the Bangladesh haplotype
19 (0.2%) (Fig. 2D). If the specimen from Sri Lanka corresponds to the nominal species,
20 the haplotype group from Bangladesh may be the “real” *E. hexadactylus*, in which
21 case other haplotypes from the Western Ghats are considered distinct species. As for

1 the genus *Euphlyctis*, another species, *E. ghoshi*, has been identified only from
2 Manipur, India (Chanda, 1990). However, as genetic analysis has never been
3 performed for this species, one of the Indian *Euphlyctis* haplotypes found here may
4 correspond to that of *E. ghoshi*. As for *H. chinensis*, we did not use specimens from
5 China. However, Che et al. (2007) also showed two distinguishable *H. chinensis*
6 haplotypes (with 9.3% and 3.0% sequence divergence for 12S and 16S rRNA genes,
7 respectively) from Hainan and Yunan, China, and the haplotypes matched our Hchi-
8 Th4 haplotype (0% sequence divergence in 16S rRNA gene; Fig 2B) and other *H.*
9 *chinensis* haplotypes (1.1%; Fig. 2B), respectively. The type locality of this species is
10 unclear, but is possibly in the vicinity of Canton, China (Frost, 2007), and Hainan is
11 very close to Canton. Thus, our results imply that *H. chinensis* as currently recognized
12 contains two distinct species; one species (Hchi-Th4) (the nominal species) might
13 occupy the wide coastal region of Southeast Asia, and the other seems to inhabit
14 southeastern China.

15 Although the distribution of *H. crassus* in Bangladesh was unclear (Islam et al.,
16 2000), we could find *H. crassus* in the Sundarban mangrove forest of Khulna,
17 Bangladesh. It is also noteworthy that the physical distance between Sundarban,
18 Khulna (Bangladesh) and Assam (India) is large (about 1100 km) (Fig. 2B), but the
19 haplotypes of *H. crassus* from these two populations (Hcra-Ba and -In) have almost
20 the same nucleotide sequence. This low divergence might represent recent population
21 expansion through the Ganges-Brahmaputra delta (Table 2).

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

7

8 4.2. Divergence times and possible events causing *Hoplobatrachus* and *Euphlyctis* 9 divergence

10 It is generally proposed that several Asian ranid (= Natatanuran sensu Frost et
11 al., 2006) lineages occurred in the Indian subcontinent after the split from
12 Gondwanaland (starting around 150 Ma) and migrated to Asia via subcontinental drift
13 and collision with Eurasia (e.g., Roelants et al., 2004; van der Meijden et al., 2005;
14 Bossuyt et al., 2006). The Dicroglossini group (including *Hoplobatrachus* and
15 *Euphlyctis*) is included in this explanation (e.g., Bossuyt and Milinkovich, 2001). In
16 this study, we could not clarify the phylogenetic position of African *H. occipitalis*;
17 however, the separation of this species from other Asian *Hoplobatrachus* and
18 *Euphlyctis* taxa was estimated at around 25 Ma (Table 3 and Fig. 4). Similar
19 separation times for this African taxon have been estimated from several studies (25–8
20 Ma, Kosuch et al., 2001; approx. 10 Ma, Vences et al., 2003), and Kosuch et al.
21 (2001) suggested that the split of African *H. occipitalis* and Asian taxa was not

1 correlated with Gondwanan vicariance (i.e., “Out of Africa” hypothesis), but rather *H.*
2 *occipitalis* returned from Asia to Africa after the India-Eurasia collision (out of Asia).
3 Furthermore, the separation between the genera *Hoplobatrachus* and *Euphlyctis* has
4 been estimated as 30–25 Ma in at least two independent studies (Roelants et al., 2004;
5 Bossuyt et al., 2006). Thus, the ancestors of *Hoplobatrachus* and *Euphlyctis* would
6 have occurred in the Indian subcontinent before the India-Eurasia collision (23–20
7 Ma; Alam et al., 2003; Uddin and Lundberg, 2004).

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

1 2003; Uddin and Lundberg, 2004) (Fig. 5B), suggesting that the ancestors of *H.*
2 *crassus*, *H. tigerinus*, and *E. hexadactylus* could not have immediately spread to North
3 and Southeast Asian areas at the time of their split. Although *H. crassus*, *H. tigerinus*,
4 and *E. hexadactylus* currently show a wide distribution, major speciation events in
5 *Hoplobatrachus* and *Euphlyctis* might have occurred in the Indian subcontinent.

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

12 South Asian biogeography is marked by a disjunct distribution pattern of
13 closely related organisms. Such a pattern has been reported for many animals
14 (mammals, birds, freshwater fish, amphibians, reptiles and insects) and plants
15 (Karanth, 2003; Gaston and Zacharias, 1996; Das, 1996; 2002; Daniel, 2002) in this
16 area. The formation of this unique distribution pattern is believed to have begun in the
17 Middle Miocene (18–11 Ma) (Ashton and Gunatilleke, 1987). Before this period,
18 humid forest extended continuously from Northeast to Southern India as well as to
19 Bangladesh (Poole and Davies, 2001). However, by Upper Siwalik times (before 5.1–
20 1.6 Ma, Fig. 5C), aridification occurred and the tropical forest was largely replaced by
21 savanna in central India; the dried zone was presumed to be a barrier for many

1 organisms (Karanath, 2003). Interestingly, the estimated branching ages of the
2 Western Ghats, Indian and Southeast Asian haplotypes of *H. tigrinus* (6.7 ± 1.8 Ma),
3 and *E. cyanophlyctis* (7.1 ± 1.7 Ma) seem to match the period of dry-zone formation.
4 This might suggest that before this period the ancestors of these taxa were widely
5 distributed in South and Southeast Asia; however, aridification of central India
6 blocked the gene flow between the West India and Southeast Asian areas. In *E.*
7 *cyanophlyctis* taxa, the Western Ghats haplotypes (Ecya-In) split at 7.1 Ma from the
8 Sri Lanka and Bangladesh haplotypes and the latter split at 6.0 Ma. Although central
9 India had dried up, the eastern coast remained wet during this period [and Sri Lanka
10 was intermittently connected to the Indian mainland during the Pleistocene (> 1.0 Ma;
11 Bossuyt et al., 2004)] (Fig. 5C). The split ages of *E. cyanophlyctis* taxa may suggest
12 that, unlike central India, the eastern side of India might have been a corridor for
13 amphibian migration during the late Miocene. The presence of very similar *E.*
14 *hexadactylus* haplotypes in both Sri Lanka and Bangladesh (see above) might support
15 this idea. Two *E. hexadactylus* haplotypes from the Western Ghats (Ehex-In2 from
16 Adyar and Ehex-In3 from Mudigere) split around 5.2 Ma, and this period is also
17 consistent with the drying age of central India. However, as in eastern India, it is
18 considered that tropical forests expanded in the Western Ghats region during this
19 period (Karanath, 2003). Thus, the divergence between Ehex-In2 (Adyar) and Ehex-In3
20 (Mudigere) haplotypes does not appear to have been caused by a vicariance

1 geographic event or environmental change (i.e., vicariant divergence) but by range
2 expansion.

