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Phylogenetic Relationships among *Hoplobatrachus rugulosus* in Thailand as Inferred from Mitochondrial DNA Sequences of the Cytochrome-*b* Gene (Amphibia, Anura, Dicroglossidae)

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A fragment (564 bp) of the mitochondrial cytochrome-*b* (*Cyt-b*) gene was studied for 73 individual rice field frogs (*Hoplobatrachus rugulosus*) from 18 geographical locations (populations) within Thailand. Sequence analysis revealed the presence of 12 haplotypes, with five haplotypes being represented in two or more populations, and the other seven being population-distinct haplotypes. Phylogenetic analysis by maximum parsimony, maximum likelihood, and neighbor joining analyses all placed the 12 haplotypes into two distinct and well-separated clades with high bootstrap support, reflecting the high sequence divergences between the clades (25.3–32.3%). The mountain ranges and the Isthmus of Kra are likely to have played important roles in hindering gene flow among *H. rugulosus* populations in Thailand. From the sequence divergence values, the two clades of *H. rugulosus* can be classified into two distinct species, and therefore, the strains of *H. rugulosus* bred in farm stocks should be restricted to a population of one clade so as to avoid cross breeding between the two clades.

Key words: rice field frog, *Hoplobatrachus rugulosus*, cytochrome-*b*, captive breeding, farm stocks, Isthmus of Kra

INTRODUCTION

The genus *Hoplobatrachus* belongs to the family Dicroglossidae, and has representative species in Asia and Africa (Frost, 2011). Currently, this genus is recognized as consisting of four species; *H. rugulosus*, *H. tigrinus*, *H. occipitalis*, and *H. crassus*. Of these, *H. rugulosus* is widespread from East Asia to Myanmar, Laos, Vietnam, Cambodia, and Thailand (Chan-ard, 2003), and is the only species of this genus that can be found throughout Thailand. Because this species is an edible and economically valuable husbandry animal, it has been farmed for more than 20 years in Thailand. However, since the stocks bred in farms are still routinely taken from the natural environment throughout the country, they are dependent upon natural local populations. Moreover, transportation of animals for husbandry may be playing a part in anthropogenic mixing and/or establishment of alien populations locally.

The classification of *H. rugulosus* based on morphology has been confused in Thailand. It was first reported in 1917 (Smith, 1917) based on the morphology of the tadpoles. Two distinct tadpoles were reported: one with a long snout

and elongated body, and the other with a shorter snout and more rounded body. Smith (1917) remarked that these two forms were similar to larval *H. (as Rana) tigrinus*. Taylor (1962) reported the occurrence of *H. rugulosus* (as *R. rugulosa*) and *H. tigrinus* (as *R. tigrina pantherina*) in Thailand and noted them to be similar to each other. He [Taylor (1962)] noted that *H. rugulosus* has a smaller body and shorter limbs than does *H. tigrinus*, and that *H. rugulosus* is restricted to the northern and northeastern regions of Thailand, while *H. tigrinus* occurs all over the country. However, Dubois (1992) considered that previous records of *R. tigrina* from Thailand were based on *H. rugulosus*. Thus, Chan-ard (2003) recognized only one species, *H. rugulosus*, from all parts of Thailand. Although Kosuch et al. (2001) suggested using the name *H. chinensis* instead of *H. rugulosus*, such an idea is not universally accepted. We follow Frost (2011) and use the name *H. rugulosus* in this paper.

The growth of molecular approaches has led to the reorganization of amphibian taxonomy (Matsui et al., 2005; Frost et al., 2006; Li et al., 2008; Matsui et al., 2010). As mentioned above, *H. rugulosus* has been classified as a single species in Thailand based on morphological data (Dubois, 1992), but the analysis of molecular data of this species by Alam et al. (2008: as *H. chinensis*) showed a high sequence divergence (average divergences of 13.4%, 5.5% and 2.7% for *Cyt-b*, 12S rRNA and 16S rRNA gene fragments, respec-

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tively) among geographical populations from Thailand. Although their study suggests the potential division of *H. rugulosus* into more than one species, the study was inadequate inasmuch as the samples were collected from only three populations and did not cover regions throughout Thailand. Thus, the species level classification of *H. rugulosus* in Thailand remains unclear.

