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Genetic Divergence in a Bornean Fanged Frog *Limnonectes leporinus* (Dicroglossidae, Anura)

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Abstract: A previous population genetic study using mitochondrial COI gene reported a Bornean dicroglossid frog *Limnonectes leporinus* to be highly diversified. However, our mtDNA phylogeny using samples collected from throughout the species distribution and longer sequences of 12S-16S rRNA genes indicates the species not markedly diversified within the island, conforming to the fact that it is also not diversified morphologically. The divergence times among local samples are thought to be younger than some congeneric species, and it is estimated that the species arose relatively new and subsequently rapidly dispersed within the island.

Key words: MtDNA phylogeny; Fanged frog; Borneo; Genetic variability; Evolution

INTRODUCTION

Limnonectes leporinus Andersson, 1923 is a large-sized riparian frog endemic to Borneo, and is widely distributed throughout the island including states of Sabah and Sarawak of Malaysia, Brunei, and Kalimantan of Indonesia. The species was long treated as *L. blythii*

(Boulenger, 1920) (e.g., Inger, 1966 as *Rana*), but is now recognized as a species distinct from *L. blythii* occurring from Myanmar through western and peninsular Thailand to Sumatra (Inger and Tan, 1996).

The species is especially abundant in primary forest, and seen on the banks of small streams or large rivers (Inger, 1966). This contrasts to the habitat of another large-sized species, *L. malesianus* (Kiew, 1984), which is commonly caught in logged rain forest, secondary growth, or clearings surrounded by

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forest, but only rarely is caught in primary forest (Inger, 1966, as *Rana macrodon* Duméril and Bibron, 1841). The species breeds in the shallows near the edge of streams with unique habits of constructing breeding nests (Inger, 1966). Furthermore, females of this species frequently call during mating, whereas males utter calls only in response to female calls (as *Rana blythii*: Emerson, 1992) unlike many other frogs, including its relative *L. blythii* (as *Rana*: Matsui, 1995). Because of its large and stocky body, local people often consume the species.

Morphologically, the species is uniform and displays no geographic variation in any morphological characters previously analyzed (Inger 1966). In contrast, Zainuddin (2004) reported *L. leporinus* to be genetically divergent by analyzing short sequences of CO1 gene of mitochondrial (mt) DNA. If this is the case, discordance between morphological and genetic variations exists in this species. However, the samples studied by Zainuddin (2004) are limited in geographic range, and more information is necessary to assess patterns of genetic variation. We therefore reassessed genetic variation in *L. leporinus* from wider area of Borneo using longer sequences of 12S–16S rRNA regions of mtDNA, and found that the species does not exhibit particularly marked genetic divergence.

MATERIALS AND METHODS

The DNA sequence data were obtained from tissues frozen or preserved in 99% ethanol (Table 1). Our taxon sampling includes 28 individuals of *Limnonectes* from Borneo, including 19 samples of *L. leporinus* from nine localities in Sabah, Sarawak, and Kalimantan (Fig. 1), and two outgroup taxa, *Fejervarya iskandari* Veith, Kosuch, Ohler, and Dubois, 2001 and *Occidozyga martensii* (Peters, 1867). Voucher specimens are stored at the BORNEENSIS Collection of University Malaysia Sabah (BORN), Graduate School of Human and Environmental Studies, Kyoto University (KUHE), Museum Zoologicum

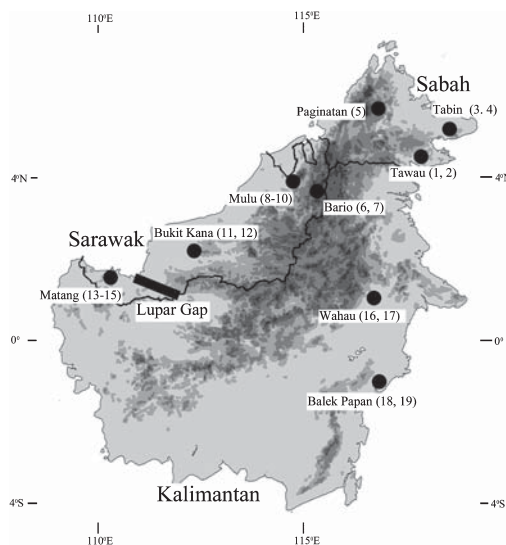


FIG. 1. Map of Borneo showing sampling localities of *Limnonectes leporinus*. Numbers in parenthesis correspond to those shown in Table 1.