3 In this study, we investigated the divergence patterns of Asian *Hoplobatrachus*
4 and *Euphlyctis* taxa based on estimated divergence times and paleogeological events,
5 and proposed that (1) major speciation events of these anuran taxa might have
6 occurred in South Asian areas, (2) the formation of Bangladesh land may have
7 allowed the spread of frog taxa to Southeast Asian areas, and (3) the aridification of
8 central India might have affected the gene flow of widely distributed species. These
9 results might be useful as a guideline for biogeographical studies in this region. At the
10 same time, we could not specify the causes of some speciation events (e.g., *H. crassus*,
11 *H. tigerinus*, and *E. hexadactylus*) due to lack of detailed investigation in East India, a
12 possible corridor connecting South and Southeast Asian anuran fauna. Further
13 extensive sampling at this area is needed to clarify the evolutionary process of these
14 frog taxa.

15

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6

7 **Supplementary Materials**

8 Supplementary Tables 1~3 are available.

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21

1 **Figure Legends**

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

4

5 Fig. 2. Distribution of haplotypes for each *Hoplobatrachus* and *Euphlyctis* species.

6 The average nucleotide divergence of 16S rRNA gene between haplotypes is denoted.

7 Dotted circles unite similar haplotypes (< 1% nucleotide difference), and solid lines

8 show the phylogenetic relationships among haplotypes (= Fig. 3). (A) *H. tigerinus*, (B)

9 *H. crassus* and *H. chinensis*, (C) *E. cyanophlyctis*, (D) *E. hexadactylus*. Abbreviations

10 for haplotype are followings: Ba from Bangladesh, In from India, Sr from Sri Lanka,

11 Th from Thailand, Ve from Vietnam, La from Laos. Yu from Yunan, and Ha from

12 Hainan. Yunan and Hainan haplotypes (Yu and Ha) of *H. chinensis* and Sri Lanka

13 haplotype (Sr) of *E. hexadactylus* are from DDBJ database (Accession Nos.

14 DQ458251, DQ458250, and AF215389, respectively).

15

16 Fig. 3. Maximum likelihood (ML) tree ($-\ln L = 10414.34$) based on the nucleotide

17 sequence of 1,544 bp of mitochondrial (Cyt *b* + 12S rRNA + 16S rRNA) genes with

18 GTR + I + G substitution model from 28 haplotypes (Table 1) of *Hoplobatrachus* and

19 *Euphlyctis* species with *M. okinavensis* as an outgroup. The Bootstrap support (above

20 50%) is given in order for ML/MP (100/100). Asterisks represent Bayesian posterior

1 probability (BPP; * > 95% and ** > 99%). The scale bar represents branches in terms
2 of nucleotide substitutions per site for the ML tree.

3

4 Fig. 4. Estimated divergence time. The range of 95% credibility interval is indicated
5 by grey rectangles. The phylogenetic relationships were assumed on the ML and BI
6 results (= Fig. 3). Arrows show the fixed reference points used here. The divergence
7 time between Mantellidae and Rhacophoridae of 92.6–53.6 million years ago (Ma)
8 was estimated by Bossuyt and Milinkovitch (2001), and the divergence between
9 *Hoplobatrachus* and *Euphlyctis* of 30–25 Ma was by two recent studies (Bossuyt et al.,
10 2006; Roelants et al., 2004).

11

12 Fig. 5. Summary of paleogeography in the Indian subcontinent. Collision of Indian
13 tectonic plate with Eurasian plate and subsequent geographic events are shown. (A)
14 Subduction and formation of Himalayas and Indo-Burman Ranges during the early
15 Miocene (22-20 Ma). (B) Formation of Bengal basin and filled by sedimentation
16 during the middle Miocene (20-14 Ma). (C) Map of Asia showing dry and wet zones
17 (10-1.6 Ma). Hatched and grey areas represent the wet zone (over 250 cm of rainfall)
18 and dry zone (rainfall between 50 cm and 100 cm), respectively. Here, Sri Lanka is
19 shown as being connected to South India, because it was geologically part of the
20 Deccan plate and was separated from India by a shallow strait that might have served
21 as a land bridge during times of lowered sea level. This land bridge might have

- 1 facilitated the movement of flora and fauna between peninsular India and Sri Lanka.
- 2 A-B modified after Uddin and Lundberg (2004) and Alam et al. (2003), and C
- 3 modified after Karanth (2003).
- 4