In this study, we investigated the phylogenetic relationships of *H. rugulosus* from 18 distinct natural populations across the six geographic regions of Thailand using a 564 bp fragment of the mitochondrial *Cyt-b* gene in order to clarify whether the Thailand populations are comprised of a single polymorphic species, or include two or more cryptic species.

MATERIALS AND METHODS

Specimens

A total of 73 individual adult *H. rugulosus* were collected throughout Thailand during the rainy season (April–October) from 2007 to 2009. These samples were collected from 18 geographically separate localities (assumed populations) distributed across the six biogeographic regions (Nabhitabhata and Chan-ard, 2005), i.e., North, Northeast, Central, West, East, and South Thailand (Table 1; Fig. 1). The liver was removed from each individual frog and preserved in absolute ethanol for subsequent DNA extraction, while some of the samples were deposited as voucher specimens at Chulalongkorn University Museum of Natural History. Sample sizes in each locality are given in Table 1.

DNA extraction

Total DNA was extracted from each liver tissue sample using standard protocols of chemical reagent and enzymic digestion [0.1 M Tris–NaCl pH 8.0, 0.2 M EDTA, 1% (w/v) SDS, 1 mg/ml (30 U/mg) proteinase K] followed by phenol/chloroform extraction, ethanol precipitation and washing (Hillis et al., 1996). Air-dried DNA pellets were dissolved in TE and used as a template to amplify a partial fragment of the *Cyt-b* gene by the polymerase chain reaction (PCR).

Table 1. Specimens used (N), *Cyt-b* haplotypes and GenBank accession numbers.

Region	Locality ¹	N	Haplotype number ²	Accession number of <i>cyt-b</i>
North	Nan (1)	3	H1 (3)	AB514482
Northeast	Udon Thani (2)	5	H2 (5)	AB514494
	Sakon Nakhon (3)	5	H2 (5)	AB514489
	Mukdahan (4)	4	H2 (4)	AB514481
	Nakhon Ratchasima (5)	6	H2 (6)	AB514485
	Wang Nam Khiao (6)	4	H3 (4)	AB514495
	Ubon Ratchathani (7)	5	H4 (4), H5	AB514492, AB514493
	Central	Lopburi (8)	2	H7 (2)
	Nakhon Nayok (9)	5	H7 (3), H8 (2)	AB514483, AB514484
West	Tak (10)	6	H7 (2), H9 (4)	AB514490, AB514491
	Phetchaburi (11)	5	H7 (5)	AB514486
East	Chonburi (12)	4	H7, H8 (3)	AB514477, AB514478
	Sa-Kaeo (13)	4	H4 (2), H6 (2)	AB514475, AB514476
	Chanthaburi (14)	4	H4 (4)	AB514480
	Trad (15)	1	H10	AB514496
South	Chumphon (16)	3	H11 (3)	AB514479
	Phang-nga (17)	5	H12 (5)	AB514487
	Songkhla (18)	2	H12 (2)	AB514488
TOTAL	18	73		

¹locality number in Fig. 1 in parenthesis. ²Numbers of individual in parenthesis.

PCR and direct sequencing

PCR amplification of the partial fragment of the *Cyt-b* gene was performed using the L-14841 (5'-CTC CCA GCC CCA TCC AAC ATC TCA GCA TGA TGA AAC TTC G-3') and CB3-H (5'-GGC AAA TAG GAA GTA TCA TTC TG-3') primer pair (Kosuch et al., 2001). Each 25 µl PCR mixture contained 10–20 ng total DNA, 250 µM of each dNTPs, 1 µM of each primer, 2.5 mM of MgCl₂, 1xPCR buffer and 1 unit *Taq* DNA polymerase (Fermentas™). The PCR thermal cycles started with 95°C for 5 min, followed by 35 cycles of 94°C for 45 s, 53°C for 45 s and 72°C for 1 min and the final extension was 72°C for 10 min. The PCR products were purified via spin columns (Macherey-Nagel™) and directly sequenced with an ABI 3100 sequencer (Perkin Elmer) in both directions using the same corresponding primers as those used for the PCR amplification. The consensus sequences of each of the 12 haplotypes found from the 73 samples from the different populations can be accessed from the DDBJ/EMBL/GenBank nucleotide sequence databases with accession numbers AB514475–AB514496 and AB539960.