Bogoriense (MZB), and University of Indonesia (UI).

Methods for DNA extraction, and amplification and sequencing of the mtDNA fragments are same as those reported by Matsui et al. (2010). The resultant new sequences were deposited in GenBank (Accession numbers KP318703–318725: Table 1). We reconstructed phylogenetic (maximum likelihood [ML] and Bayesian inference [BI]) trees from 2489 base pairs (bp) of partial sequences of mitochondrial 12S and 16S rRNA genes.

We estimated divergence times among haplotypes, by preparing a data set adding published sequences (Matsui et al., 2010) of *L. fujianensis* Ye and Fei, 1994 from continental China (AB526311) and Taiwan (AB526317) for internal calibration (see below). We estimated the divergence times using the same methods as those reported by Kuraishi et al. (2013).

For this data set, each external and internal calibration point was used to estimate the dates of cladogenetic events. As an external calibration point, the divergence time of 82.7 [95% credibility interval (CI) 55–118] million years before present (MYBP) between

TABLE 1. Sample of *L. leporinus* and other Bornean species of *Limnonectes* used for DNA analysis in this study together with the information on voucher, collection locality, and GenBank accession numbers. Voucher abbreviations: BORN=BORNEENSIS Collection, University Malaysia Sabah, KUHE=Graduate School of Human and Environmental Studies, Kyoto University; MZB=Museum Zoologicum Bogoriense; UI=University of Indonesia.

Species	Voucher	Locality	GenBank
1 <i>L. leporinus</i>	BORN 22023	Tawau, Sabah, Borneo, Malaysia	KP318703
2 <i>L. leporinus</i>	BORN 22174	Tawau, Sabah, Borneo, Malaysia	KP318704
3 <i>L. leporinus</i>	BORN 22202	Tabin, Sabah, Borneo, Malaysia	KP318705
4 <i>L. leporinus</i>	BORN 22204	Tabin, Sabah, Borneo, Malaysia	KP318706
5 <i>L. leporinus</i>	BORN 22337	Paginatan, Sabah, Borneo, Malaysia	KP318707
6 <i>L. leporinus</i>	KUHE 53540	Bario, Sarawak, Borneo, Malaysia	KP318708
7 <i>L. leporinus</i>	KUHE 53576	Bario, Sarawak, Borneo, Malaysia	KP318709
8 <i>L. leporinus</i>	KUHE 53647	Mulu, Sarawak, Borneo, Malaysia	KP318710
9 <i>L. leporinus</i>	KUHE 53663	Mulu, Sarawak, Borneo, Malaysia	KP318711
10 <i>L. leporinus</i>	KUHE 54477	Mulu, Sarawak, Borneo, Malaysia	KP318712
11 <i>L. leporinus</i>	KUHE 53691	Bukit Kana, Sarawak, Borneo, Malaysia	KP318713
12 <i>L. leporinus</i>	KUHE 53692	Bukit Kana, Sarawak, Borneo, Malaysia	KP318714
13 <i>L. leporinus</i>	KUHE 53486	Matang, Sarawak, Borneo, Malaysia	AB981420
14 <i>L. leporinus</i>	KUHE 53756	Matang, Sarawak, Borneo, Malaysia	KP318715
15 <i>L. leporinus</i>	KUHE 54544	Matang, Sarawak, Borneo, Malaysia	KP318716
16 <i>L. leporinus</i>	MZB BJE 02519	Wahau, East Kalimantan, Borneo, Indonesia	KP318717
17 <i>L. leporinus</i>	MZB BJE 02522	Wahau, East Kalimantan, Borneo, Indonesia	KP318718
18 <i>L. leporinus</i>	MZB16332	Balek Papan, East Kalimantan, Borneo, Indonesia	KP318719
19 <i>L. leporinus</i>	MZB16333	Balek Papan, East Kalimantan, Borneo, Indonesia	KP318720
20 <i>L. ingeri</i>	KUHE 53634	Mulu, Sarawak, Borneo, Malaysia	KP318721
21 <i>L. malesianus</i>	KUHE 17707a	Matang, Sarawak, Borneo, Malaysia	KP318722
22 <i>L. paramacrodon</i>	BORN 09154	Tawau, Sabah, Borneo, Malaysia	AB981418
23 <i>L. ibanorum</i>	KUHE 53666	Mulu, Sarawak, Borneo, Malaysia	KP318723
24 <i>L. palavanensis</i>	KUHE 54429	Penrissen, Sarawak, Borneo, Malaysia	AB981419
25 <i>L. finchi</i>	BORN 12533	Mahua, Sabah, Borneo, Malaysia	KP318724
26 <i>L. "kuhlii"</i>	BORN 22645	Kinabalu, Sabah, Borneo, Malaysia	AB526323
27 <i>L. "kuhlii"</i>	KUHE 12025	Matang, Sarawak, Borneo, Malaysia	AB526322
28 <i>L. hikidai</i>	KUHE 10654	Matang, Sarawak, Borneo, Malaysia	AB981413
29 <i>Fejervarya iskandari</i>	UI unnumbered	Banyuwangi, east Java, Indonesia	AB526324
30 <i>Occidozyga martensii</i>	KUHE 19790	Chachoengso, Thailand	KP318725

