Zoological Studies **49**(5): 688-702 (2010)

Zoological Studies

Genetic Structure of *Hylarana erythraea* (Amphibia: Anura: Ranidae) from Malaysia

Ramlah Zainudin[1,2,*], Shukor Mohd Nor[2], Norhayati Ahmad[2], Badrul Munir Md-Zain[2], and Mustafa Abdul Rahman[1]

[1]*Molecular Ecology Laboratory, Faculty of Resource Science and Technology, Univ. Malaysia Sarawak, Kota Samarahan, Sarawak 94300, Malaysia*

[2]*School of Environment and Natural Resources Science, Faculty of Science and Technology, Univ. Kebangsaan Malaysia, Bangi, Selangor 43600, Malaysia*

(Accepted March 31, 2010)

Ramlah Zainudin, Shukor Mohd Nor, Norhayati Ahmad, Badrul Munir Md-Zain, and Mustafa Abdul Rahman (2010) Genetic structure of *Hylarana erythraea* (Amphibia: Anura: Ranidae) from Malaysia. *Zoological Studies* **49**(5): 688-702. We studied the genetic structure and evolutionary relationships among populations of *Hylarana erythraea* in Sarawak, Malaysian Borneo (Borneo Heights of Padawan, Sadong Jaya, and Bario) and central Peninsular Malaysia (Tasik Chini of Pahang) using the partial cytochrome oxidase subunit 1 (CO1) gene of mitochondrial (mt)DNA. Two distinct geographical clades were observed, i) the 1st clade, haplogroup 2 included 7 divergent haplotypes in Bario, whereas ii) the 2nd clade (haplogroup 1) contained 16 haplotypes of the remaining populations. Gene flow estimators indicated high numbers of migrants per generation and panmictic populations of this species, except for a low number of migrants per generation and genetic isolation of the Bario population. The species was estimated to have undergone population expansion either for the whole population or for each population as shown by small and nonsignificant values of the sum of the standard deviation of the observed and expected mismatch distributions and Harpending raggedness index. However, multimodal distributions were seen in the scatterplot of mismatch distributions for the entire populations of Sadong and Bario. Geographical subdivision might explain the anomalies in the mismatch distribution for these populations. Furthermore, a large negative value and significant test of Fu' Fs in the Bario population suggested recent expansion and are indicative of dispersal from ancient Sunda Shelf populations (Pahang, Sadong, and Borneo Heights) to East Sarawak (Bario). The results suggested that populations of *H. erythraea* were subdivided where populations in central Peninsular Malaysia and western Borneo were more closely related than those in western Borneo were to those of eastern Borneo. The study implied that a feature in the landscape of Borneo (the Lupar line) created a greater barrier than repeated intervening ocean between glacial periods. Our study also supports the notion that a widely distributed frog species includes different evolutionary lineages that are possibly cryptic species. http://zoolstud.sinica.edu.tw/Journals/49.5/688.pdf

Key words: *Hylarana erythraea*, mtDNA CO1, Population expansion, Population subdivision.

The green paddy frog *Hylarana erythraea* Schlegel is a small to medium sized frog with bright to dark green color dorsally and laterally (Inger and Stuebing 2005). Although the species occurs in natural grasslands and open areas, the frog is an important indicator of habitat disturbance in Malaysia, as it is mostly found in disturbed habitats created by man, such as flooded paddy fields, plantations, and irrigation ditches. The species is widely distributed in Southeast Asia (Borneo, Peninsular Malaysia, Singapore, Indonesia, Thailand, the Philippines, Vietnam, Laos,

*To whom correspondence and reprint requests should be addressed. Tel: 6082582972 or 60128492960 (cell). Fax: 6082583160. E-mail:zramlah@frst.unimas.my

Cambodia, and Myanmar), hence suggesting complexity within the species as observed in other widely distributed Southeast Asian frogs (e.g., Stuart et al. 2006, Inger et al. 2009). However, no diagnostic morphological differences were reported among populations.

Previous studies on the ecology of *H. erythraea* included those of Alcala (1955), on breeding and early development, and of Inger and Greenberg (1963), who studied the reproductive cycle in a sample from Sarawak, Malaysia. A more-comprehensive ecological study on the Philippine population (Brown and Alcala 1970) reported variations in the sex ratio and lifespan of *H. erythraea*. However, no detailed studies from many other aspects, including molecular ones, of this species have ever been conducted. Because *H. erythraea* is a habitat indicator, it is an important species to explore, especially its genetic differentiation at the population level.

Stuart et al. (2006) showed the presence of sympatric evolutionary lineages of cryptic species in 2 forest-dwelling ranid frogs (*Odorrana livida* and *Hylarana chalconota*) and suggested that such a pattern may be common to frogs in Southeast Asia. Namely, there might not be a single frog species that is geographically widespread, which dwells in the forests of this region. If species are confined to a single population, specific mitochondrial (mt)DNA variants should be confined to specific populations. Since previous study on 3 riparian forest frogs (*H. chalconota*, *Limnonectes kuhli*, and *L. leporinus*) from Sarawak revealed relatively rare migration events, ancient haplotypes, and the genetic break at the Lupar barrier (Ramlah 1998), it was hypothesized that *H. eryhtraea*, being a species commensal with man, widely distributed and living in non-forested areas, may have experienced panmictic populations and undergone population expansions. Thus, the aims of this paper were to infer population subdivision, the genetic structure, evolutionary neutrality, and population expansion of *H. erythraea* among 4 populations from Sarawak and Peninsular Malaysia.

MATERIALS AND METHODS

Sample description and collection locations

Sarawak is made up of 2 geologically distinct areas, approximately divided by the Lupar River. West Sarawak forms part of the ancient Sunda Shelf with rocks older than 80 my (Cretaceous and older) while central and northern Sarawak are dominated by younger rocks (Late Cretaceous and Tertiary) (Hazebroek and Kassim 2001). Since *H. erythraea* is a widely distributed frog that lives in disturbed habitats, natural grasslands, and open areas, 4 localities were chosen that represent the ancient Sunda Shelf (Pahang and part of western Sarawak) and the eastern part of Borneo (Bario) so that past fragmentation could be inferred (Fig. 1). The study areas generally consist of flat lowland forests except for the highlands of Bario and Borneo Heights (1000 m in elevation). Sadong Jaya (1°31'05"N, 110°43'09"E) lies along the eastern side of Batang Sadong and consists of a large expanse of mangrove and peat swamp forests. Now, 98% of the total area has been turned into palm oil plantations where most of the species were caught. In contrast, the Borneo Highlands Resort (1°10'22"N, 110°12'57"E), stands at 1000 m, and is surrounded by virgin rainforest that is over 1.5 my old, the second oldest tropical rainforest in the world. The frogs were caught on banks of the lake adjacent to the hotel resort. Bario (3°45'N, 115°27'E) which is known as the Kelabit Highlands, is situated in the upper north of Sarawak Borneo. The area is a plateau with an elevation of approximately 1,200 m, and forms the uppermost catchments of the Sg Baram watershed, consisting of 3 major forests: primary rainforest surrounding the native settlements and agricultural areas, and heath and secondary forests located adjacent to villages. Most of the frogs were caught at paddy plantations and ponds at native settlements and agricultural areas. Tasik Chini, Pahang (3°26'38"N, 102°54'58"E), on the other hand, is located in central Peninsular Malaysia and consists of a mixture of freshwater swamps and old secondary forests. The frogs were caught on the bank of the freshwater lake.

In total, 36 adult frogs (Table 1) were collected by a visual encounter survey, and all ecological data were recorded on site. Adult specimens were tagged, and the frog thigh muscle or toe was cut, and the tissue specimen immediately put in a cryovial containing EDTA buffer. The vials were numbered to match the specimen tags. Specimens were preserved in 4% formalin and brought back to the Univ. Malaysia Sarawak for further analysis. The specimens were put in 70% alcohol as voucher specimens.

DNA extraction, polymerase chain reaction (PCR), and sequencing

The entire mtDNA genome was extracted using a Genispin™ Tissue DNA Kit (BioSynTech, Subang, Jaya) following the manufacturer's protocol. Double-stranded DNA samples were amplified using a polymerase chain reaction (PCR) Biometra machine. The components of a standard PCR protocol using *Taq* DNA polymerase were as follows; 10x PCR buffer containing Tris HCl (pH 7.5-9.0), [50mM] KCl or $(NH_4)_2SO_4$, [2mM] $MgCl_2$, 0.2 mM dNTPs, 0.1-1.0 μM of each primer, 2.0-2.5 units of *Taq* polymerase, the DNA template, and sterile deionized water. The primers used (Palumbi et al. 1991) were COI-e, 5-CAGTAAATAACGGGA ATCAGTG-3; and COI-f, 5-CCTGCCGGAGGAG GTGAYCC-3. Double-stranded PCR amplification was carried out with the following parameters: 94°C denaturation for 5 min, 94°C for 45 s, 50°C annealing for 1 min, and 72°C extension for 1.5 min for 30 cycles, followed by an extension at 72°C for 7 min. The PCR products were purified using a Genespin PCR Purification Kit. All PCR products were sequenced directly on an ABI 377 automatic sequencer (First Base Laboratories Sdn Bhd., Kuala Lumpur). Finally all sequences were deposited in GenBank under accession nos. EU569726-EU569761.