Phylogenetic analysis

A total of 23 samples, comprised of the 12 haplotypes (Table

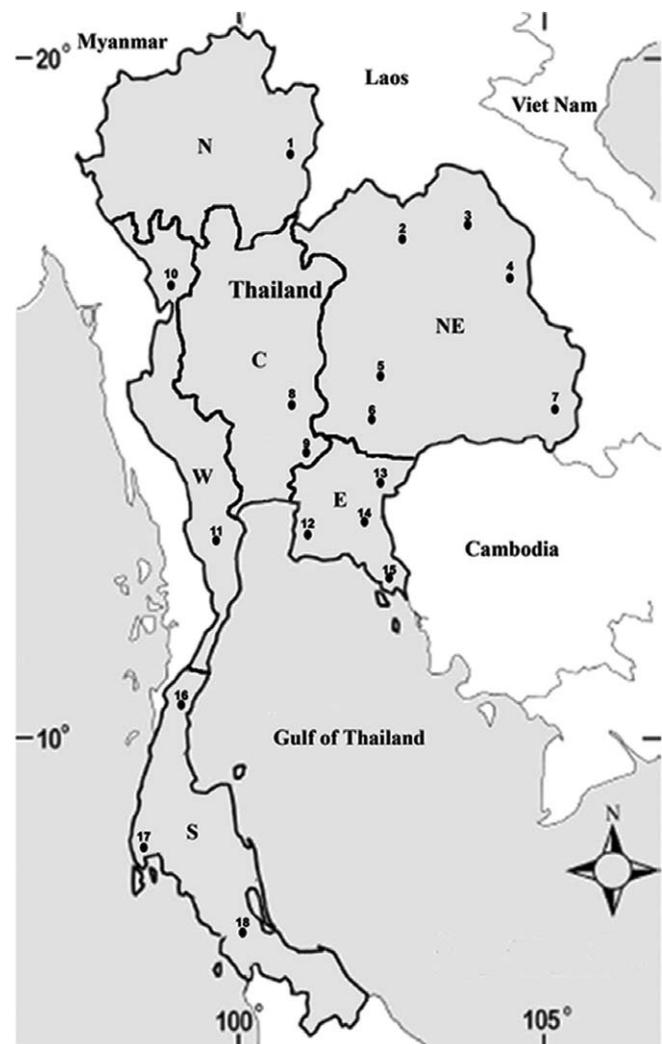


Fig. 1. A map of Thailand showing collection localities of *H. rugulosus* samples. For the names associated with the locality numbers, see Table 1. Geographical regions indicated on the map are; N = North, NE = Northeast, C = Central, W = West, E = East and S = South.

1) plus the two outgroup sequences were aligned using the default parameters of CLUSTALX (Thompson et al., 1997) and then imported to MacClade version 4.06 (Maddison and Maddison, 2000) to create the NEXUS format files. Phylogenetic relationships were constructed using maximum parsimony (MP), maximum likelihood (ML) and the neighbor joining (NJ) distance based analyses. MP, ML and NJ were performed using a heuristic search setting with random-addition sequences and tree bisection-reconnection (TBR) branch swapping in PAUP* 4.0b10 (Swofford, 2003). For the MP analysis, all characters were weighted equally, and gaps were treated as missing data. For the ML analysis, the best-fit model of sequence evolution was determined using Akaike Information Criterion (AIC) (Akaike, 1974) in Modeltest version 3.7 (Posada and Crandall, 1998). For the distance-based NJ analysis, the NJ tree was based on the HKY+G model. The bootstrap technique was used to test the reliability of the MP, ML, and NJ trees with 1000, 100, and 1000 replicates, respectively. Tree topologies with bootstrap values of 70% or greater were regarded as sufficiently resolved (Huelsenbeck and Hillis, 1993), and those between 50 and 70% as weakly supported. Pairwise comparisons of corrected sequence divergences [Kimura-2 parameter (K2p) distances (Kimura, 1980)] were also calculated with PAUP.