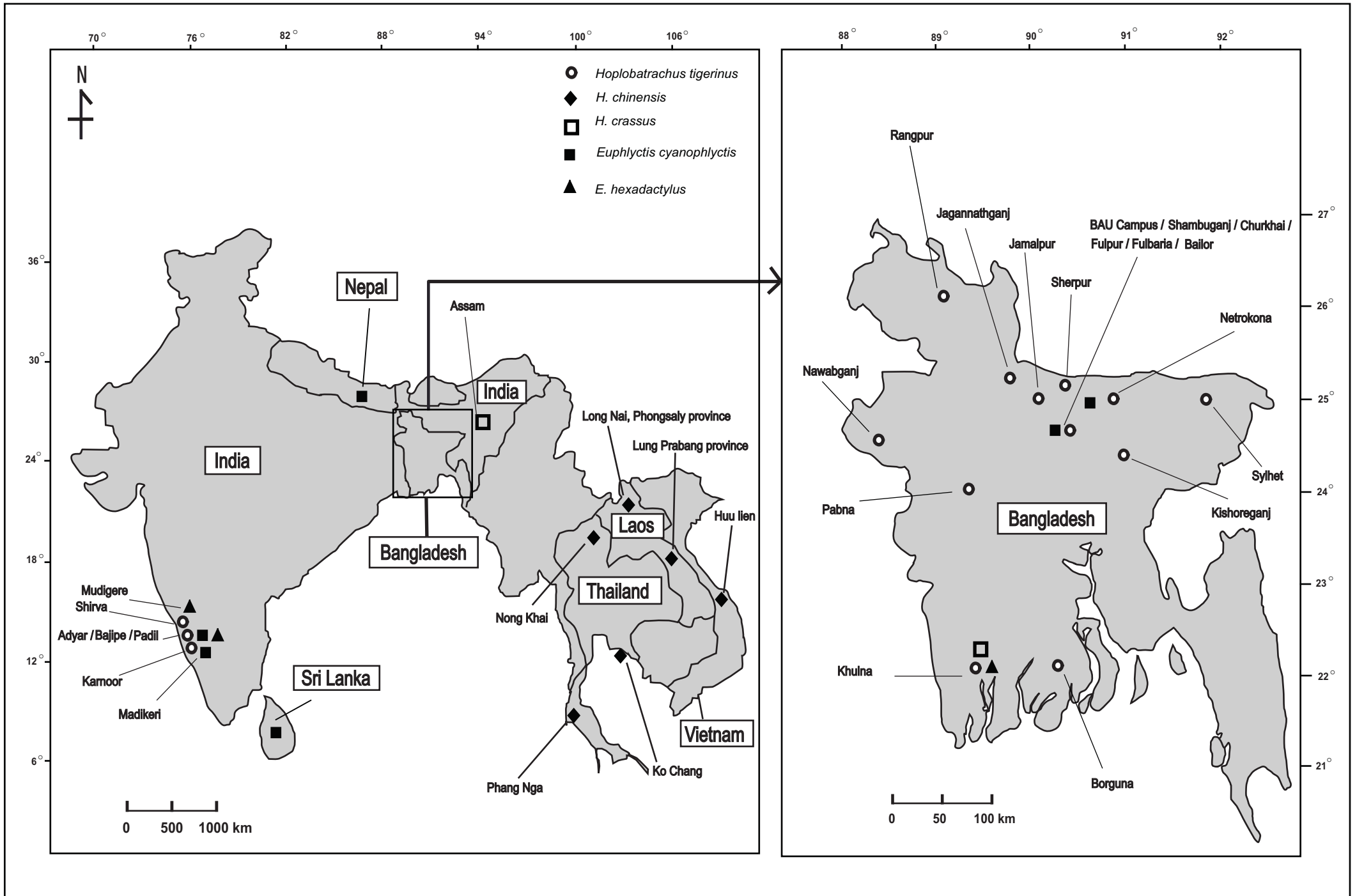


Fig. 1.