Data analyses

We followed the procedures of Matsui et al. (2005) to align the sequences, obtain comparative data to reconstruct the phylogenetic tree, and assign a degree of confidence to the trees. The sequences were aligned with Clustal X (Thompson et al. 1997) and translated to amino acid sequences in MEGA 4.1 (Kumar et al. 2008). Stop codons were removed from the aligned sequences, and the final sequences were aligned again using Clustal X (Thompson et al. 1997). The aligned data were then transformed into a distance matrix with the Hasegawa-Kishino-Yano (HKY) model, which takes into account unequal rates of evolution of transitions and transversions and allows unequal base frequencies (Hasegawa et al. 1985). Maximum-likelihood (ML) and Bayesian analyses were based on the substitution model and phylogenetic parameters (HKY+G) identified as optimal by the Bayesian information criterion (BIC) in jModeltest (Posada 2008). For confidence levels of the maximum-parsimony (MP) and ML analyses using PAUP4.0b10 (Swofford 2000), only bootstrap values of ≥ 70% were regarded as sufficiently resolved topologies (Huelsenbeck and Hillis 1993) and those of 50%-70% as tendencies. For the Bayesian analyses using MrBayes (Huelsenbeck and Ronquist 2001),

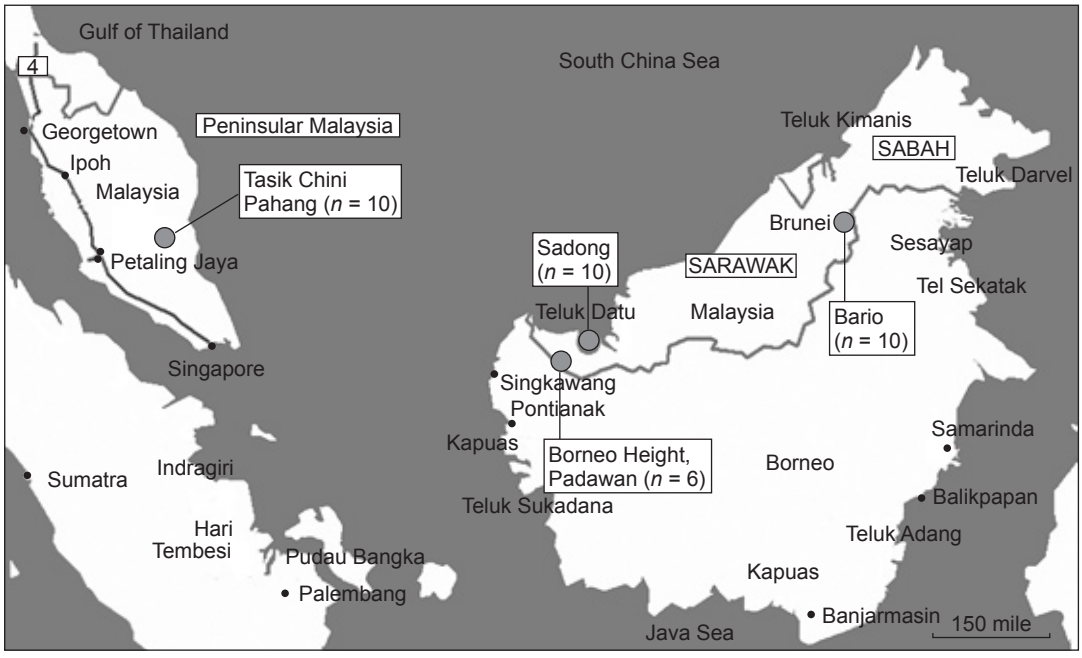

Fig. 1. Map of Malaysia showing study locations and sample sizes of *Hylarana erythraea* collected for the present study. *n*, sample size.

the same substitution model as used for the ML analysis was used with 2 simultaneous metropolis-coupled Monte-Carlo Markov chains that were run for 946,000 generations or until the probability of split frequencies (*p*) fell below 0.01. A tree was sampled every 100 generations, and a consensus topology was calculated for 7095 trees by omitting the first 2,365 trees (as burn-in). The confidence level of tree nodes was indicated by posterior probabilities which represent the true probabilities of the clades (Rannala and Yang 1996). Posterior probabilities of ≥ 98% were considered significant (Leache and Reeder 2002).

Measures of population genetic parameters such as genetic diversity (the probability that 2 randomly chosen mtDNA sequences differed in the sample) and nucleotide diversity (per nucleotide site, i.e., the probability that 2 randomly chosen homologous nucleotides differed in the sample; Nei 1987) were estimated from the mtDNA dataset using DNASP 4.0 (Rozas et al. 2003). Estimates of nucleotide divergence among populations, after accounting for nucleotide diversity within populations (Da), were also generated using DNASP 4.0. To test for a relationship between the corrected genetic distances and geographic

Table 1. Samples of *Hylarana* analyzed for DNA (cytochrome oxidase subunit 1; COI) sequence variation with GenBank accession number, locality, GPS reading, field voucher, and identified haplotypes

Species	Haplotype	Locality	GPS reading	Field voucher	GenBank accession no.
H. erythraea	Hap_1	Sadong	1°31'05.21"N 110°43'09.12"E	RZ240	EU569726
H. erythraea	Hap_10	Sadong	1°31'05.21"N 110°43'09.12"E	RZ244	EU569727
H. erythraea	Hap_16	Sadong	1°31'05.21"N 110°43'09.12"E	RZ253	EU569728
H. erythraea	Hap_3	Sadong	1°31'05.21"N 110°43'09.12"E	RZ233	EU569734
H. erythraea	Hap_14	Sadong	1°31'05.21"N 110°43'09.12"E	RZ238	EU569735
H. erythraea	Hap_10	Sadong	1°31'05.21"N 110°43'09.12"E	RZ252	EU569736
H. erythraea	Hap_12	Sadong	1°31'05.21"N 110°43'09.12"E	RZ228	EU569757
H. erythraea	Hap_13	Sadong	1°31'05.21"N 110°43'09.12"E	RZ245	EU569758
H. erythraea	Hap_10	Sadong	1°31'05.21"N 110°43'09.12"E	RZ251	EU569759
H. erythraea	Hap_11	Sadong	1°31'05.21"N 110°43'09.12"E	RZ254	EU569760
H. erythraea	Hap_2	Kubah NP	1°36'44.61"N 110°11'38.47"E	RZ107	EU569733
H. erythraea	Hap_5	Tasik Chini Pahang	3°26'37.54"N 102°54'58.23"E	NA0251	EU569738
H. erythraea	Hap_6	Tasik Chini Pahang	3°26'37.54"N 102°54'58.23"E	NA0252	EU569745
H. erythraea	Hap_7	Tasik Chini Pahang	3°26'37.54"N 102°54'58.23"E	NA0290	EU569746
H. erythraea	Hap_5	Tasik Chini Pahang	3°26'37.54"N 102°54'58.23"E	NA0311	EU569747
H. erythraea	Hap_5	Tasik Chini Pahang	3°26'37.54"N 102°54'58.23"E	NA0314	EU569748
H .erythraea	Hap_4	Tasik Chini Pahang	3°26'37.54"N 102°54'58.23"E	NA0253	EU569737
H. erythraea	Hap_8	Tasik Chini Pahang	3°26'37.54"N 102°54'58.23"E	NA0316	EU569749
H. erythraea	Hap_5	Tasik Chini Pahang	3°26'37.54"N 102°54'58.23"E	NA318	EU569750
H. erythraea	Hap_9	Tasik Chini Pahang	3°26'37.54"N 102°54'58.23"E	NA312	EU569761
H. erythraea	Hap_15	Borneo Height, Padawan	1°10'22.38"N 110°12'56.74"E	RZ68	EU569756
H. erythraea	Hap_10	Borneo Height, Padawan	1°10'22.38"N 110°12'56.74"E	RZ94	EU569752
H. erythraea	Hap_10	Borneo Height, Padawan	1°10'22.38"N 110°12'56.74"E	RZ150	EU569754
H. erythraea	Hap_10	Borneo Height, Padawan	1°10'22.38"N 110°12'56.74"E	RZ177	EU569755
H. erythraea	Hap_10	Borneo Height	1°10'22.38"N 110°12'56.74"E	RZ175	EU569751
H. erythraea	Hap_19	Bario	3°45'N 115°27'E	RZ49	EU569732
H. erythraea	Hap_18	Bario	3°45'N 115°27'E	RZ07	EU569731
H. erythraea	Hap_17	Bario	3°45'N 115°27'E	RZ04	EU569729
H. erythraea	Hap_17	Bario	3°45'N 115°27'E	RZ05	EU569730
H. erythraea	Hap_23	Bario	3°45'N 115°27'E	RZ28	EU569739
H. erythraea	Hap_21	Bario	3°45'N 115°27'E	RZ36	EU569740
H. erythraea	Hap_20	Bario	3°45'N 115°27'E	RZ43	EU569741
H. erythraea	Hap_22	Bario	3°45'N 115°27'E	RZ48	EU569742
H. erythraea	Hap_17	Bario	3°45'N 115°27'E	RZ03	EU569743
H. erythraea	Hap_23	Bario	3°45'N 115°27'E	RZ01	EU569744

distances, the normalized Mantel statistics, Z, was calculated (Mantel 1967). Significance levels of Z values were obtained using 1000 permutations of the matrices computed in Arlequin vers. 3.00 (Excoffier et al. 2005).