Dicloglossinae and Occidozyginae, assumed by Zhang et al. (2013) was used, while the divergence of *L. fujianensis* from Taiwan and continental China as 1.25 (CI 1.05–1.45) MYBP (Jang-Liaw and Chou, 2011) were set as internal calibration points.

RESULTS

Of 2489 nucleotide sites, 184 were variable and 165 were phylogenetically informative within the ingroup. The best substitution model was GTR+G with gamma shape parameter (G) of 0.256 for ML and 0.278 for BI. The

likelihood values (-lnLs) of the ML and BI trees were 14637.283 and 14679.184, respectively.

Phylogenetic analyses employing ML and BI methods yielded identical topologies, and only the ML tree is presented in Fig. 2. Monophyly of Bornean *Limnonectes* with respect to the outgroup *Fejervarya* and *Occidozyga* was strongly supported (MLBS=99%, BPP=1.00). Within Bornean *Limnonectes*, *L. hikidai* Matsui and Nishikawa, 2014 and two samples of *Limnonectes "kuhlii"* formed a well-supported (99%, 1.00) sister clade to the clade of the remaining species (99%,

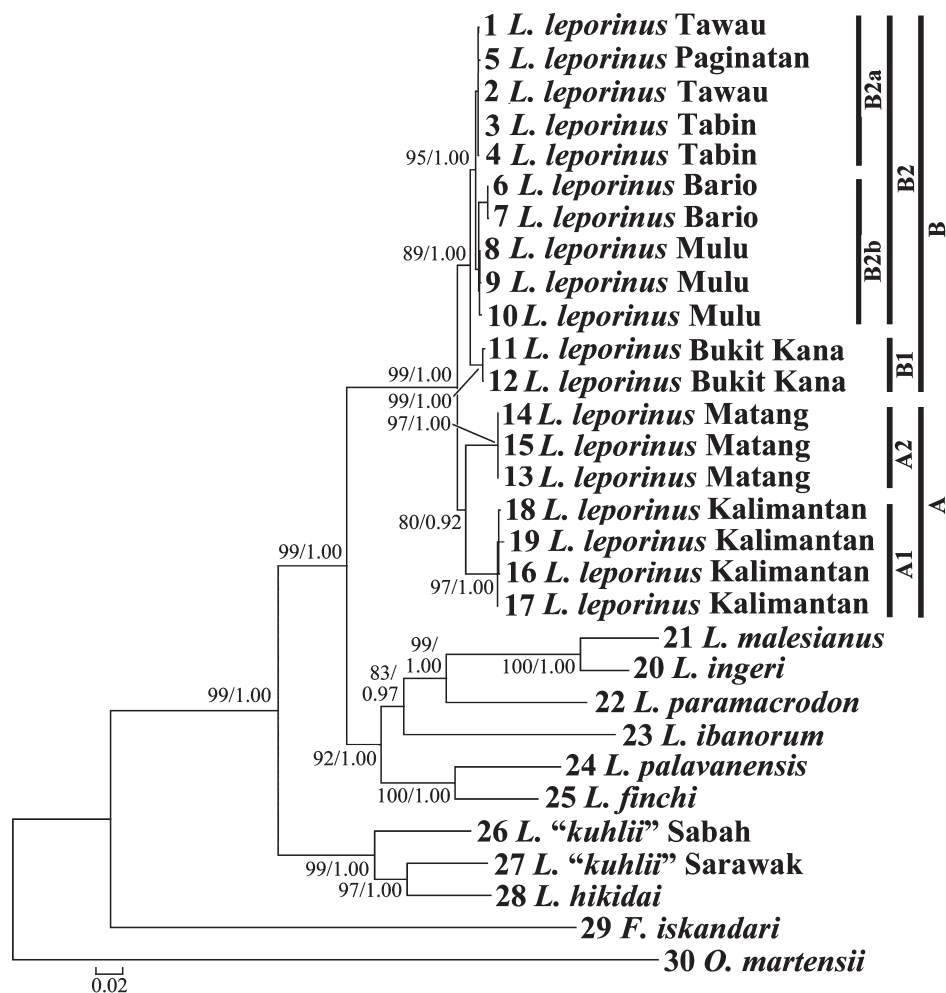


FIG. 2. ML tree from a 2489 bp sequence of mitochondrial 12S rRNA and 16S rRNA genes for samples of *Limnonectes* frogs from Borneo. Numbers above or below branches represent bootstrap supports for ML inference and Bayesian posterior probability (ML-BS/BPP).

1.00). This latter clade was composed of sister clades of *L. leporinus* (99%, 1.00) and the other species (92%, 1.00), in which *L. malesianus* and *L. ingeri* (Kiew, 1978), once confused with *L. leporinus*, were nested.

Our phylogenetic analyses show that the *L. leporinus* lineage split into two major clades early in its evolutionary history (Clades A and B in Fig. 2). The weakly supported Clade A (80%, 0.92) was further split into two sister clades (A1 and A2). Clade A1 (97%, 1.00) included samples from Kalimantan, and Clade A2 (97%, 1.00) included samples from

Matang of southwestern Sarawak. Clade B (89%, 1.00) also was composed of two sister clades, Clade B1 (99%, 1.00) and B2 (95%, 1.00). Clade B1 was composed of samples from Bukit Kana, central western Sarawak, whereas Clade B2 was split into two sister clades, Clade B2a (98%, 1.00) and Clade B2b (96%, 1.00). Clade B2a consisted of samples from eastern Sabah (Tawau, Tabin, and Paginatan), while Clade B2b included samples from northern Sarawak (Bario and Mulu).

Uncorrected p-distance within *L. leporinus* varied from 0% to 4.0% (Table 2). The dis-