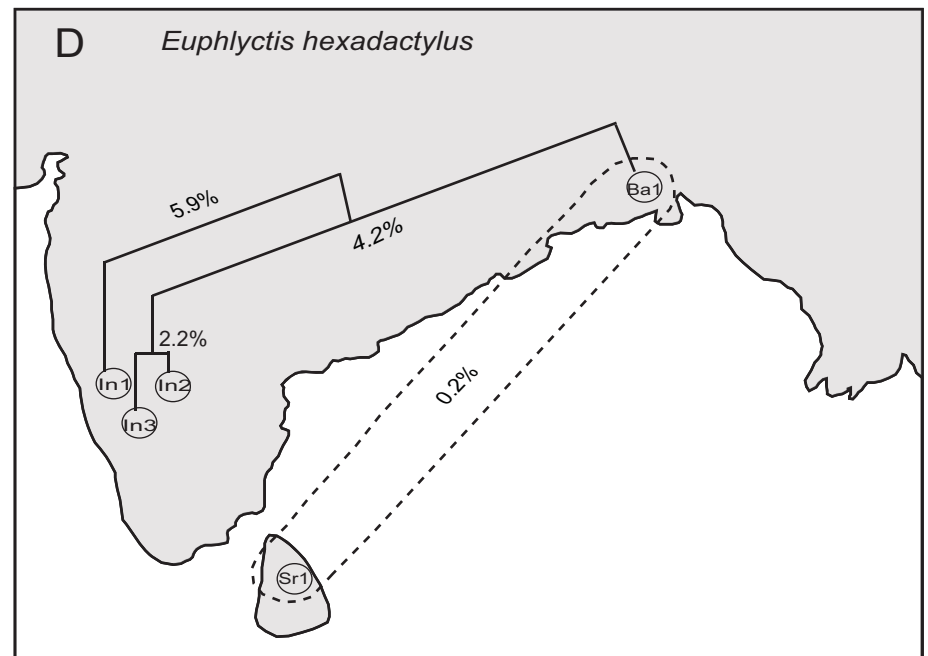
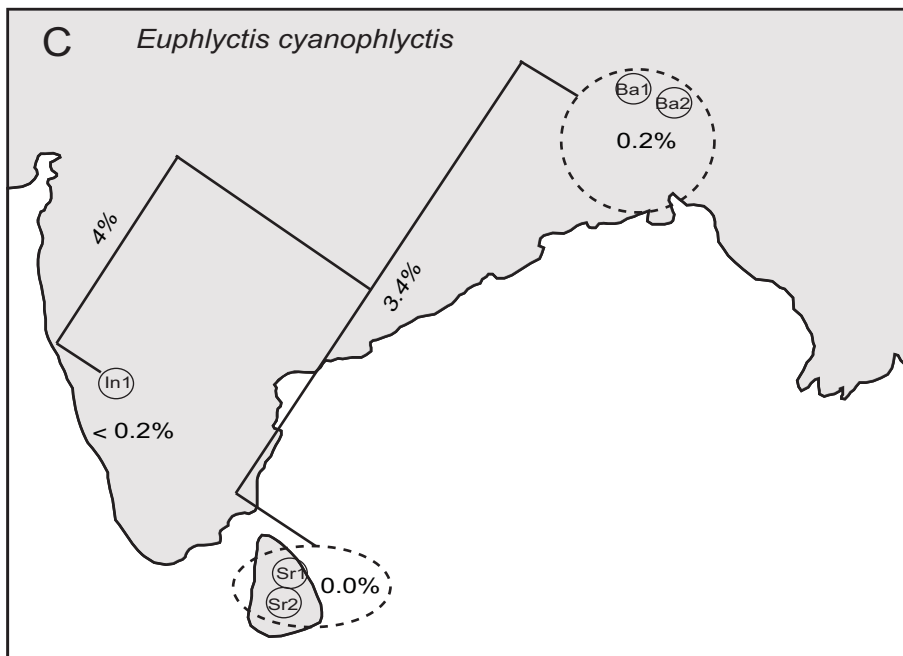
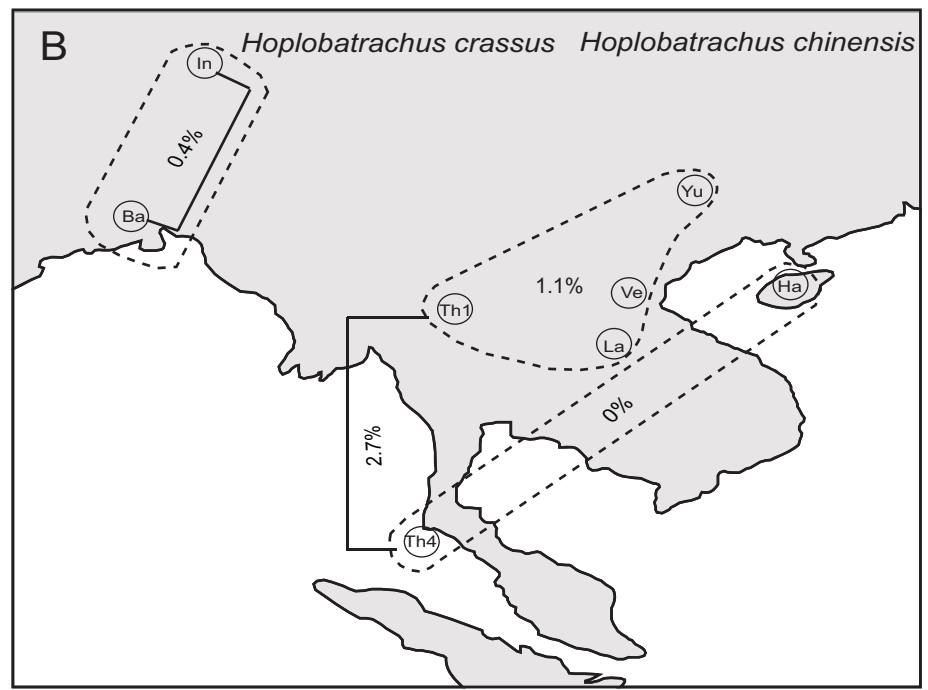
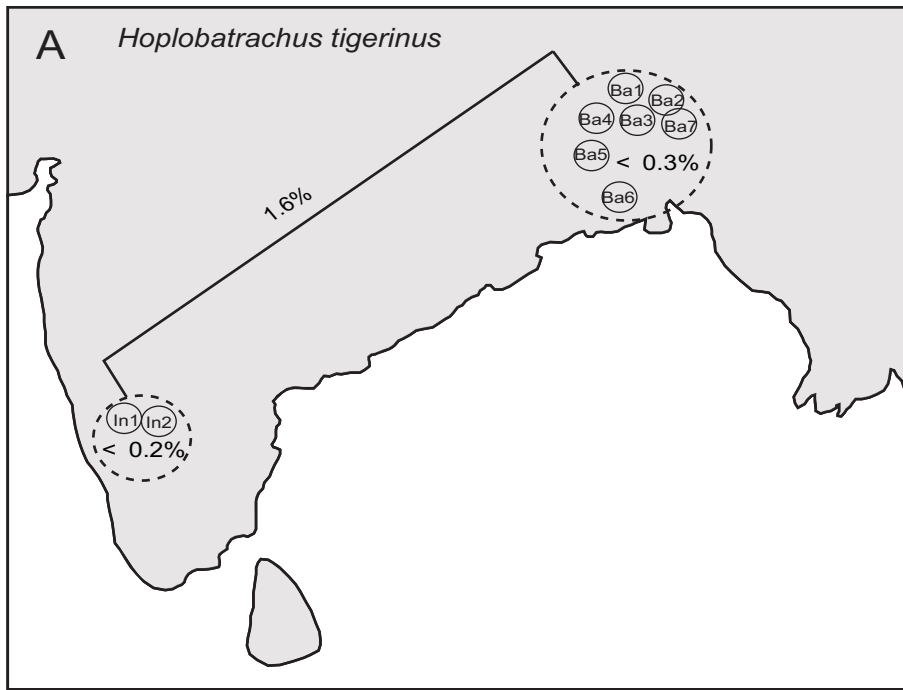


Fig. 2.