The demographic history was examined by Tajima's test of neutrality, D (Tajima 1989), and Fu's Fs statistics test (Fu 1997), to test for deviation of sequence variation from evolutionary neutrality. A population that has experienced population expansion may result in rejection of the null hypothesis of neutrality of Tajima's D, or a large negative value of Fu's Fs. All analyses were computed in Arlequin vers. 3.00 (Excoffier et al. 2005).

In order to investigate the extent of population differentiation, individuals were divided into broad geographical groups, based on the ML topology of the mtDNA phylogeny. These groupings were compared by analysis of molecular variance (AMOVA, Excoffier et al. 2005) to estimate the extent of genetic differentiation among populations, Φ_{ST}. Statistical significance was tested using 1000 permutations as implemented in Arlequin vers. 3.0 (Excoffier et al. 2005). The null hypothesis of no correlation between genetic differentiation and geographic distances was tested by permuting Mantel's non-parametric test (Mantel 1967) with 1000 permutations in Arlequin vers. 3.0 (Excoffier et al. 2005).

An inference of population expansion events was performed using a mismatch distribution analysis (Rogers and Harpending 1992, Rogers 1995) using Arlequin vers. 3.0 with 1000 permutations (Excoffier et al. 2005) and site-frequency spectra (Donnelly et al. 2001) as implemented in DnaSP 4.0 (Rozas et al. 2003). The parsimony criterion was used to reconstruct haplotype relationships of *H. erythraea*, assuming that differences at any given site between 2 randomly drawn haplotypes were unlikely to have arisen from more than 1 mutational step (Alexandrino et al. 2002). A minimum-spanning network (MSN) was generated using Network 4.5.0.2 (Bandelt et al. 1999) to illustrate this relationship.

RESULTS

In total, 441 bp of the partial CO1 gene of 36 sequences of *H. erythraea* with 2 outgroups, *H. baramica* and *L. kuhli*, were successfully obtained. The overall frequency distributions of nucleotides at the 1st, 2nd, and 3rd codon positions were as follows: A = 25.5%, 18.5%, and 24.9%; C = 18.7%, 28.1%, and 33.4%; G = 27.4%, 15.1%, and 8.9%; and T = 28.0%, 38.0%, and 33.0%, respectively. A compositional nucleotide bias analysis revealed no significant bias (p = 1.00) across the *H. erythraea* haplotypes. Of the 441 bp of the partial CO1 gene, 81 sites were variables with 12 singleton sites, leaving 69 (85%) potentially parsimoniously informative characters, indicating that the gene is a reliable marker to infer genetic variations at the population level and is consistent with Ramlah's (1998) findings for other Bornean frog species. Translation of DNA sequences into amino acid indicated 51 variable nonsynonymous substitution sites (34.7%) among *H. erythraea* sequences, and 48 acid amino replacements (32.6%) were phylogenetically informative.

Among the 36 individuals sequenced, 23 haplotypes were identified as having only 1 haplotype (Hap_10) shared by 2 populations (Sadong and Borneo Heights: Table 2). Two haplogroups (haplotype groups) were apparent: haplogroup 1 consisted of haplotypes from Sadong, Borneo Heights, and Pahang, and haplogroup 2 consisted of haplotypes from Bario, in the eastern part of Borneo. Sadong being the most polluted (mostly due to pesticides from oil palm plantations) had the highest number of unique haplotypes with 8 haplotypes in the 10 individuals sampled.

Corrected sequence divergences (HKY distances, data not shown) among the mtDNA CO1 haplotypes ranged 0.0%-4.3% (within population) and 0.0%-12.3% (among populations), indicating large disparities in differentiation within and among populations. Additionally, nucleotide diversity (π) was also low within populations, ranging 0.7%-1.5% (Table 3). Among populations, π values of 0.7%-1.4% and net nucleotide divergences (Da) of 0.87%-2.37% were lower in populations from western Sarawak and Pahang than from Bario, indicating little genetic differentiation among these 3 populations. Between those populations and Bario, much higher Da values (9.7%-10%) were found (Table 4)

There was a lack of a significant relationship (only at $p < 0.5$) between Da and geographic distance among the 4 populations of *H. erythraea* (r = 0.362, p = 0.323 Mantel test, 1000 permutations). Phylogenetic trees of the NJ (not shown), MP (not shown), ML (not shown), and Bayesian (Fig. 2) analyses all revealed monophyly of *H. erythraea*, with 100% (NJ), 100% (MP), and

100% (ML) bootstrap support and 1.00 (BPP) with respect to the outgroup species, *L. kuhlii* and *H. baramica*. Two distinct geographical clades were observed with long branches separating the 2 major clades and short-terminal branches for each population. The 1st clade, haplogroup 2, included a divergent 7 haplotypes from Bario (100%, 100%, 98%, and 1.00), while the 2nd clade (haplogroup 1)

Table 2. Segregating sites (67 bp) in the 441-bp segment of the cytochrome oxidase subunit 1 (COI) gene defining 23 different haplotypes and 2 haplogroups, and their distributions across 4 populations of *Hylarana erythraea*

Haplotype	Nucleotide positions	Locality[a]				HG
	[1111111111222222222233333333334444444444555555555566666666]					
	[12345678901234567890123456789012345678901234567890123456789012345678901234567]	1	2	3	4	
Hap_1	ATTCAAAAACACAAAACTCAGCCTTAGGCAACAGTCCAGGGTCCTGATAGTACACTCCCGTTAACTC	1	-	-	-	1
Hap_2	.......GT..................T..........G..............................	-	1	-	-	1
Hap_3	..........................T.........................G...........C...	1	-	-	-	1
Hap_4	....G.......GT......A.....T.T.....C..G..AA.....C..G..........G.C...	-	-	1	-	1
Hap_5	......GGT...G.TT....A....CT.T.G...C.....A......CG.GG..........C...	-	-	1	-	1
Hap_6	......GGT...G.TT....A....CT.T.G...C.....A......CG..G..........C...	-	-	5	-	1
Hap_7	......GGT...G.TT....A....CT.T.G.C.C.....A......CG..G..........G...	-	-	1	-	1
Hap_8	......GGT...G.TT....A....CT.T.G...C.....A......CG..G..........G...	-	-	1	-	1
Hap_9	......GGT...G.TT....A....CTCT.G...C.....A......CG..G..........C...	-	-	1	-	1
Hap_10	......GGT...G.TT.........CT...G..................G..G..........C...	3	4	-	-	1
Hap_11	......GGT...G.TT.........CT...G.C..............G..G..........C...	1	-	-	-	1
Hap_12	......GGT...G.TT.........CT...G......G..........G..G..........C...	1	-	-	-	1
Hap_13	......GGT...G.TT.........CT...G..................G..G.G.G.......C...	1	-	-	-	1
Hap_14	......GGT...G.TT.........CT...G..T..............G..G..........C...	1	-	-	-	1
Hap_15	C.....GGT...G.TT.........CT...G..................G..G..........C...	-	1	-	-	1
Hap_16	.....CGGT...G.TT.........CT...G..................G..G..........C...	1	-	-	-	1
Hap_17	.C.T.GGGTT.TG.TTTCGGATTCCCT.TGGT...GT.CA..TTCAG..A.GT.T.TTTAC.GCT.T	-	-	-	3	2
Hap_18	.C.T.GGCTT.TG.TTTCGGATTCCCT.TGGT...GT.CA..TTCAG..A.GT.T.TTTAC.GCT.T	-	-	-	1	2
Hap_19	CC.T.GGGTT.TG.TTTCGGATTCCCTCTGGT...GT.CA..TTCAG..A.GT.T.TTTAC.GCT.T	-	-	-	1	2
Hap_20	TC.T.GGGTT.TG.TTTCGGATTCCCT.TGGT...GT.CA..TTCAG..A.GT.T.TTTAC.GCT.T	-	-	-	1	2
Hap_21	G.CT.GGGTTCTG.TTTCGGATTCCCTCTGGT...GT.CA..TTCAG..A.GT.T.TTTAC.GCT.T	-	-	-	1	2
Hap_22	.C.T.GGGTT.TG.TTTCGGATTCCCTCTGGT...GT.CA..TTCAG..A.GT.T.TTTAC.GCTAT	-	-	-	1	2
Hap_23	CC.T.GGGTT.TG.TTTCGGATTCCCT.TGGT...GT.CA..TTCAG..A.GT.T.TTTAC.GCT.T	-	-	-	2	2

[a]Locality information: 1, Sadong Jaya, Sarawak; 2, Borneo Highland, Sarawak; 3, Pahang, Peninsular Malaysia; 4, Bario, Sarawak. HG, haplogroup.