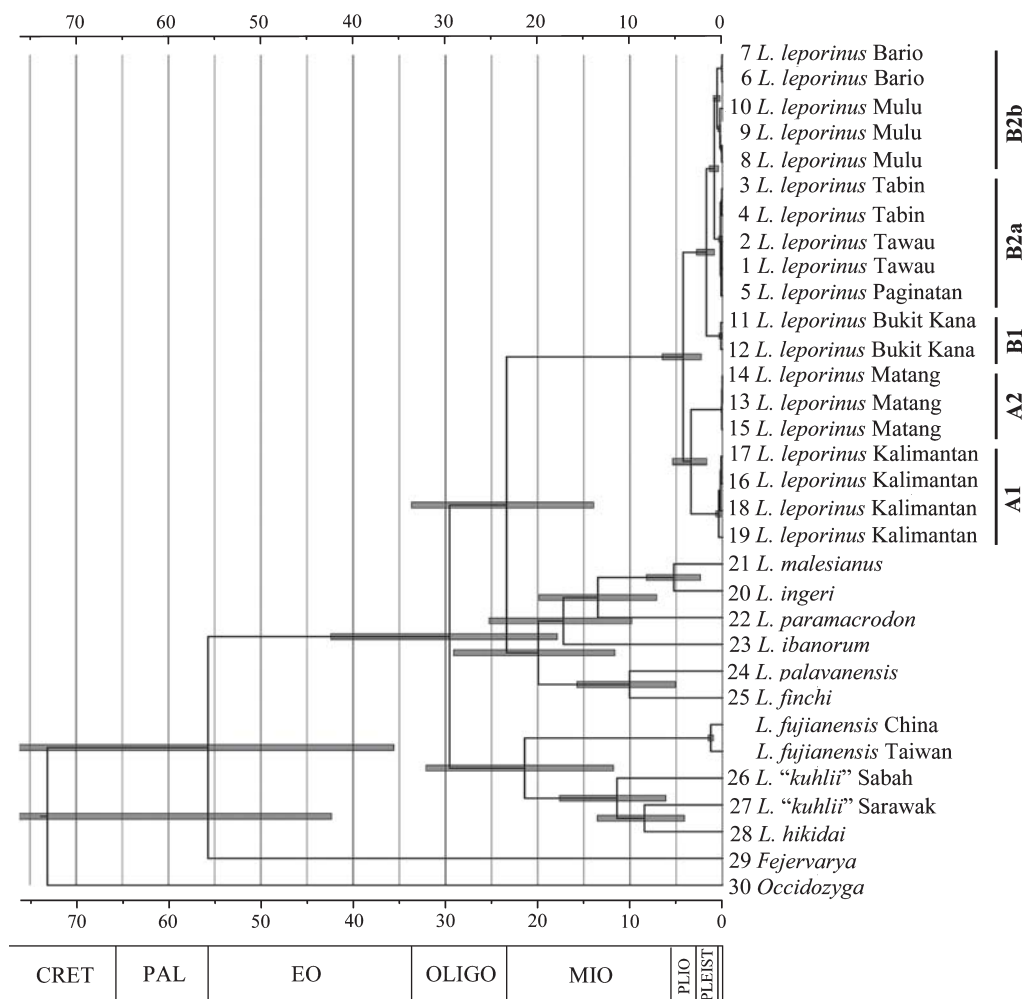


FIG. 3. Estimated times in million years before present (MYBP) of the main divergences of *Limnonectes* frogs from Borneo. Two samples of *L. fujianensis* used for internal calibration are included.

tances between *L. leporinus* and other Bornean *Limnonectes* compared varied from 11.1% to 14.4%. Between the other Bornean *Limnonectes*, the minimum distance 5.0% was observed between *L. ingeri* and *L. malesianus*, which was much smaller than 11.1% between *L. leporinus* and *L. finchi* (Inger, 1966). Between taxa of other *Limnonectes* employed, the distance between two samples of *Limnonectes* “*kuhlii*” from Sabah and Sarawak was as large as 8.3%, and was much larger than the value observed in *L. leporinus* (<4.0%).

The divergence times estimated largely overlapped in the confidence intervals between many

lineages, precluding a confident conclusion regarding the timing of differentiation (Fig. 3). Our data suggest that *L. leporinus* diverged from the common ancestor of *L. malesianus*, *L. ingeri*, *L. paramacrodon* (Inger, 1966), *L. ibanorum* (Inger, 1964), *L. palavanensis* (Boulenger, 1894), and *L. finchi* at 23.4 (CI 14.0–33.7) MYBP. In the clade sister to *L. leporinus*, divergence of *L. malesianus* and *L. ingeri* is estimated to have begun at 5.3 (CI 2.6–8.4) MYBP. The first divergence within *L. leporinus* was estimated to have occurred at 4.3 (CI 2.3–6.5) MYBP, when two major clades A and B split. Within Clade A, Clades

A1 and A2 split at 3.4 (CI 1.7–5.3) MYBP, while Clades B1 and B2 in Clade B split at 1.7 (CI 0.9–2.8) MYBP. Within Clade B2, divergence between Clade B2a (Sabah) and B2b (northern Sarawak) occurred at 0.89 (CI 0.44–1.4) MYBP, followed by divergence within B2a (Sabah) at 0.59 (CI 0.27–0.96) MYBP and within B2b (northern Sarawak) at 0.22 (CI 0.07–0.41). Divergence within terminal clades was young, 0.40 (CI 0.14–0.70) MYBP within Kalimantan and as recent as 0.057 (CI 0.002–0.15) MYBP within the Matang population.