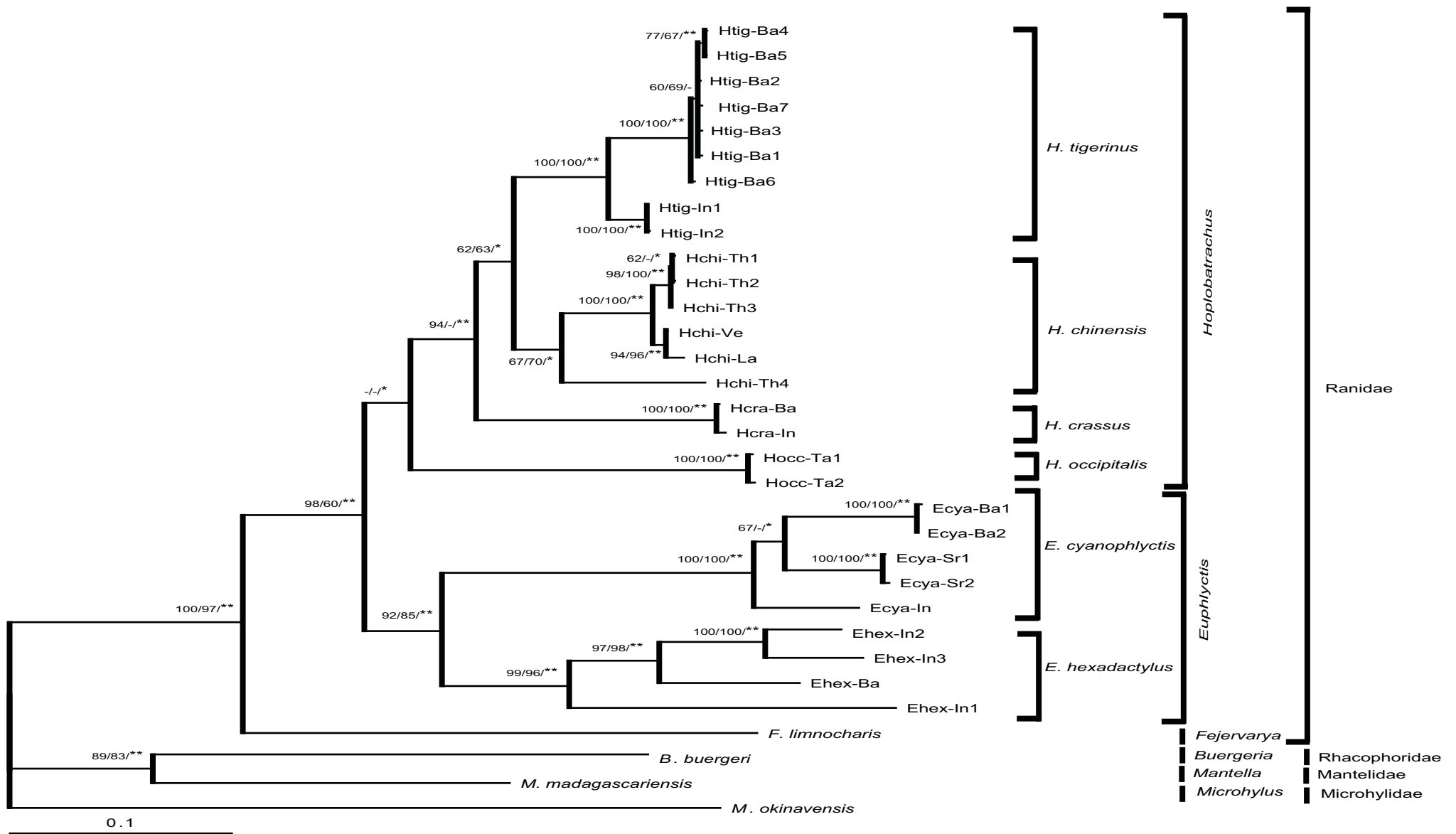


Fig. 3.

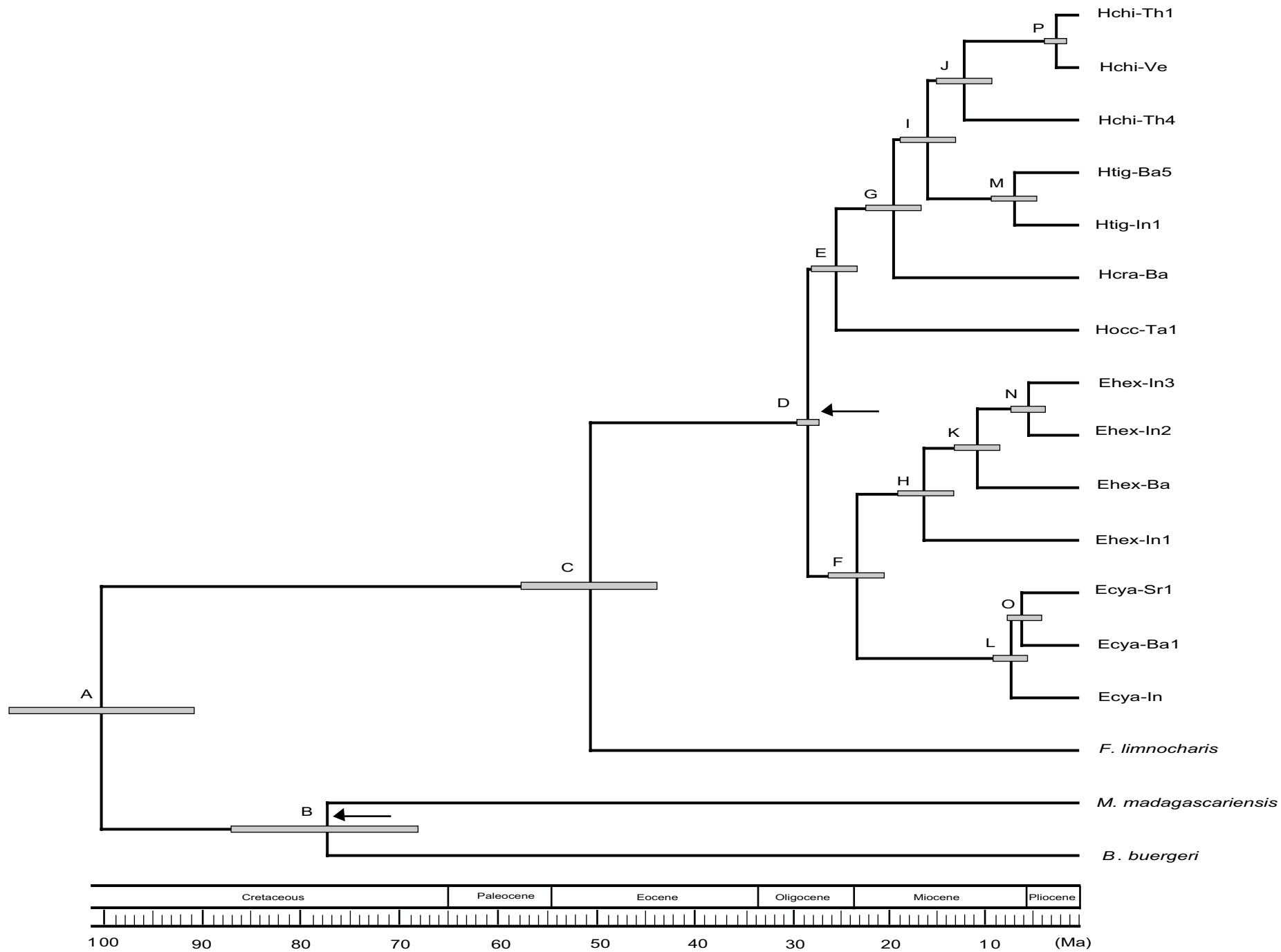


Fig. 4.