Table 3. Measures of haplotypes and nucleotide diversity within populations of *Hylarana erythraea* analyzed by location

Locality	*N*	No. of haplotypes	Percent (%) pairwise divergence[a]	Gene diversity[b]	Nucleotide diversity (π)[a,b]
Sadong	10	8	0.46-4.05	1.00 ± 0.045	0.015 ± 0.003
Borneo Heights	6	3	0.00-1.98	0.60 ± 0.215	0.007 ± 0.004
Pahang	10	6	0.00-4.26	0.93 ± 0.077	0.011 ± 0.008
Bario	10	7	0.00-1.91	0.98 ± 0.054	0.009 ± 0.001

N, number of individuals. [a]Estimated using the HKY distance (Hasegawa et al. 1985). [b]Sites with gaps were completely excluded.

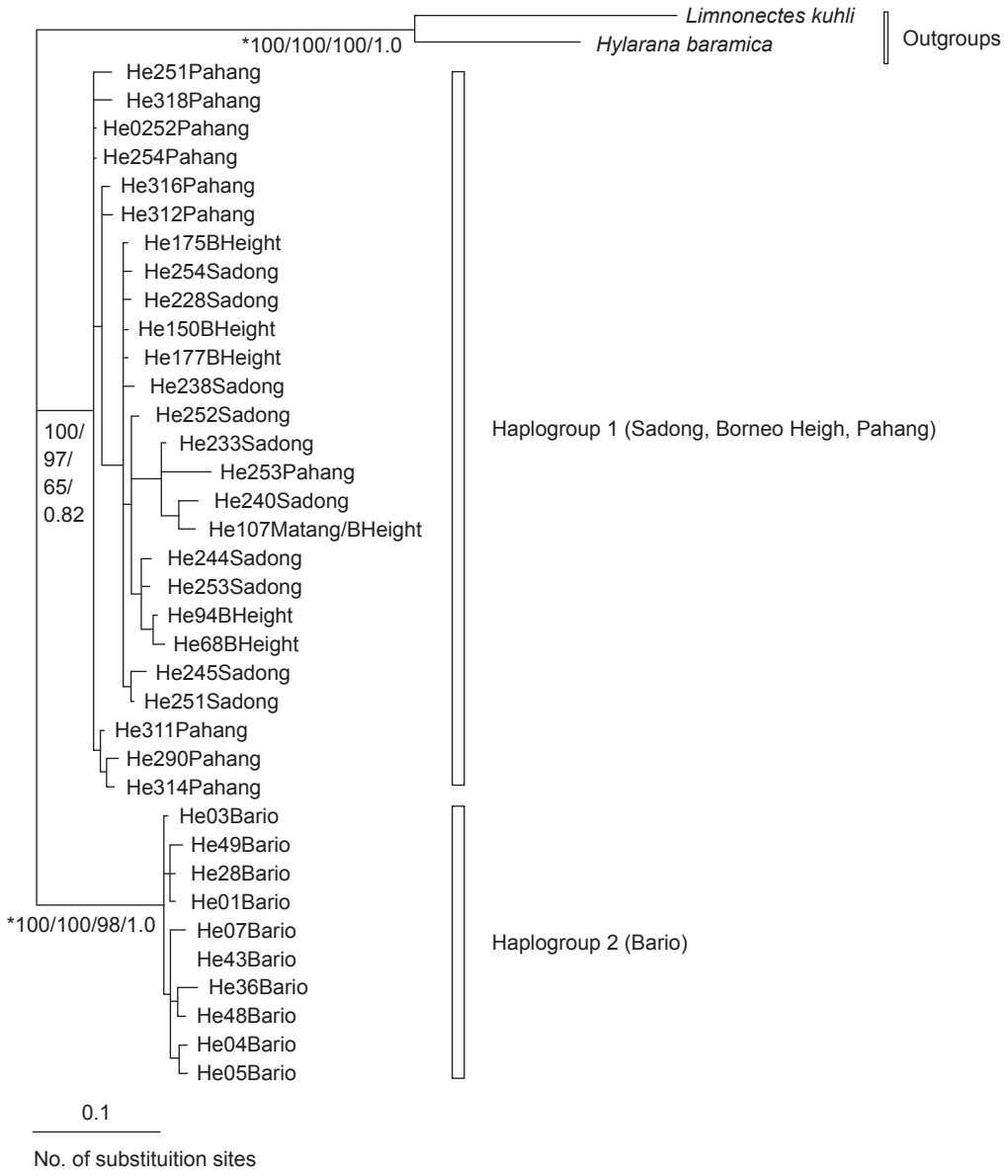

Fig. 2. Bayesian inference of the 50% majority rule consensus tree of cytochrome oxidase subunit 1 (CO1) mtDNA of *Hylarana erythraea*. Bootstrap values and Bayesian posterior probabilities (BPPs) are accordingly indicated below the branch nodes (*NJ, neighbor-joining; MP, maximum-parsimony; ML, maximum-likelihood; and BPP).

Table 4. Measures of nucleotide diversity (π) and net nucleotide divergence among populations of *Hylarana erythraea* analyzed by location

Locality	Distance (KM)	Nucleotide diversity (π)[a,b]	Net nucleotide divergence (Da)
Sadong-Borneo Heights	71.8	0.0114	0.0087
Sadong-Pahang	895	0.0136	0.0237
Sadong-Bario	532	0.0126	0.0995
Borneo Heights-Pahang	847	0.0073	0.0195
Borneo Heights-Bario	604	0.0075	0.1008
Pahang-Bario	1322	0.0101	0.102

[a]Estimated using the Kimura 2-parameter distance (Kimura 1980). [b]Sites with gaps were completely excluded.

consisted of all 16 haplotypes from the remaining populations with low significance value of ML and Bayesian posterior probabilities (100%, 97%, 65%, and 0.82). The MSN (Fig. 3) within this limit resulted in 2 networks, identified as haplogroup 1 (Sadong, Borneo Heights, and Tasik Chini) and haplogroup 2 (Bario), and this was consistent with the topologies of the phylogenetic trees. In addition, the phylogenetic trees and network indicated that haplogroup 1 (Sadong, Pahang, and Borneo Heights) was basal compared to the Bario population with respect to the outgroups.

The mismatch distribution of pairwise nucleotide differences among CO1 sequences either for the entire population or each population, supported population expansion hypotheses following the expected distribution under a sudden (recent) expansion model (Slatkin and Hudson 1991, Rogers and Harpending 1992) and the spatial expansion model (range expansion with high levels of migration between neighboring demes (Ray et al. 2003, Excoffier et al. 2005) as seen in the small SSD values (0.02-0.24) and a lack of significance observed (p = 0.14-0.78) for both expansion models (Table 5). Small values for Harpending's raggedness index (0.02-0.083) along with a lack of significance (p = 0.19-0.83) indicated unimodal interpretation of the mismatch distributions in the species. However, scatterplots of the mismatch distribution for the entire population (Fig. 4A) and each population (Fig. 5A-D) indicated multimodal mismatch distributions. The allelic frequency spectra for the entire population, on the other hand, revealed an excess of singleton mutations and deviated from expected frequencies of no population change (Fig. 4B).

mtDNA of CO1 sequences from the Borneo Heights and Pahang populations was far from neutral and was estimated to have evolved under a non-random process such as demographic expansion or contraction as shown by the significance of the Tajima test (p = 0.03 and 0.02, respectively, Table 5). In addition, Fu's Fs value (Table 5) was negative (-0.91 to -3.00 except for the Borneo Heights population) with a lack of significance (p = 0.3-0.45) suggesting the presence of rare haplotypes, but was not significant to infer either population expansion or genetic hitchhiking (Fu 1997). The small negative value and a significant test of Fu' Fs for the Bario population indicated recent population expansion.