The partial *Cyt-b* gene sequence of congeneric *H. tigrinus* (AB 274044: Alam et al., 2008) and confamilial diglossid *Fejervarya limnocharis* (AY 158705: Liu et al., 2005) were used as outgroups to construct the phylogenetic trees.

RESULTS

Cyt-b partial gene fragment sequence characteristics

All 73 samples yielded 564 bp of *Cyt-b* gene fragment, including 79 variable and 102 parsimoniously informative sites. Alignment of these 73 *Cyt-b* gene sequences revealed a total of 12 different haplotypes. Five haplotypes (H2, H4, H7, H8, and H12) were represented in two or more populations, and the other seven (H1, H3, H5, H6, H9, H10, and H11) were distinct haplotypes for Nan, Wang Nam Khiao, Ubon Ratchathani, Sa-Kaeo, Tak, Trad and Chumphon, respectively (Table 1). H2 was the most frequently sampled haplotype, found in 20 of 73 individuals (27.4%) and in samples from four of the 18 localities. These four localities are all located in the northeastern region. Haplotypes found in more than one geographical region were observed in three cases, for H4 (northeastern and eastern regions), H7 (central, western and eastern regions), and H8 (central and eastern regions). In addition, different haplotypes were found in the same population in four of the 18 localities (Nakhon Nayok, Tak, Chonburi and Sa-Kaeo).

Phylogenetic analyses

For the phylogenetic analysis, Modeltest suggested the HKY + G model as the best-fit model for our data, with the proportion of invariable sites (I) as 0, a gamma distribution shape parameter (G) of 0.2555, a transition/transversion (Ti/Tv) ratio of 5.9860 and equilibrium base frequencies of: A = 0.2406, C = 0.3099, G = 0.1599 and T = 0.2896.

The MP, ML and NJ based phylogenetic analyses yielded similar topologies, so only the ML tree ($-\ln L = 1500.2120$) is shown in Fig. 2. MP analysis resulted in four equally parsimonious trees (CI = 0.877, RI = 0.959), and along with ML and NJ analyses, revealed two major clades among the 12 haplotypes.

The first clade (Clade A) (100% support) consisted of four haplotypes found in populations from the western, cen-