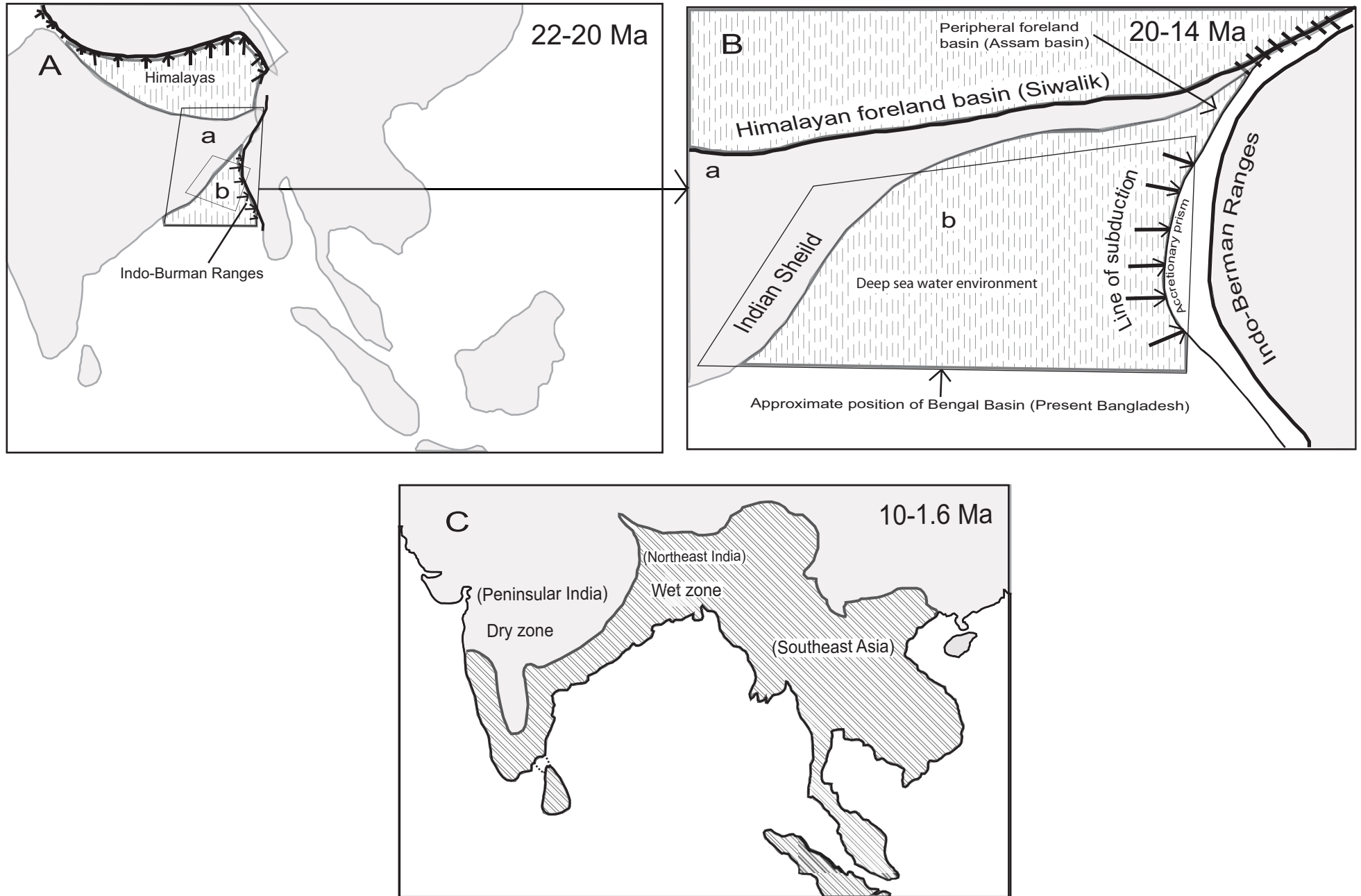


Fig. 5.

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

Species	Collecting station		No. of frogs used	Haplotype abbreviation *	No. of Haplotypes (Accession Number)			GPS information	
	Country	Locality			Cyt <i>b</i>	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	
	India	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	
		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)
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	
<i>Hoplobatrachus crassus</i>	Vietnam	Huu Lien	4	Hchi-Ve	3 (AB290603 ~ AB290605)	1 (AB290430)	2 (AB290414, AB20415)	21° 40' 13" N, 106° 23' 09" E	
	Bangladesh	Sundarban , Khulna	1	Hcra-Ba	1 (AB274143)	1 (AB273158)	1 (AB272595)	22° 21' 0" N, 89° 18' 0" E	
<i>Hoplobatrachus occipitalis</i>	India	Assam	1	Hcra-In	1 (AB290597)	1 (AB290425)	1 (AB290413)	26° 09' 56" N, 92° 50' 29" E	
	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	
		Gajni, Sherpur	2	-	1 (AB274157)	-	-	25° 0' 0" N, 90° 0' 0" E	
	India	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		

* Haplotypes used for combined data set

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	16S rRNA
<i>H. tigrinus</i>	Intrapopulation	Htig-Ba	0.6 (0.2 – 0.9)	0.2 (0 – 0.4)	0.3 (0 – 0.4)
		Htig-In	0.4	0.0	0.2
<i>H. chinensis</i>	Interpopulation	Htig-Ba vs. Htig-In	9.3 (9.1 – 9.8)	1.8 (1.7 – 1.9)	1.6 (0 – 1.7)
	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)
	Ecya-In vs. Ecya-Sr	15.1 (15.0 – 15.2)	3.9 (3.8 – 4.0)	2.0	
<i>E. hexadactylus</i>	Interpopulation	Ehex-Ba vs. Ehex-In	16.9 (10.0 – 20.1)	8.6 (4.4 – 13.0)	4.8 (2.2 – 6.3)
		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. tigrinus</i>	4.0 (0.2 – 9.8)	0.8 (0 – 1.9)	0.8 (0 – 1.7)	
	<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)	