The AMOVA analysis (Table 6) showed that most of the diversity (87.53%) was found among groups, which had significantly differentiated (p = 0.00). The variation within populations (12.47%) was also supported (p = 0.000), suggesting an unequal rate of evolution among lineages in the same populations. The estimated Φ_{ST} values (Table 7) among the grouped populations remained significant in the pairwise genetic differentiation except for the Borneo Heights and Sadong populations. The species from western Sarawak and Pahang populations also revealed low levels of nucleotide (Nst) and population subdivision (F_{ST}) with high levels of migrants per generation (N_m), indicating either recurrent gene flow or past historical associations among them. The species showed its highest gene flow between Sadong and Borneo Heights (N_m = -7.48, undefined, Table 8). On the other hand, populations from Bario appeared to be isolated from the other 3 populations (Nst or F_{ST} > 0.9) with low levels of migrants per generation (N_m = 0.03-0.05, Table 8). Overall gene flow estimators indicated high numbers of migrants per generation and panmictic populations of *H. erythraea*, except for the Bario population.

DISCUSSION

Historical associations might explain the genetic break among *H. erythraea* from western Sarawak (Sadong and Borneo Heights), Peninsular Malaysia, and east of the Lupar gap (Bario). The Lupar gap was supposedly formed by the Lupar River 10-15 mya (Hutchinson 1996, Fig. 6). This might have been due to the tectonic evolution of Borneo that created the Lupar line problem (Hutchinson 1996, Fig. 6) which divided Sarawak into 2 geologically distinct areas and thus limited the dispersal and restricted gene flow among frog populations. A study of *Opsariichthys bidens* in China (Li et al. 2009) using cytochrome b oxidase also revealed 2 distinct clades among populations of the species, and the divergence corresponded to the time of the 2nd uplift of the Qinghai-Tibet Plateau, the emergence of the East Asian monsoon, and the Epoch-6 event.

The Lupar Valley (gap) consists of a large river (Batang Lupar) and extensive swamp forests surrounding the area. This creates a great barrier for dispersal of frogs that are intolerant of acidic environments and those which are stream or pond dwellers. Since *H. erythraea* is a pond dweller and never found in swampy areas except when turned into plantations, the Lupar Valley may have served as a barrier to its dispersal. The Lupar gap

barrier to the COI mtDNA gene was also seen in 2 species of Bornean riparian frogs, *L. kuhli* and *L. leporinus* (Ramlah 1998). Another study by Jalil et al. (2008) revealed riverine effects on the mitochondrial structure of Sarawak orangutans (*Pongo pygmaeus*) at 2 spatial scales in Borneo. Thus, this feature of the Borneo landscape may have led to genetic differentiation among species populations.

On the other hand, isolation by distance (IBD) can also be a factor contributing to the high genetic divergence as supported by the AMOVA (Table 5) when comparing genetic differentiation of Sadong and/or Borneo Heights populations from those of Bario or Pahang. The frogs could not have easily migrated between Sarawak or Peninsular Malaysia due to barriers like topography and seawater except during the last ice age. The

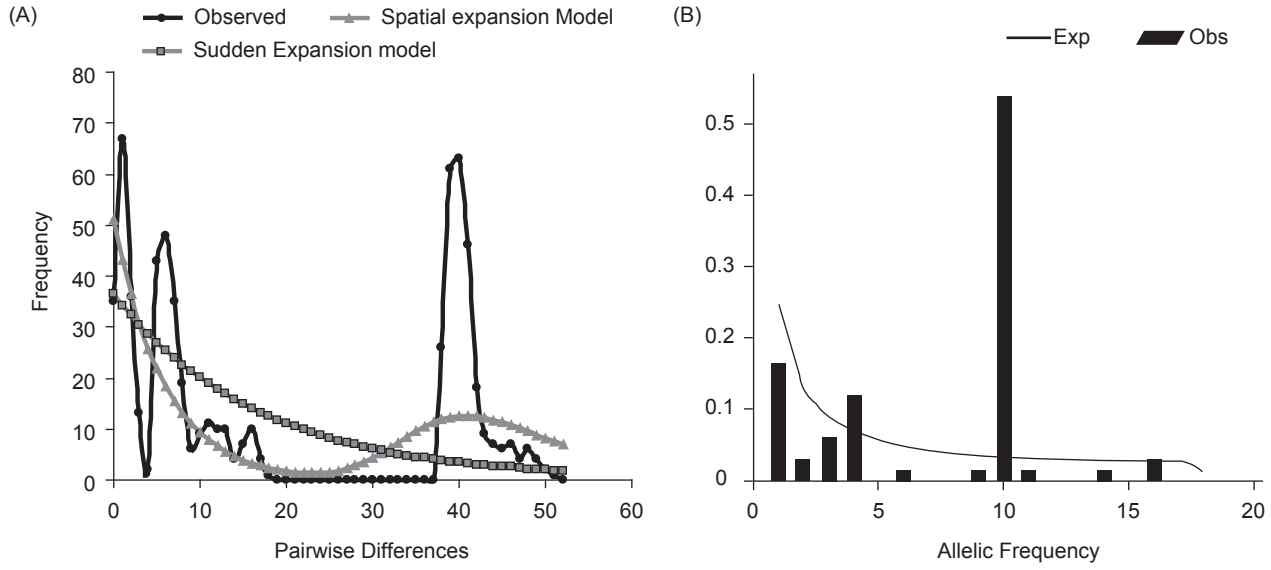

Fig. 3. Population expansion signatures in mitochondrial cytochrome oxidase subunit 1 (CO1) sequences data of *Hylarana erythraea*. (A) Mismatch distribution of observed frequencies of pairwise differences among CO1 sequences and expected frequencies under the sudden and spatial expansion models; (B) allele frequency spectrum indicating an excess of singleton mutations in CO1 sequences.

Table 5. Summary statistics of cytochrome oxidase subunit 1 (COI) mtDNA sequence variation in 4 populations of *Hylarana erythraea* from Sarawak and Pahang

Population	*N*	H	% sdiv	D	Fs	Sudden expansion		Spatial expansion	
						SSD	*r*	SSD	*r*
Sadong Jaya, Sarawak	10	8	0.46-4.05	-1.12	-2.42[a]	0.05	0.08	0.04	0.08
				(p = 0.13)	(p = 0.05)	(p = 0.14)	(p = 0.58)	(p = 0.26)	(p = 0.50)
Borneo Heights, Sarawak	6	3	0.00-1.98	-1.42**	1.81	0.10	0.20	0.07	0.20
				(p = 0.03)	(p = 0.84)	(p = 0.25)	(p = 0.78)	(p = 0.41)	(p = 0.83)
Pahang, Peninsular Malaysia	10	6	0.00-4.26	-1.70**	-0.73	0.02	0.08	0.24	0.083
				(p = 0.02)	(p = 0.30)	(p = 0.39)	(p = 0.66)	(p = 0.31)	(p = 0.65)
Bario, Sarawak	10	7	0.00-1.91	-0.51	-3.00**	0.03	0.14	0.03	0.15
				(p = 0.32)	(p = 0.02)	(p = 0.29)	(p = 0.29)	(p = 0.19)	(p = 0.24)
Entire population	36	23	0.00-14.6	1.17	-0.91	0.04	0.02	0.03	0.02
				(p = 0.83)	(p = 0.45)	(p = 0.5; 0.49)	(p = 0.47)	(p = 0.39)	(p = 0.75)

N, number of sequences analyzed; H, number of haplotypes; sdiv, pairwise sequence divergence (estimated using the HKY distance (Hasegawa and Yano 1985)); D, Tajima's statistic (P (D_{simul} < D_{obs}), Tajima 1989); SSD, sum of squared deviations of the observed and expected mismatch with *p* values in parentheses; *r*, raggedness statistic (Harpending 1994) with *p* values in parentheses; Fs, Fu's statistic (Fu 1997). ** Significance (p < 0.05) was determined using coalescent simulations in Excoffier (2004). [a]Sites with gaps were completely excluded.

species might have dispersed all across Borneo and Peninsular Malaysia especially during the last ice age, but each population may have evolved independently through time. The results suggest that populations of *H. erythraea* from western Sarawak are panmictic with populations from Pahang, and correspond to the notation that Borneo was once connected to Peninsular Malaysia which formed Sundaland during the last ice age (11,000 yr ago) in the Pleistocene epoch. This can be seen in channels on the seabed (Fig. 6) probably representing Pleistocene river courses that once existed in Sundaland. However, relationships among populations of *H. erythraea* on

