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Phylogenetic relationships of megophryid frogs of the genus *Leptobrachium* (Amphibia, Anura) as revealed by mtDNA gene sequences

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ABSTRACT

By investigating genealogical relationships, we estimated the phylogenetic history and biogeography in the megophryid genus *Leptobrachium* (sensu lato, including *Vibrissaphora*) from southern China, Indochina, Thailand and the Sundaland. The genealogical relationships among the 30 named and unnamed taxa were estimated using 2009 bp of sequences from the mitochondrial DNA genes 12S rRNA, $tRNA^{val}$, and 16S rRNA using maximum parsimony, maximum likelihood, and Bayesian inference methods. The genus *Leptobrachium* was a well-supported monophyletic group that contained two major clades. One clade had three subclades primarily from disjunct regions including Borneo, Peninsular Malaysia and Java, and Thailand. The Bornean subclade included one species each from the Philippines and Sumatra. The other major clade consisted of two subclades, one from Indochina and the other from southern China (*Vibrissaphora*). Divergence times estimated an old evolutionary history of each subclade, one that could not be explained by the geohistory of Southeast Asian major landmasses.

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1. Introduction

The megophryid genus *Leptobrachium* Tschudi, 1838 (type species *L. hasseltii* from Java) is diagnosed by having a broad head and thin limbs (Dubois and Ohler, 1998). The genus is often considered to contain two subgenera, *Vibrissaphora* Liu, 1945, with adult males bearing spines on upper lip, and *Leptobrachium*, which is without such spines (Ohler et al., 2004). *Vibrissaphora* is distributed in southern China and Indochina, and it includes five to seven species (Frost, 2009; Rao and Wilkinson, 2008; Fei et al., 2009). Subgenus *Leptobrachium* is distributed from Indochina to Sundaland and it contains 15–17 species (Frost, 2009; Fei et al., 2009). Using DNA sequence data, both Zheng et al. (2008) and Rao and Wilkinson (2008) place *Vibrissaphora* within the genus *Leptobrachium*, and neither group of researchers recognizes subgenera. However, this taxonomic conclusion is made without exploring the extent of genetic diversity within Sundaland and neither study includes the type species of the genus.

Sundaland contains the Malay Peninsula and the Indonesian islands of Sumatra, Java, Bali, Borneo, and smaller islands west of the Makassar and Lombok straits. All of these areas are linked by the shallow-water (<200 m) Sunda Shelf, which was exposed during periods of low sea level in the Pleistocene. The eastern boundary of Sundaland is Wallace's Line where the Indomalayan and Australasian faunas meet. Studies of some anuran lineages from Sundaland indicate that species distributions and phylogenies are strongly related to the geological history of this region (e.g., Emerson et al., 2000; Inger and Voris, 2001; Brown and Guttman, 2002; Matsui et al., 2010).

Seven species of *Leptobrachium* are known from Sundaland and peninsular Thailand as follows: *L. hasseltii* Tschudi, 1838; *L. montanum* Fischer, 1885; *L. abbotti* (Cochran, 1926); *L. hendricksoni* Taylor, 1962; *L. nigrops* Berry and Hendrickson, 1963; *L. gunungense*

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Malkmus, 1996, and *L. smithi* Matsui, Nabhitabhata, and Panha, 1999. Among these species, *L. montanum, L. abbotti*, and *L. gunungense* are endemic to Borneo and *L. smithi* is mainly distributed in Thailand and the northern part of Peninsular Malaysia, as well as through Myanmar to Assam, India (Sengupta et al., 2001). *Leptobrachium hendricksoni* is distributed from southern Thailand through the Malay Peninsula to western Borneo and Sumatra, and *L. nigrops* occurs on the Malay Peninsula, Belitung Island, and western Borneo. How and when they obtained their present-day distributions in the Sunda Islands are biogeographically interesting topics.

In *Leptobrachium*, taxonomic problems span from the supraspecific category to the species level. The name *L. hasseltii* was applied to many Southeast Asian populations (Inger, 1954, 1966; Berry, 1975) until Inger et al. (1995) clarified that *L. hasseltii* from Borneo was not conspecific with the Javanese population. They applied the names *L. montanum* and *L. abbotti* to Bornean species. However, some populations, such as those from the Philippines, are still referred to as *L. hasseltii* because of the absence of a taxonomic reassessment. Iskandar (1998) and Matsui et al. (1999) suggested that true *L. hasseltii* was likely restricted to Java and adjacent islands, and Dubois and Ohler (1998) suggested the possible occurrence of more than one species of *Leptobrachium* on Java, based on a large extent of variation in female body size of *Leptobrachium* (sensu lato). Such taxonomic arguments still need to be clarified.

These uncertainties mainly derive from the use of a few specimens from limited ranges. Intra- and interspecific variation is inadequately assessed. Some critical characteristics useful for species identification are lost upon preservation of museum vouchers. Molecular data and phylogenetic analyses can enable the identification of species and a higher level taxonomy in *Leptobrachium*. In this study, we use mtDNA gene sequences from various populations of *Leptobrachium*, especially from Sundaland, to evaluate the taxonomic status of many populations and hypothesize the evolutionary history of the species in this genus.