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>		<i>H. occipitalis</i> + <i>Euphlyctis</i>		Polytomy (<i>H. occipitalis</i> , <i>Hoplobatrachus</i> and <i>Euphlyctis</i>)		
	Estimated time \pm SD	95% interval	Estimated time \pm SD	95% interval	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	80.2 \pm 8.0	62.4 — 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	5 \pm 1.4	2.7 — 8.1	2.4 — 8.5
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	10.1 \pm 2.1	6.5 — 14.5	5.8 — 15.3
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	15.4 \pm 2.4	11.0 — 20.5	9.8 — 21.8
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	5.6 \pm 1.4	3.2 — 8.8	3.2 — 9.5
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	6.6 \pm 1.6	4.1 — 10.3	4.1 — 11.1
Between <i>E. hexadactylus</i> and <i>E. cyanophlyctis</i> (Basal <i>Euphlyctis</i>) (F)	23.4 \pm 2.5	18.5 — 28.2	20.1 \pm 2.5	15.3 — 25.1	22.2 \pm 2.5	17.3 — 27.3	15.3 — 28.2
Between <i>H. occipitalis</i> and the other <i>Euphlyctis</i> (Basal <i>Euphlyctis</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	7.4 \pm 2.0	4.2 — 11.9	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	2.6 \pm 1.0	1.1 — 5.0	1.0 — 5.7
Between another Thailand and other populations of <i>H. chinensis</i> (J)	12 \pm 2.4	7.8 — 17.1	14.3 \pm 2.7	9.4 — 20.1	13.2 \pm 2.5	8.7 — 18.6	7.8 — 20.1
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	17.4 \pm 2.6	12.6 — 22.8	11.3 — 24.5
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	21.2 \pm 2.5	16.4 — 26.3	14.6 — 27.9
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>Euphlyctis</i> + <i>H. occipitalis</i>	-	-	-	-	28.5 \pm 1.2	25.5 — 30.0	25.5 — 30.0
Between <i>Hoplobatrachus</i> and <i>Euphlyctis</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>Euphlyctis</i> (C)	51.7 \pm 6.1	40.3 — 64.6	53.5 \pm 6.8	41.2 — 67.8	53.7 \pm 6.4	41.7 — 67.0	40.3 — 67.8
Between Dicroglossidae and Mantellidae + Rhacophoridae (A)	103.3 \pm 8.2	87.6 — 119.5	104.9 \pm 8.0	89.5 — 120.7	104.8 \pm 7.9	89.4 — 120.7	87.6 — 120.7

Electronic supplement 1
Voucher Number of the specimens used in this experiment

Species	Collecting station		No. of frogs used	Laboratory Voucher Number (Frog specimens/DNA)	
	Country	Locality			
<i>Hoplobatrachus tigerinus</i>	Bangladesh	BAU Campus, Mymensingh	29	53582, 20877 ~ 20886, 53066 ~ 53068, 53087, 53589, 53131, 51001 ~ 51003, 51008, 51009, 51012, 51013, 51021, 51026, 51031 and two live in IABHU	
		Shambhuganj, Mymensingh	20	20909 ~ 20928	
		Fulbaria, Mymensingh	11	20826 ~ 20836	
		Fulpur, Mymensingh	11	20898 ~ 20908	
		Churkhai, Mymensingh	9	20817 ~ 20825	
		Netrokona	7	20929 ~ 20935	
		Kishoreganj	1	20033	
		Jamalpur	16	20847 ~ 20856, 53075, 53080, 53084, 53623, 53169, 53662	
		Jagannathganj, Jamalpur	14	20807 ~ 20816, 53078, 53081, 53082, 53179	
		Rangpur	15	20867 ~ 20876, 53063, 53076, 53085, 53086	
		Pabna	6	20801 ~ 20807	
		Nawabganj	2	52010, 52015	
		Borguna	11	20887 ~ 20897	
		Sundarban, Khulna	15	53534, 20857 ~ 20866, 53058 ~ 53061, 53074	
		Sylhet	15	20837 ~ 20846, 53064, 53065, 53077, 53079, 53083	
		India	Padil, Mangalore, Western Ghats	2	20030, 20031
			Bajipe, Mangalore, Western Ghats	5	20107, 20108, 20332, 20337, 20338
			Karnoor, Western Ghats	2	20137, 20138
			Shirva, Western Ghats	2	20324, 20325
			Nong Khai	2	34062 ~ 34063
Thailand	Ko Chang	2	34060 ~ 34061		
	Phang Nga	1	20631		
	-	2	20625, 20626		
Laos	Luang Prabang Province	1	20647		
	Long Nai, Phongsaly Province	2	20696, 20697		
Vietnam	Huu Lien	4	20627 ~ 20630		
<i>Hoplobatrachus crassus</i>	Bangladesh	Sundarban, Khulna	1	20865	
	India	Assam	1	20698	
<i>Hoplobatrachus occipitalis</i>	Tanzania	-	4	53167, 53168, 53184, 20699	
<i>Euphlyctis cyanophlyctis</i>	Bangladesh	BAU Campus, Mymensingh	6	22103, 22104, 22115 ~ 22117, 22120	
		Bailor, Mymensingh	6	22109 ~ 22114	
		Gajni, Sherpur	2	22122, 22123	
	India	Padil, Mangalore	1	20003	
		Bajipe, Mangalore, Western Ghats	1	20109	
		Madikeri, Western Ghats	1	20021	
		Karnoor, Western Ghats	3	20131, 20135, 20136	
		Assam	1	20658	
	Nepal	-	2	20608, 20609	
	Sri Lanka	-	2	20656, 20657	
	Bangladesh	Sundarban, Khulna	3	22138 ~ 22140	
		India	Adyar, Mangalore, Western Ghats	7	20007, 20008, 20017, 20018, 20222, 20223, 20224
		Bajipe, Mangalore, Western Ghats	3	20103, 20104, 20328	
	Mudigere, Western Ghats	1	20214		
	Total			252	

Voucher No. 20801 ~ 20985 are preserved in Bangladesh Agricultural University, Fisheries Biology and Genetics, Bangladesh (BAUFBG).