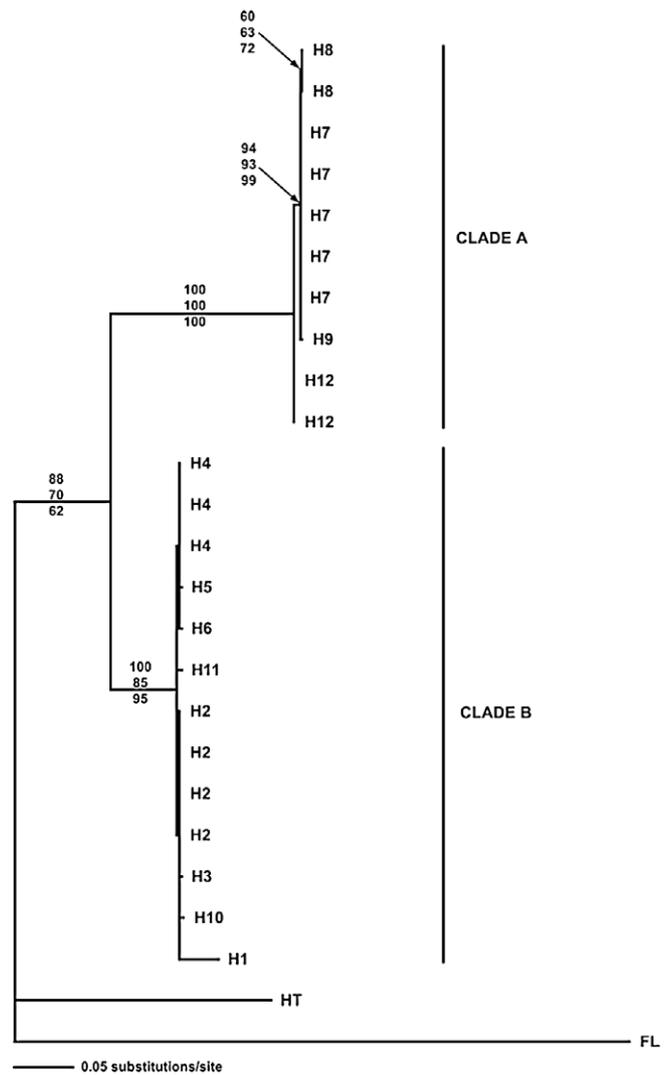


Fig. 2. Maximum likelihood tree of 564 bp of the *Cyt-b* gene for the 23 *H. rugulosus* samples (12 haplotypes), and outgroup taxa, *H. tigrinus* (HT) and *F. limnocharis* (FL). Bootstrap support (as%) are given in the order for MP, ML and NJ from 1000, 100 and 1000 replicates, respectively.

tral, and southern regions (except Chumphon), plus Chonburi from the eastern region, and can be further divided into three groups (1–3). Group 1 consisted of haplotype H8 from the central and eastern regions, but had low bootstrap support values (60, 63, and 72%). Group 2, with strong bootstrap support (94, 93, and 90%), consisted of haplotype H7 from the central, western, and eastern regions, plus haplotype H9 from the western region, which was sympatric with haplotype H7. The last group (Group 3) was represented by haplotype H12 from Phang-nga and Songkhla in the southern region.

The second clade (Clade B), also with strong bootstrap support (100, 85, and 95%), consisted of eight haplotypes found in the northern and northeastern regions and some (3/4) eastern populations as well as one of the southern populations. The three potential subdivisions of this clade [(i) H1, (ii) H4, H5 and H6 and (iii) H2, H3, H10 and H11] revealed no significant bootstrap support and so are treated

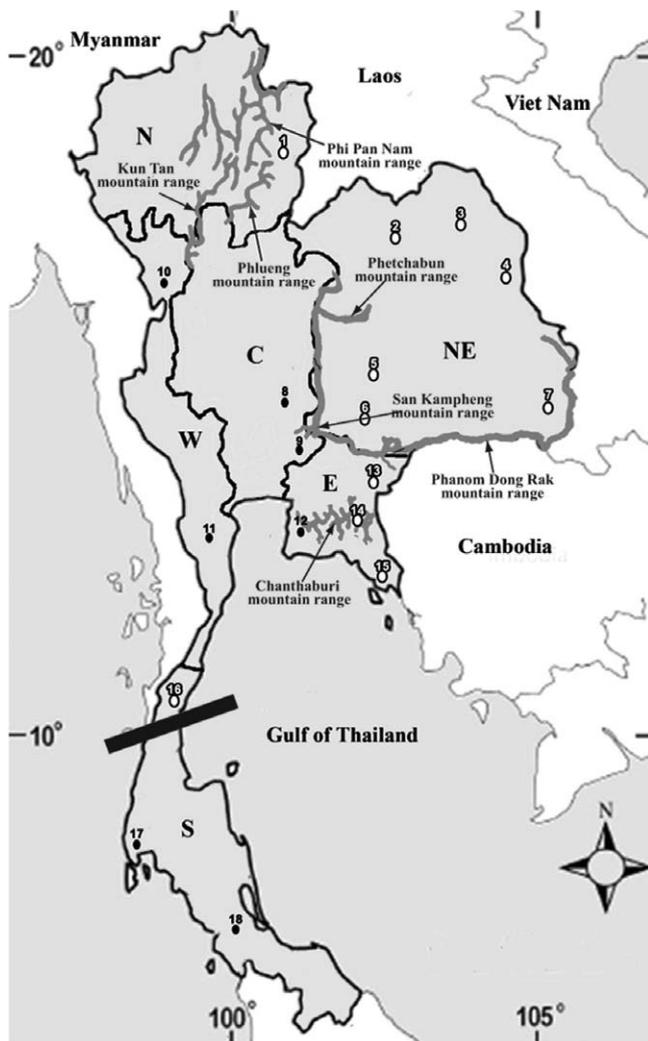


Fig. 3. Map showing the mountain ranges in Thailand. Locality numbers are as given in Table 1. Closed and open circle symbols refer to the populations that are grouped into clade A and clade B, respectively (see Fig. 2). The solid line indicates the location of the Isthmus of Kra.

as just a single group.