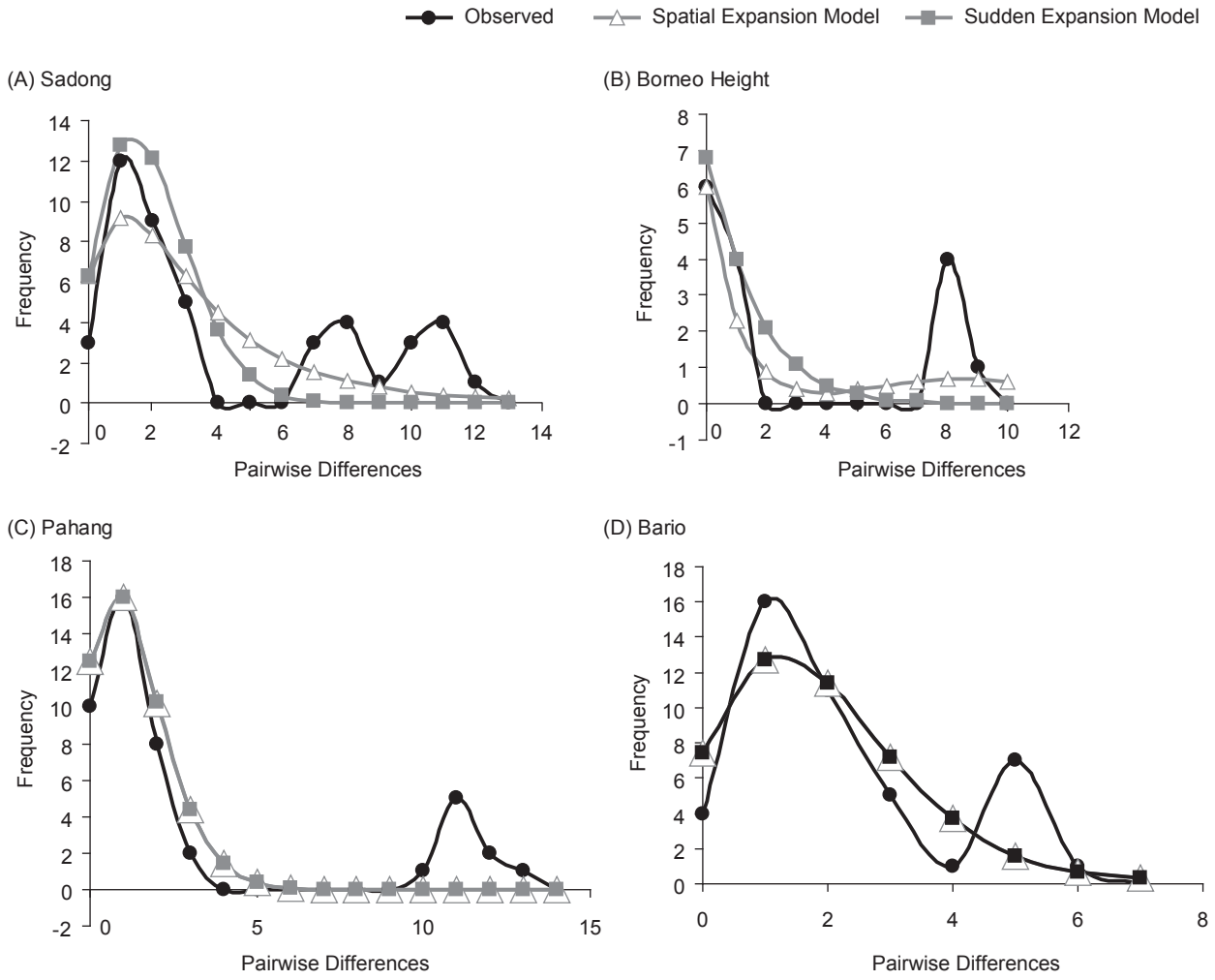

Fig. 4. Mismatch distribution for Sarawak *Hylarana erythraea* at each locality. The dark line represents the observed and light lines represent the expected distributions for each model.

Table 6. Measures of geographical population differentiation in *Hylarana erythraea* based on an analysis of molecular variance approach with cytochrome *c* oxidase subunit 1 data

Source of variation	Variance component	Percent (%) variation	Fixation index, Φ	p^a
Among populations	11.09	87.53	0.88	0.00 ± 0.00*
Within populations	1.58	12.47	0.87	0.00 ± 0.00*

*Significant ($p < 0.05$). [a]Probability of finding a more-extreme variance component of the Φ index than that observed by chance alone after 1000 permutations.

the ancient Sunda Shelf (Sadong, Borneo Heights, and Pahang) were not resolved due to low significant values of the ML and Bayesian posterior probabilities. Only isolation of the Bario population was supported by the phylogenetic trees. This suggests that a feature in the landscape of Borneo (the Lupar line) created a greater barrier than the repeated intervening ocean between glacial periods. Furthermore, from the phylogenetic trees and network, the Bario population can be regarded as a derivative end product population of Western Sarawak and Pahang, further suggesting that the species may have dispersed from the ancient Sunda Shelf (Western Sarawak and Pahang

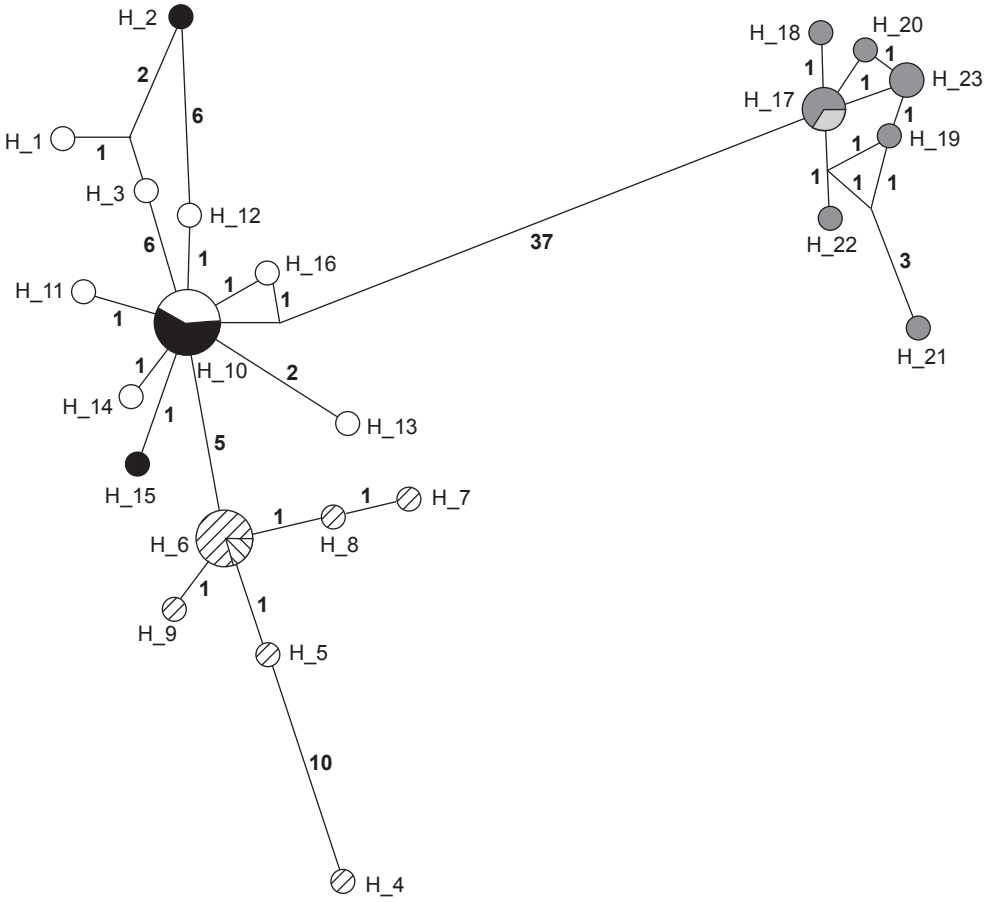

Fig. 5. The minimum-spanning network (MSN) generated by Network 4.5.0.2 (Bandelt et al. 1999) illustrating the relationships of the green paddy frog *Hylarana erythraea* in Pahang (Peninsular Malaysia) and Sarawak (East Malaysia). Each circle represents a haplotype, and the diameter is scaled to the haplotype frequency. Note that backward diagonal circles indicate Pahang haplotypes, the white circles indicate Sadong haplotypes, black circles indicate haplotypes of Borneo Heights, and dark gray indicates Bario haplotypes. Bold numbers on the lines connecting haplotypes indicate number of mutational steps.