Voucher No. 34060 ~ 34063 are preserved in Kyoto University, Japan (KU)

Voucher No. 20933 ~ 20988, 22103 ~ 22140, 51001 ~ 51031, 53063 ~ 53087, 53131 ~ 53184, 53623 ~ 53662 are preserved in Institute for Amphibian Biology, Hiroshima University, Japan (IABHU)

Voucher No. 20608 ~ 20699 are preserved in Musée National d' Histoire Naturelle, France (MNHNF)

Voucher No. 20003 ~ 20031, 20103 ~ 20138, 20214 ~ 20224, 20324 ~ 20338 are preserved in the Rondano Biodiversity Research of St. Aloysius College (RBRL), India

Electronic supplement 2
 Primers used in the present study for PCR amplification

Gene	Primer	Sequence (5' - 3')	Length (PCR Product)	Source
<i>Cyt b</i>	Fow-1-1	ACMGGHYTMTTYYTRGCHATRCAYTA	0.64 kbp	Sano et al. (2005)
	Rev-1	TADGCRAAWAGRAARTAYCAYTCNGG		Kurabayashi (Unpublished)
12S rRNA	FS01	ACGCTAAGATGAACCCTAAAAAGTTCT	2.5 kbp	Sumida et al. (1998)
	RFR60	ACTTACCATGTTACGACTTGC		Sumida et al. (1998)
	R51	GGTCTGAACTCAGATCACGTA		Sumida et al. (1998)
16S rRNA	F51	CCCGCCTGTTTACCAAAAACAT	0.6 kbp	Sumida et al. (2002)
	R51	GGTCTGAACTCAGATCACGTA		Sumida et al. (2002)

Electronic supplement 3

Comparison of log-likelihood scores among the alternative tree topologies using AU, KH and SH tests in combined data set of mtDNA genes

Tree topology	Method	-lnL difference	P-value		
			au	kh	sh
Candidate trees for the position of <i>H. occipitalis</i> (Hocc-Ta)					
(Micro,((Fejer,((Hocc-Ta, (Hcra-Ba,((Hchi-Th4,(Hchi-Th1,Hchi-Ve)),(Htig-Ba6, Htig-In1))))),(Ecy-a-In1,(Ecy-a-Sr1,Ecy-a-Ba1)),(Ehex-In1,(Ehex-Ba,(Ehex-In3,Ehex-In2))))),(Mante, Burge)))	ML, BI	-0.4	0.682	0.537	0.987
(Micro,((Fejer,((Hcra-Ba,((Hchi-Th4,(Hchi-Th1,Hchi-Ve)),(Htig-Ba6,Htig-In1))),((Hocc-Ta,((Ecy-a-In1,(Ecy-a-Sr1,Ecy-a-Ba1)),(Ehex-In1,(Ehex-Ba,(Ehex-In3, Ehex-In2))))))),((Mante,Burge)))		0.8	0.658	0.463	0.969
(Micro,((Fejer, (Hocc-Ta((, (Hcra-Ba,((Hchi-Th4),(Hchi-Th1,Hchi-Ve)),(Htig-Ba6, Htig-In1))),((Ecy-a-Ba1,(Ecy-a-In1,Ecy-a-Sr1)),(Ehex-In1,(Ehex-Ba,(Ehex-In3,Ehex-In2))))))),((Mante, Burge)))	MP	11.8	0.093	0.101	0.101
Candidate trees for the relationships among <i>E. cyanophlyctis</i> (Ecy-a-Sr1, Ecy-a-In1, Ecy-a-Ba1)					
((Ecy-a-In1,(Ecy-a-Sr1,Ecy-a-Ba1))	ML, BI	-0.4	0.682	0.537	0.987
((Ecy-a-Ba1,(Ecy-a-Sr1,Ecy-a-In1))	MP	1.6	0.576	0.347	0.945
((Ecy-a-Sr1,(Ecy-a-Ba1,Ecy-a-In1))		2.9	0.365	0.215	0.910
Candidate trees for the relationships among <i>H. tigrina</i> and <i>H. chinensis</i> (Hcra-Ba, Hchi-Th4, (Hchi-Th1, Hchi-Ve), (Htig-Ba6, Htig-In1))					
(Hcra-Ba,((Hchi-Th4,(Hchi-Th1,Hchi-Ve)),(Htig-Ba6, Htig-In1))))	ML, BI	-0.4	0.682	0.537	0.987
(Hcra-Ba,(Hchi-Th4,((Hchi-Th1,Hchi-Ve)),(Htig-Ba6,Htig-In1))))		4.2	0.545	0.274	0.845
(Hcra-Ba,((Hchi-Th1,Hchi-Ve),(Hchi-Th4,(Htig-Ba6,Htig-In1))))		8.8	0.006*	0.057	0.622
((Hcra-Ba,(Hchi-Th4,(Hchi-Th1,Hchi-Ve)),(Htig-Ba6,Htig-In1))))		1.5	0.612	0.381	0.949
((Hchi-Th4,(Hcra-Ba,(Hchi-Th1,Hchi-Ve)),(Htig-Ba6,Htig-In1))))		8.5	0.059	0.135	0.620
((Hchi-Th1,Hchi-Ve),(Hchi-Th4,Hcra-Ba),(Htig-Ba6,Htig-In1))))		7.8	0.200	0.160	0.655
((Hchi-Th1,Hchi-Ve),(Hcra-Ba,(Hchi-Th4,(Htig-Ba6,Htig-In1))))		14.9	4e-065*	0.019*	0.254
((Hchi-Th1,Hchi-Ve),(Hchi-Th4,(Hcra-Ba,(Htig-Ba6,Htig-In1))))		9.1	0.052	0.106	0.587

((Hchi-Th1,Hchi-Ve),(Hchi-Th4,Hcra-Ba),(Htig-Ba6,Htig-In1)))	13.7	0.003*	0.037*	0.313
(Hchi-Th4,(Hcra-Ba,((Hchi-Th1,Hchi-Ve),(Htig-Ba6,Htig-In1))))	8.7	0.167	0.155	0.598
(Hchi-Th4,((Hchi-Th1,Hchi-Ve),(Hcra-Ba,(Htig-Ba6,Htig-In1))))	8.1	0.255	0.148	0.644
(Hchi-Th4,((Hcra-Ba,(Hchi-Th1,Hchi-Ve)),(Htig-Ba6,Htig-In1)))	13.5	0.020*	0.042*	0.324
((Hchi-Th4,(Hchi-Th1,Hchi-Ve)),(Hcra-Ba,(Htig-Ba6,Htig-In1)))	2.1	0.549	0.322	0.935
((Hcra-Ba,(Hchi-Th1,Hchi-Ve)),(Hchi-Th4,(Htig-Ba6,Htig-In1)))	14.7	0.043*	0.024*	0.266
((Hchi-Th1,Hchi-Ve),(Htig-Ba6,Htig-In1),(Hchi-Th4,Hcra-Ba))	8.7	0.145	0.156	0.599

* The values were not significant (< 0.05) among any of the compared topologies. Haplotype abbreviation after Table 1.