DISCUSSION

Emerson et al. (2000) analyzed partial sequences of 12S and 16S rRNA genes of Southeast Asian fanged frogs and tentatively identified four species groups, one of which (Species group 2) included *L. leporinus* from Danum Valley of Sabah, Brunei, and Barito Ulu in Mentaya Hulu of Kalimantan Selatan, whereas *L. blythii*, with which *L. leporinus* was long confused (e.g., Inger, 1966), was in a different group (Species group 4), supporting Inger and Tan's (1996) revision. Slightly later, Evans et al. (2003) studied *L. leporinus* from tRNA^{phe}, 12S rRNA, tRNA^{val}, and 16S rRNA genes of three specimens from Sabah, Kutai National Park of Kalimantan Timur, and Kalimantan Selatan (Sabah and Kalimantan Selatan specimens identical with those used by Emerson et al., 2000). Neither of these papers described details of genetic diversity among samples of *L. leporinus*, but their phylogenetic tree supports monophyly of the species and little divergence among individuals.

In contrast, Zainuddin (2004) reported *L. leporinus* to be genetically very divergent. Using ca. 500 bp of CO1, that study found high levels of sequence divergence in 22 individuals of *L. leporinus* from Sabah, Brunei, and Sarawak (Batang Ai, Matang, Gading, and Padawan). The sequence divergences (in Kimura's 2-parameter distance) were $3.7 \pm 2.1\%$ (0.4–8.7%) within and $6.1 \pm 3.7\%$ (0.4–12.3%) among populations. The maximum inter-

population divergence, 12.3%, corresponding to 11.2% in uncorrected p-distance, was observed between individuals from Batang Ai and Matang. Zainuddin (2004) found high values of haplotype and nucleotide diversity within the Matang population, in which one individual had the haplotype extremely different from the others. We suspect this individual was misidentified since our three other samples from Matang shared an identical haplotype.

Zainuddin (1998, 2004) also studied Bornean *L. "kuhlii"* and found even higher interpopulation diversity (0.2–21%). That study found a genetic break between populations in both species, *L. leporinus* and *L. "kuhlii"* between western Sarawak (Gading, Matang, and Padawan) and eastern Sarawak (Batang Ai of Sarawak, Sabah, and Brunei). *Limnonectes leporinus* and *L. "kuhlii"* inhabit riparian habitat along streams with moderate to steep gradients. Such streams do not occur in the Lupar gap separating the Matang-Gading-Padawan areas from Batang Ai (Fig. 1), rather the Lupar gap (Lupar Valley) consists of a river and extensive swamp forests that formed 10–15 MYBP (Hutchinson, 1996). In contrast, no extensive lowland gap separates Batang Ai from Brunei, and Sabah. Thus, Zainuddin (1998) ascribed the sharp west-east genetic break in both species to the presence of Lupar gap. Our results support this hypothesis with Matang samples in Clade A2 sharply split from central (Bukit Kana) and northern Sarawak (Mulu and Bario) samples in Clade B. However, since Zainuddin (1998, 2004) did not treat samples from Kalimantan, the "west-east break" applies only within Malaysian Borneo. There is a chain of high mountain ranges between Malaysian and Indonesian Borneo (see Fig. 1), which interrupts Zainuddin's (1998, 2004) "west-east break" by the Lupar gap. From the current topology, our samples from Kalimantan (Clade A1) seem to be less isolated by geographic barriers from samples from Sabah (Clade B2a) than from southwestern Sarawak samples (Clade A2). However, our result indi-

cated sister clade relationship of Clades A1 and A2, although the support was not strong. These results indicate past migrations between southwestern Sarawak and East Kalimantan populations through the lowland south of the high mountain chain, and not between populations from eastern Sabah and East Kalimantan.