Table 7. Genetic differentiation matrix of populations calculated by Φ_{ST}. *p* values are shown in parenthesis (below the diagonal)

	Sadong	Borneo Heights	Pahang	Bario
Sadong	-			
Borneo Heights	-0.09 (0.92 ± 0.01)	-		
Pahang	0.57 (0.00 ± 0.00)*	0.61 (0.00 ± 0.00)*	-	
Bario	0.92 (0.00 ± 0.00)*	0.94 (0.00 ± 0.00)*	0.94 (0.00 ± 0.00)*	-

*Significant ($p < 0.05$) with 1000 permutations.

populations) to Bario, which is also supported by the large negative value with a high significant test (Fu's Fs = -3.00, p = 0.02) of the Bario population, implying a recent demographic expansion.

High genetic divergence of 11.3% between western Sarawak and the east of Sarawak (RZ233 Sadong vs. RZ04 Bario) compared to 3.8% for western Sarawak and Pahang, Peninsular

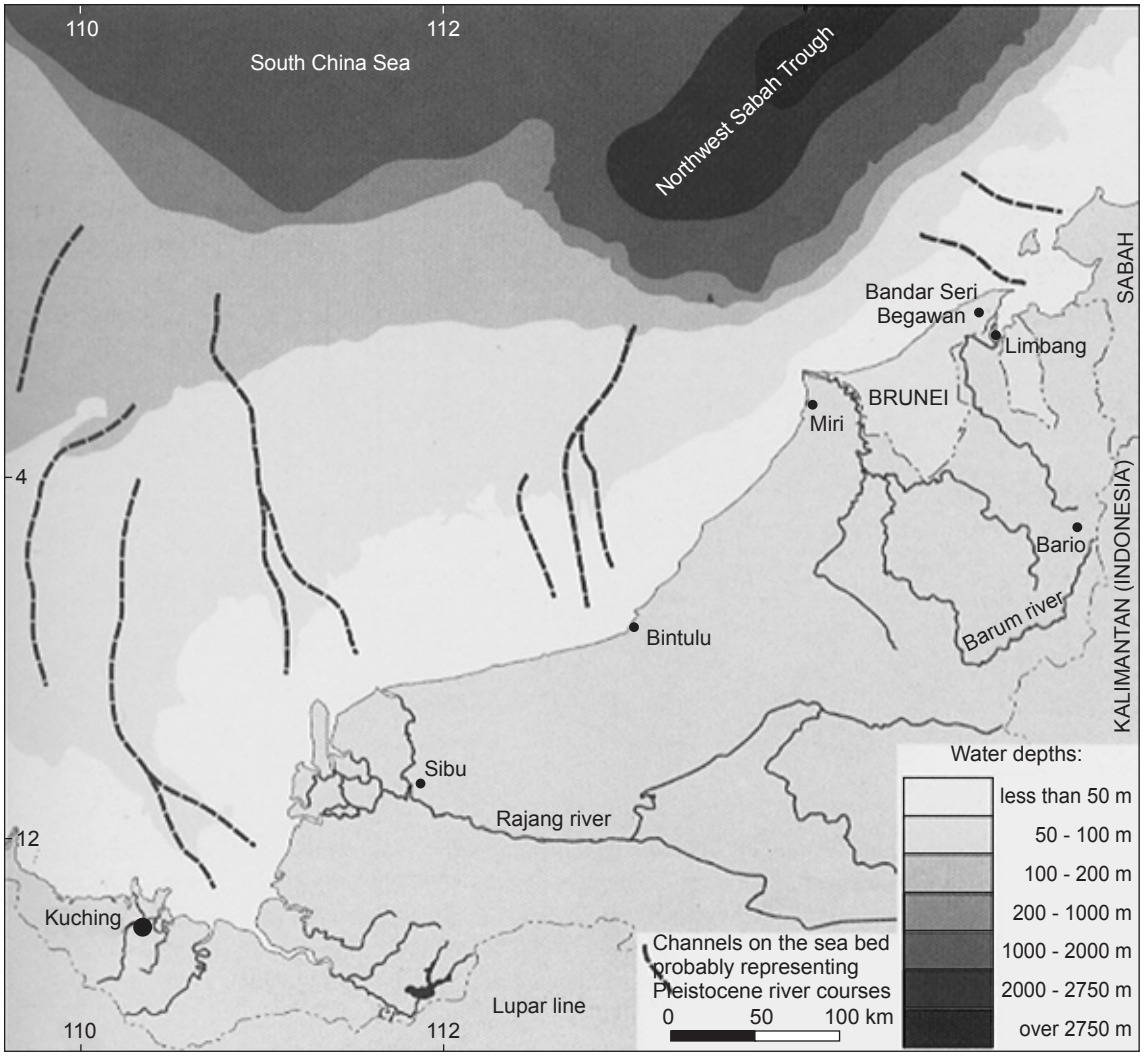

Fig. 6. Sarawak geological map showing the Lupar line and water depths off Sarawak's coast (Hazebroake and Kassim 2001).

Table 8. Measures of nucleotide subdivision (N_{st}), population subdivision (F_{ST}), and gene flow (number of migrants, N_m) among 4 populations of *Hylarana erythraea*

Locality	Distance (KM)	Nucleotide subdivision (N_{st})[a]	Estimate of population subdivision (F_{ST})[b]	Number of migrants per generation (N_m)[b]
Sadong-Borneo Heights	71.8	-0.07	-0.07	-7.48
Sadong-Pahang	895	0.48	0.47	0.55
Borneo Heights-Pahang	847	0.61	0.60	0.33
Sadong-Bario	532	0.91	0.91	0.05
Borneo Heights-Bario	604	0.94	0.94	0.03
Pahang-Bario	1322	0.93	0.93	0.04

[a]Estimated using Lynch and Crease (1990). [b]Estimated using Hudson et al. (1992).

Malaysia (RZ233 Sadong vs. NA318 Pahang) suggests that the population of western Sarawak is more closely related to Peninsular Malaysia than with the population of eastern Sarawak. The result is consistent with Esa et al. (2008) who found shared haplotypes between Batang Ai Sarawak and Peninsular Malaysia that reflected the historical connection of drainages between the regions in the Pleistocene glaciation period. Furthermore, the highest genetic divergence among *H. erythraea* (14.6% between NA253 Pahang and RZ36 Bario) may be an indicator of genetically cryptic species (Bradley and Baker 2001), and additional study is needed in terms of the species status.

The Western Sarawak areas (Sadong and Borneo Heights) were identified as rather distinct in other studies on flora (Ashton and Hall 1992, Ridder-Numan 1998, Wong 1998, Potts et al. 2002) and fauna (MacKinnon and MacKinnon 1986) of Borneo. Thus, different phylogeographic and zoogeographic areas presumably reflect differences in geographical history, Pleistocene land connections, and geographical barriers to dispersal of species (MacKinnon et al. 1996).

Species of the Borneo Heights and Pahang populations have experienced demographic expansion and deviated from the neutrality theory (with a significant Tajima's test), but the overall mtDNA of CO1 sequences exhibited a multimodal distribution which is common for populations at demographic equilibrium and reflects a highly stochastic shape of the evolutionary lineages. Although sudden population growth results in a unimodal mismatch distribution in a geographically homogeneous population, the pattern can dramatically differ when there is geographical subdivision (Hartl 2004). This is true in our study, since the Bario population was significantly divided from the remainder of the populations. With small migration rates between subpopulations, the mismatch distribution can become multimodal (Hartl 2004). Geographical subdivision might explain some of the anomalies in the mismatch distributions that were observed for these populations.

In conclusion, our study revealed 2 distinct geographical clades; i) the 1st clade, haplogroup 2, included 7 divergent haplotypes of *H. erythraea* from Bario, whereas ii) the 2nd clade (haplogroup 1) contained all 16 haplotypes of *H. erythraea* from Sadong Jaya, Borneo Highlands, Sarawak, and Pahang, Peninsular Malaysia. Gene flow estimators indicated high numbers of migrants per generation and panmictic populations of *H. erythraea*, except for a low number of migrants per generation and genetic isolation for the Bario population. The study implied that a feature in the landscape of Borneo created a greater barrier than repeatedly intervening ocean between glacial periods. The results also support the notion that widely distributed frog species exhibit different evolutionary lineages, and genetically distinct species exist. The results obtained underscore the need for a complete sequence of DNA regions or multigenes with the same rates of evolution in order to elucidate the evolutionary relationships of geographically separated populations through more-extensive samplings spanning wider geographical ranges. Consequently, the complete DNA sequences can be used for DNA baseline data for future management and conservation of Sarawak frogs of the genus *Hylarana*.

Acknowledgments: We would like to thank Dr. R.F. Inger, Prof. M. Matsui, and Prof. I. Das for their valuable advice. We also would like to thank everyone who was directly and indirectly involved in this project. Funding for this study was provided by a UNIMAS research grant 01(124)513(05)(12). Lastly, our gratitude also goes to the Sarawak Forest Department, Sarawak Forest Corporation, and PERHILITAN for the permits for sample collection.