The sequence divergence between samples within each population of Ubon Ratchathani, Nakhon Nayok, Tak, Chonburi, and Sa-Khao was small (0.2%) (Table 2). The sequence divergences between the four and eight haplotypes within clade A and clade B, respectively, were also small (0–0.7% and 0–0.9%, respectively), although the sequence divergences between haplotype H1 (Nan) and the other seven haplotypes within clade B were somewhat larger (3.8–4.2%). In contrast, the sequence divergences between clade A and B were large (25.3–32.3%). Divergence of the outgroup species from our *H. rugulosus* haplotypes were very high (37.9–47.5% in *H. tigerinus* and 55.7–62.5% in *F. limnochoris*).

DISCUSSION

The phylogenetic analysis of the mitochondrial *Cyt-b* gene fragment showed that *H. rugulosus* can be divided into two distinct clades. The first clade (Clade A) consists of pop-

ulations from the western, central, and southern regions (except Chumphon), plus Chonburi from the eastern region of Thailand. The second clade consists of populations from the northern, northeastern, and eastern regions (except Chonburi), plus one population (Chumphon) from the southern region. A similar result was found in a previous study that separated the populations of *H. chinensis* (= *H. rugulosus*) collected from the southern region (Phang-nga) from the northeastern (Nong Khai) and the eastern (Ko Chang, Trad) regions of Thailand. The later study was based on the sequence divergence of three mitochondrial DNA fragments (*Cyt-b*, 12S rRNA and 16S rRNA genes) (Alam et al., 2008). Thus, there is no significant isolation by geographic distance that alone explains the subgroups within the A or the A/B clades, except for comparing populations between the northern and southern regions of Thailand.

Based on the geographic topology of Thailand, the northeastern region is separated from the central and eastern regions by four mountain ranges. The Phetchabun, Dong Phya Yen, and the western part of the San Kampheng mountain ranges separate the northeastern region from the central region, while the Phanom Dong Rak and the eastern part of the San Kampheng mountain ranges separate the northeastern and eastern regions (Fig. 3). Most of the mountain ranges are higher than 1,200 m a.s.l., except for the Phanom Dong Rak mountain range, which is about 600 m a.s.l. In general, the location of the mountain ranges in Thailand correlates well with the observed *Cyt-b* clades across the region. The Sa-Khao, Chanthaburi, and Trad populations, which are located near Chonburi (CBR), were placed in the same phylogenetic group as the northeastern populations. This is reasonable, as the Chanthaburi mountain range may serve as a natural barrier between the regions (Fig. 3) preventing gene flow. Certainly, this mountain range is higher than 1,600 m a.s.l., and so could restrict movement between the flanking regions significantly reducing or preventing gene flow between Chonburi and the other eastern populations. If this is the case, we would expect to see similar genetic segregation between these areas in other species with related dispersal abilities and habitat requirements. In contrast, the altitude of the Phanom Dong Rak mountain range (600 m a.s.l.) is considerably lower than the Chanthaburi mountain range and so, in this scenario, movement, and hence gene flow, between the Sa-Khao, Chanthaburi and Trad populations could be envisaged to occur with the northeastern populations.

There are two reports that support the role of mountain ranges in obstructing gene flow in amphibians (Hagemann and Pröhl, 2007; Zhang et al., 2010). The migration of animals has important consequences for the genetic pool of populations, potentially increasing or maintaining genetic variation in the populations involved. However, the home range of amphibians is commonly reported to occur over short-distances (< 0.5 km) (Zug, 1993), and there is no report on the dispersal of *H. rugulosus* across mountain ranges higher than 1,000 m a.s.l. In accord with this, the genetic structure of *H. rugulosus* in Thailand appears to be related to the geographical topology of the country, as described above (and see Figs. 2, 3). Therefore, these mountain ranges may act as natural barriers obstructing gene flow among the regions.