2. Materials and methods

2.1. Sampling design

We examined a total of 80 partial DNA sequences of the mitochondrial DNA genes 12S rRNA, $tRNA^{val}$, and 16S rRNA from 30 species/subspecies of the genus *Leptobrachium* (sensu lato) and six outgroup species (Figs. 1 and 2 and Table 1). Our own sampling of *Leptobrachium* consisted of 60 specimens of 21 taxa mainly obtained from Sundaland and Indochina, including several undescribed or unidentified taxa including the following: *Leptobrachium* sp. 1, a Sumatran species that has distinct eye color (Hamidy and Matsui, 2010); *Leptobrachium* sp. 2, a Philippine species often called *L. hasseltii*; *Leptobrachium* sp. 3 from Gwa, Myanmar, catalogued as *L. hasseltii* (Zheng et al., 2008); *Leptobrachium* sp. 4 from Pilok, Thailand, a dried carcass whose identification was difficult; and *Leptobrachium* sp. 5, which resembles *Vibrissaphora* but lacks male labial spines. We used *Leptolalax heteropus* (Boulenger, 1900) and *Pelodytes punctatus* (Daudin, 1802) as outgroup taxa. DNA sequences from GenBank were obtained for 11 Indochinese and Chinese species of *Leptobrachium* (sensu lato) and the following four outgroup species: *Oreolalax rhodostigmatus* Hu and Fei in Liu, Hu, and Fei, 1979 (EF397248: Fu et al., 2007), *Megophrys nasuta* (Schlegel, 1858) (DQ283342: Frost et al., 2006), *Pelobates fuscus* (Laurenti, 1768) (DQ283113: Frost et al., 2006), and *Scaphiopus holbrookii* (Harlan, 1835) (DQ283156: Frost et al., 2006). *Leptolalax* Dubois, 1980 and *Oreolalax* Myers and Leviton, 1962 are the sister group of *Leptobrachium* (Lathrop, 1997; Zheng et al., 2004, 2008; Fu et al., 2007), whereas *Pelobates* Wagler, 1830 (Pelobatidae Bonaparte, 1850), *Pelodytes* Bonaparte, 1838 (Pelodytidae Bonaparte, 1850), and *Scaphiopus* Holbrook, 1836 (Scaphiopodidae Cope, 1865) form a monophyletic sister group with Megophryidae (García-París et al., 2003; Dubois, 2005).

Voucher specimens/tissues are stored in ABTC (Australian Biological Tissue Collection, South Australian Museum), BOR (BORNEENSIS collection, University Malaysia Sabah), IEBR (Institute of Ecology and Biological Resources, Hanoi, Vietnam), KUHE (Kyoto University, Graduate School of Human and Environmental Studies), MDK (Department of Conservation and Ecotourism, Faculty of Forestry, Bogor Agricultural Institute), MNHN (Museum National d'Histoire Naturelle, Paris), MZB (Museum Zoologicum Bogoriense), ROM (Royal Ontario Museum), SP (Sabah Parks), UKM (University Kebangsaan Malaysia), UM (University Malaya), and UTA (Department of Biology, University Texas at Arlington).

2.2. Preparation of DNA, PCR and DNA sequencing

We obtained tissues from frozen or ethanol (95–99%) preserved specimens and extracted total genomic DNA using standard Phenol–chloroform extraction procedure (Hillis et al., 1996). We homogenized tissues in 0.6 ml STE buffer containing 10 mM Tris/HCl, pH 8.0, 100 mM NaCl and 1 mM EDTA, pH 8.0. We added Proteinase K (0.1 mg/ml) to the homogenate solutions and digested proteins for 4–12 h at 55 °C. The solution was treated with phenol and chloroform/isoamyl alcohol and DNA was precipitated with ethanol. DNA precipitates were dried and then resuspended in 0.6 ml TE (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and 1 μl was subjected to polymerase chain reaction (PCR). The PCR cycle included an initial denaturation step of 5 min at 94 °C and 33 cycles of denaturation for 30 s at 94 °C, primer annealing for 30 s at 48–50 °C, and extension for 1 min 30 s at 72 °C. Primers used in PCR are shown in Table 2. The PCR products purified using polyethylene glycol (PEG, 13%) precipitation procedures were used directly as templates for Cycle Sequencing Reactions with fluorescent-dye-labeled terminator (ABI Big Dye Terminators v.3.1 cycle sequencing kit). The sequencing reaction products were purified by ethanol precipitation following the manufacture's protocol and then run on an ABI PRISM 3130 genetic analyzer. All samples were sequenced in both directions using the same primers as for PCR.

2.3. Phylogenetic analysis

Aligned, concatenated sequences of 12S rRNA, $tRNA^{val}$, and 16S rRNA yielded a total 2009 bp positions. We used Chromas Pro software (Technelysium Pty Ltd., Tewntin, Australia) to edit the sequences, and align them using the ClustalW option of Bioedit (Hall, 1999). The initial alignments were then checked by eye and adjusted slightly. Phylogenetic trees were constructed using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI). MP trees, obtained using PAUP*4.0b10 (Swofford, 2002), involved a heuristic search with the tree bisection recognition (TBR) branch-swapping algorithm, and 100 random additions replicates. Transitions and transversions were equally weighted, and gaps were treated as missing data. ML analysis were performed by Treefinder version June 2007 (Jobb et al., 2004), with the general time-reversible (GTR) model of DNA evolution with a gamma shape parameter (G), identified as the best-fitting model under the Akaike information criterion implemented in Kakusan 3 (Tanabe, 2007). BI and Bayesian posterior probabilities (BPP) were estimated using MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001), under the GTR model with G and proportion of invariable sites (I), selected by MrModeltest2.2 (Nylander, 2004). BI used four simultaneous Metropolis coupled Monte Carlo Markov chains for 6,000,000 generations. We sampled a tree every 100 generations

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