REFERENCES

Alcala AC. 1955. Observation on the life history and ecology of *Rana erythraea* Schlegel, on Negros Island, Philippines. Siliman J. **2:** 175-192.

Alexandrino J, JW Jarntzen, N Ferrand. 2002. Nested clade analysis & the genetic evidence for population expansion in the phylogeography of the golden-striped salamander, *Chioglossa lusitanica* (Amphibia: Urodela). Heredity **88:** 66-74.

Ashton PS, P Hall. 1992. Comparisons of structure among mixed dipterocarp forests of north-western Borneo. J. Ecol. **80:** 459-481.

Bandelt HJ, P Forster, A Röhl. 1999. Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol. **16:** 37-48.

Bradley RD, RJ Baker. 2001. A test of the genetic species concept: cytochrome-*b* sequences and mammals. J. Mammal. **82:** 960-973.

Brown WC, AC Alcala. 1970. Population ecology of the frog *Rana erythraea* in southern Negros, Philippines. Copeia **1970:** 611-622.

Donnelly MJ, MC Licht, T Lehmann. 2001. Evidence for recent population expansion in the evolutionary history of the malaria vectors *Anopheles arabiensis* and *Anopheles gambiae*. Mol. Biol. Evol. **18:** 1353-1364.

Esa Y, SS Siraj, SK Daud, KAA Rahim, JJ Ryan, SG Tan. 2008. Mitochondrial DNA diversity of *Tor tambroides* Valenciennes (Cyprinidae) from five natural populations in Malaysia. Zool. Stud. **47:** 360-367.

Excoffier L, G Laval, S Schneider. 2005. Arlequin vers. 3.0: an integrated software package for population genetics data analysis. Evol. Bioinform. Online **1:** 47-50.

Fu YX. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics **147:** 915-925.

Guindon S, O Gascuel. 2003. A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. Systemat. Biol. **52:** 696-704.

Hartl DL. 2004. The origin of malaria: mixed messages from genetic diversity. Nat. Rev. Microb. **2:** 15-22.

Hasegawa M, H Kishino, T Yano. 1985. Dating the human-ape split by a molecular clock of mitochondrial DNA. J. Mol, Evol. **22:** 160-174.

Hazebroek HP, AM Kashim. 2001. National parks of Sarawak. *In* KM Wong, RCK Chung, eds. Kota Kinabalu, Sabah Malaysia: Natural History Publications (Borneo), pp. 16-27.

Hudson RR, M Slatkin, WP Maddison. 1992. Estimation of levels of gene flow from DNA sequence data. Genetics **132:** 583-589.

Huelsenbeck JP, JE Hill. 1993. Success of phylogenetic methods in the four taxon case. Systemat. Biol. **42:** 247-264.

Huelsenbeck JP, FR Ronquist. 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics **17:** 754-755.

Hutchinson CS. 1996. The 'Rejang accretionary prism' & 'Lupar line' problem of Borneo. *In* R Hall, Blundell P, eds. Tectonics evolution of Southeast Asia. (Geological Society of London, Special Publication no. 106). Society of America; illustrated edition, pp. 247-261.

Inger RF, B Greenberg. 1963. The annual reproductive pattern of the frog *Rana erythraea* in Sarawak. Physiol. Zool. **36:** 21-33.

Inger RF, BL Stuart, DT Iskandar. 2009. Systematics of a widespread Southeast Asian frog, *Rana chalconota* (Amphibia: Anura: Ranidae). Zool. J. Linn. Soc. **155:** 123-147.

Inger RF, RB Stuebing. 2005. A field guide to the frogs of Borneo. 2nd ed. Kota Kinabalu, Sabah Malaysia: Natural History Publications (Borneo), pp. 1-200.

Jalil MF, J Cable, J Sinyor, I Lackman-Ancrenaz, M Ancrenaz, MW Bruford, B Goosens. 2008. Riverine effects on mitochondrial structure of Sarawak orangutans (*Pongo pygmaeus*) at two spatial scales. Mol. Ecol. **17:** 2898-2909.

Kumar S, J Dudley, M Nei, K Tamura. 2008. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brie. Bioinform. **9:** 299-306.

Leache AD, TW Reeder. 2002. Molecular systematics of the eastern fence lizard (*Sceloporus undulatus*): a comparison of parsimony, likelihood, and Bayesian approaches. Systemat. Biol. **51:** 44-68.

Li GY, XZ Wang, YH Zhao, J Zhang, CG Zhang, SP He. 2009. Speciation and phylogeography of *Opsariichthys bidens* (Pisces: Cypriniformes: Cyprinidae) in China: analysis of the cytochrome *b* gene of mtDNA from diverse populations. Zool. Stud. **48:** 569-583.

Lynch M, TJ Crease. 1990. The analysis of population survey data on DNA sequence variation. Mol. Biol. Evol. **7:** 377-394.

MacKinnon J, K MacKinnon. 1986. Review of the protected areas system in the Indo-Malayan realm. Gland, Switzerland: International Union for the Conservation of Nature.

MacKinnon K, G Hatta, H Halim, A Mangalik. 1996. The ecology of Kalimantan. The ecology of Indonesian series. Hong Kong: Periplus Editions, 3, pp. 1-802.

Mantel N. 1967. The detection of disease clustering & a generalized regression approach. Cancer Res. **27:** 209-220.

Matsui M, T Shimada, H Ota, T Tanaka-Ueno. 2005. Multiple invasions of the Ryukyu Archipelago by Oriental frogs of the subgenus *Odorrana* with phylogenetic reassessment of the related subgenera of the genus *Rana*. Mol. Phylogenet. Evol. **37:** 733-742.

Mertens R. 1934. Die Amphibian und Reptilien der Deutschen Limnologischen Sunda Expedition. Arch. Hydrobiol. Suppl. Band 12 Trop. Bimmenwaser **4:** 667-701.

Nei M. 1987. Molecular evolutionary genetics. New York: Columbia Univ. Press.

Posada D. 2008. jModelTest: phylogenetic model averaging. molecular biology and evolution. Mol. Biol. Evol. **25:** 1253-1256.

Potts MD, PS Ashton, LS Kaufman, JB Plotkin. 2002. Habitat patterns in tropical rain forests: a comparison of 105 plots in Northwest Borneo. Ecology **83:** 2782-2797.

Ray N, M Currat, L Excoffier. 2003. Intra-deme molecular diversity in spatially expanding populations. Mol. Biol. Evol. **20:** 76-86.

Ramlah Z. 1998. Microgeographic variation of mitochondrial DNA in populations of Sarawak riparian frogs. MSc thesis, Univ. of Illinois at Chicago, Chicago, IL.

Rannala B, Z Yang. 1996. Probability distribution of molecular evolution trees: a new method of phylogenetic inference. J. Mol. Evol. **43:** 304-311.

Ridder-Numan JWA. 1998. Historical biogeography of Spatholobus (Leguminosae – Papilionioideae) and allies in SE Asia. *In* R Hall, D Holloway, eds. Biogeography and geological evolution of SE Asia. Leiden, the Netherlands: Ackhuys Publishers, pp. 259-277.

Rogers AR. 1995. Genetic evidence for a Pleistocene population explosion. Evolution **49:** 608-615.

Rogers AR, H Harpending. 1992. Population growth makes waves in the distribution of pairwise genetic differences. Mol. Biol. Evol. **9:** 552-569.

Rozas J, R Rozas. 1997. DnaSP vers. 2.0: a novel software package for extensive molecular population genetics analysis. Comput. Appl. Biosci. **13:** 307-311.

Rozas J, JC Sanches-DelBarrio, X Messeguer, R Rozas. 2003. DnaSP, DNA polymorphism by the coalescent & other methods. Bioinformatics **19:** 2496-3497.

Slatkin M, RR Hudson. 1991. Pairwise comparisons of mitochondrial DNA sequences in stable & exponentially growing populations. Genetics **129:** 555-562.

Stuart BL, RF Inger, HK Voris. 2006. High level of cryptic species diversity revealed by sympatric lineages of Southeast Asian forest frogs. Biol. Lett. **2:** 470-474.

Swofford DL. 2000. PAUP*. Phylogenetic Analysis Using Parsimony (*& other methods). Vers. 4.0b10. Sunderland, MA: Sinauer Associates.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The ClustalX windows interface: flexible

strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research, **24:** 4876-4882

Tajima F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics **123:** 585-595.

Wong KM. 1998. Patterns of plant endemism and rarity in Borneo and the Malay Peninsula. *In* CI Peng, PP Lowry II, eds. Rare, threatened, and endangered floras of Asia and the Pacific rim. Taipei, Taiwan: Institute of Botany, Academia Sinica Monograph Series 16, pp. 139-169.