In the case of the Chumphon samples, in spite of its location in the same broad region (southern Thailand) as the Phang-nga and Songkhla populations, the phylogenetic analysis suggested they were more closely related to the northeastern populations and some populations from the eastern region (Sa-Kaeo, Chanthaburi, and Trad) than to the other two southern populations. Chumphon is currently more than 500 km distant from eastern Thailand, and is separated by the Gulf of Thailand (< 100 m deep). However, it has been shown that both regions were connected by land until 13,000 years before the present-day (Hall, 1998; Voris, 2000; Sathiamurthy and Voris, 2006); thus, gene flow between these populations could have occurred through routes across the present Gulf of Thailand before the recent sea level rise. This explanation is supported by an allozyme analysis of the genetic diversity of *R. nigrovittata* populations in this region, where the southern Thailand population was closely related to the eastern populations (Matsui et al., 2001). Another explanation of this phenomenon is that they might have been artificially introduced (anthropogenic dispersal) from the other locations to Chumphon via local people since this species is an edible and economic animal, they are caught and transported between regions by man, some of which may have escaped or been released.

Within clade B, other than the Chumphon population, there is no clear explanation for the B1/B2 subgroup localization, although the bootstrap support for this division is weak. Subject to the additional caveats of the limited sampling locations and sizes per location, and that of anthropogenic movement, this may require analysis of the *H. rugulosus* populations within the bordering Cambodia and Laos.

Within clade A, two populations from the southern regions (Phang-nga and Songkhla), but not the third Chumphon, could be grouped in the same clade as the populations from the central and western regions, but they were separated into a different subgroup. Phang-nga and Songkhla are located to the south of the Isthmus of Kra, whilst Chumphon is to north of this zoogeographic border (Fig. 3). The Isthmus of Kra is located on the Thai-Malay Peninsula and hindered the faunal transition between the Indochinese (north) and Sundaic (south) subregions (Woodruff, 2003; Hughes et al., 2003; de Bruyn et al., 2005; Woodruff and Turner, 2009). It can be considered as a geographic barrier limiting the distribution patterns and divergence of anuran fauna between the southern and the other regions of Thailand (Inger, 1999; Inger and Voris, 2001). For this reason, the Isthmus of Kra may be expected to have led to the genetic separation between the southern populations (Phang-nga and Songkhla) and all the other populations within clade A.

Recently, molecular based analysis has led to the concept that the values of the intra- and inter-specific sequence divergence in gene sequences can be used to help identify anuran cryptic species. For example, Graybeal (1997) studied the phylogeny of bufonid frogs and suggested that the inter-specific sequence divergences of *Cyt-b* were more than 15%. The similar result was reported on the isolated cryptic species of *Microhyla*. The inter-specific sequence divergences of *Cyt-b* were greater than 16% (Matsui et al., 2005). In this study, the *Cyt-b* sequence divergences between members of clade A and B were 25.3 to 32.3%,

and the previous study revealed different morphological characters of *H. rugulosus* between the northeastern populations and the other populations. For example, the shape and color of the body, which in the northeastern populations are respectively more slender and darker than the other geographical populations (our unpublished data). Thus, these results imply that *H. rugulosus* as currently recognized contains at least two distinct species in Thailand; one species might occupy the northern, northeastern and some parts of the eastern regions, whilst the other seems to inhabit the remaining regions of Thailand. This is in accord with the morphological data reported by Taylor (1962). However, specimens from the type locality (Lantau Island, Hong Kong, China) (Frost, 2011) were not available, so it is difficult to specify which haplotype group corresponds to the nominal species and so samples from other locations in Thailand especially the northern region should be verified to confirm the classification of *H. rugulosus* in Thailand.

The results of our study, the result suggests that *H. rugulosus* should be separated into two distinct species in Thailand so the strains of *H. rugulosus* in farm stocks should be restricted to a population of one clade so as to avoid cross breeding between the two clades.

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