Our analysis, which included more individuals from a greater geographic distribution than any previous study of Bornean *Limnectes*, supports the conclusions of Emerson et al. (2000) and Evans et al. (2003) that found little diversification among samples of *L. leporinus* they studied. Our results do not corroborate the findings of Zainuddin's (2004) who concluded that *L. leporinus* is highly diversified. This discordance may be partially explained by the choice of different genes because the CO1 mtDNA gene examined by Zainuddin (2004) evolves faster than 12S and 16S rRNA genes as exemplified by species thresholds of 3–5% for 16S rRNA vs. 10% for CO1 (Vences et al., 2005a, b; Fouquet et al., 2007; Crawford et al., 2012). However, when compared with other congeneric species, the genetic diversity of *L. leporinus* is relatively low. For example, the p-distance between two *L. "kuhlii"* from different populations of Borneo was 8.3%, which value is much larger than the maximum distance within *L. leporinus* (4.0%). Matsui et al. (2010) reported similar results, where two Bornean individuals of *L. "kuhlii"* were separated by a genetic distance much greater than that between two distinct species, *L. namiyei* (Stejneger, 1901) and *L. fujianensis*.

Our finding that genetic diversity in *L. leporinus* is relatively lower than *L. "kuhlii"* from Borneo was also reported by Zainuddin (2004). However, the problem is that *L. "kuhlii"* from Borneo actually contains many genetic lineages that are putative cryptic species (Matsui et al., 2013). Thus, the most pertinent comparison should be made between each lineage of Bornean *L. "kuhlii"* and *L. leporinus*. We predict that such a comparison would also reinforce our hypothesis that the genetic diversity within *L. leporinus* is low and likely

contains not many cryptic species.

Inger (1966) hypothesized that *L. leporinus* (as *Rana blythi*) has inhabited Borneo much longer than *L. malesianus* (as *R. macrodon*). This is because *L. leporinus* is distributed throughout Borneo and does not live in disturbed vegetation. Whereas, *L. malesianus* lives in habitats that are often geographically and often ecologically peripheral. Because of the absence of *L. malesianus* from interior forests, Inger (1966) considered that the species is probably a recent invader of Borneo, and has not had time to occupy the pristine forests unlike *L. leporinus*.

Emerson et al. (2000) estimated the clade of *Limnectes* arose in the early Tertiary (after 66.0 MYBP) at a time when free faunal exchange was possible among Southeast Asia, Borneo, Sumatra, Java, and, probably, Sulawesi. Our date estimation for the origin of Bornean *Limnectes* was 29.6 (CI 18.0–42.4) MYBP, and not discordant with Emerson et al.'s (2000) estimation, which utilized larger taxon sampling of the genus from wider regions of Southeast Asia. Our divergence time estimation indicated the origin of the *L. leporinus* clade dates back around 23.4 MYBP at the end of Miocene. The most recent common ancestor of the current clades is estimated at 4.3 MYBP in the mid Pliocene, slightly later than split of *L. malesianus* and *L. ingeri* (5.3 MYBP at the beginning of the Pliocene) and much later than the divergence of the two clades of Bornean *L. "kuhlii"* (one clade from Sabah vs. another clade from Sarawak plus *L. hikidai*: 11.4 MYBP).

The Lupar gap, as noted above was already present by this time (10–15 MYBP), and might have played an important role in separating clades between southwestern (Clade A2) and central-northern (Clade B2) Sarawak, if their common ancestor had occupied a wide range within the island (Zainuddin, 1998). However, this is not fully convincing because we estimate that genetic divergences within *L. leporinus* occurred much later (4.3 MYBP) than the formation of the gap, and seem to have not affected by its presence. Subsequent

divergences of Kalimantan (Clade A1) and southwestern Sarawak (Clade A2) soon followed at 3.4 MYBP in the mid Pliocene, much earlier than the divergence of central western Sarawak (Clade B1) and northern Sarawak and Sabah (Clade B2) at 1.7MYBP in the Pleistocene. These estimates suggest the history of *L. leporinus* is not particularly old as emphasized by Zainuddin (1998, 2004).

Because we have not studied genetic variation in *L. malesianus*, which also occurs in the Malay Peninsula, we cannot evaluate Inger's (1966) hypothesis. However, our results suggest that *L. leporinus* arose in Borneo not in very old ages, as compared with *L. "kuhlii"*, but instead experienced rapid dispersal within the island. In conclusion, *L. leporinus*, although not remarkably variable in morphology, is moderately variable genetically, and possibility of cryptic diversity is not ruled out.

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