PHYLOGENETIC RELATIONSHIPS OF *LEPTOBRACHIUM HASSELTII* TSCHUDI, 1838 (AMPHIBIA, ANURA, MEGOPHRYIDAE) - DETECTION OF A POSSIBLE CRYPTIC SPECIES

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ABSTRACT

By examining mitochondrial DNA phylogeny using 2424bp of sequence data 12S rRNA, $tRNA^{val}$, and 16S rRNA genes, we evaluated the taxonomic relationships among Javan litter frogs *Leptobrachium hasseltii* from southern Sumatra, Java, and Bali. *Leptobrachium hasseltii* formed a well-supported monophyletic group, which comprised two major clades. One major clade represented the southern Sumatran and Javan populations and the other consisted of the population from Bali. The Javan and southern Sumatran clade included two subclades: the West Javan-southern Sumatran group and the Central Javan group. The genetic divergence between the two major clades (Bali vs. Java-Sumatra) suggested their separation happen at species level. Further studies using morphological and acoustic data are needed to determine the taxonomic status of Bali population.

Key words: Bali, Java, *Leptobrachium*, mitochondrial DNA, Sumatra

INTRODUCTION

The genus *Leptobrachium* was proposed by Tschudi in 1838 with type species *L. hasseltii* from Java. The genus consists of 35 species distributed from India, southern China, Indochina to Sundaland and Philippines (Frost 2016). Twelve species of *Leptobrachium* are known from Sundaland and peninsular Thailand: *L. hasseltii* Tschudi, 1838; *L. montanum* Fischer, 1885; *L. abbotti* Cochran, 1926; *L. hendricksoni* Taylor, 1962; *L. nigrops* Berry & Hendrickson, 1963; *L. gunungense* Malkmus, 1996; *L. smithi* Matsui, Nabhitabhata & Panha, 1999; *L. waysepuntiense* Hamidy & Matsui, 2010; *L. ingeri* Hamidy, Matsui, Nishikawa & Belabut, 2012; *L. kanowitense* Hamidy, Matsui, Nishikawa & Belabut, 2012; and *L. kantonishikawai* Hamidy & Matsui, 2014. The name *L. hasseltii* was once applied to many Southeast Asian populations (Inger 1954, Taylor 1962, Inger 1966, Berry 1975), although subsequent studies split the populations from Malay Peninsula and Singapore into two distinct taxa: *L. hendricksoni* (Taylor 1962) and *L. nigrops* (Berry & Hendrickson 1963). Another study by Inger *et al.* (1995) also clarified that *L. hasseltii* from Borneo was not conspecific with the Javan population. More recent taxonomic works restricted *L. hasseltii* to Java (Iskandar 1998, Matsui *et al*. 1999, Brown *et al*. 2009). However, further studies involving more samples proved that *L. hasseltii* is not only restricted to Java Island, but also occurs in the southern part of Sumatra (Matsui *et al.* 2010a, Hamidy & Matsui 2010).

Dubois & Ohler (1998) found a large extent of variation in the female body size among the Javan population of *Leptobrachium*, and suggested possible occurrence of more than one species on the island. Such taxonomic argument need clarification through molecular studies. The occurrence of *L. hasseltii* on Bali Island was first reported by Iskandar (1998) without any information on voucher specimens. MacKay (2006) showed the photographs of this species in life from Gunung Batu Karu, Bali, where we collected two tadpoles in 2010. Our morphological examination of the tadpoles showed them to be identical with the larval *L. hasseltii* from Java. To confirm this identification and to evaluate taxonomic relationship of the Bali population, we then performed a molecular study using samples of *L. hasseltii* representing the populations from its distribution area.

MATERIALS AND METHODS

Sampling design

We examined a total of 18 partial sequence data of the mitochondrial DNA genes 12S rRNA, $tRNA^{val}$, and 16S rRNA of *Leptobrachium hasseltii*, representing populations from Sumatra, Java, and Bali, and four outgroup species (*L. chapaense, L. smithi, L. nigrops* and *L. hendricksoni*; Table 1). Specimens were collected from southern Sumatra, West Java, and Bali (Fig. 1). Voucher specimens and/ or tissues are stored in Museum Zoologicum Bogoriense (MZB), Research Center for Biology-Indonesian Institute of Sciences (LIPI); Graduate School of Human and Environmental Studies, Kyoto University (KUHE); Zoological Reference Collection, Department of Zoology, National University of Singapore (ZRC); Department of Biology, University of Texas at Arlington (UTA); and Naturalis Biodiversity Center, Leiden Museum (RMNH).

Figure 1. Map of Java, southern Sumatra, and Bali showing sampling localities of *L. hasseltii* used in this study. Sample numbers are included in Table 1. (Map modified from Goggle earth, January 23, 2017).

Table 1. Sample of L. hasseltii and outgroup species used for mtDNA analysis in this study together with the information on voucher, collection locality and GenBank accession numbers

Samples	Species	Voucher	Locality	Gene Bank	Reference
1	*L. chapaense*	KUHE 19122	Thailand, Doi Intanon, Ban Khun Klang	AB530444, AB646428	Matsui *et al*. 2010, Hamidy *et al*. 2011
2	*L. smithi*	KUHE 19834	Thailand, Mae Hong Son, Phasua WF	AB530434	Matsui *et al*. 2010
3	*L. nigrops*	ZRC L SG002	Singapore, Seletar	AB719239	Matsui *et al*. 2010
4	*L. hendricksoni*	KUHE 15680	Malaysia, Peninsula, Selangor, Kuala Lumpur	AB530417	Matsui *et al*. 2010
5	*L. hasseltii*	KUHE 42807	Indonesia, Southern Sumatra, Lampung, Liwa, Kubu Perahu	AB530419	Matsui *et al*. 2010
6	*L. hasseltii*	KUHE 42808	Indonesia, Southern Sumatra, Lampung, Liwa, Kubu Perahu	AB530420	Matsui *et al*. 2010
7	*L. hasseltii*	KUHE 42809	Indonesia, Southern Sumatra, Lampung, Liwa, Kubu Perahu	LC215910	This study
8	*L. hasseltii*	MZB UN tissue	Indonesia, West Java, Gede-Pangrango National Park	AB530421	Matsui *et al*. 2010
9	*L. hasseltii*	MZB Amph 23766	Indonesia, West Java, Gede-Pangrango National Park	LC215911	This study
10	*L. hasseltii*	UTA A 53688	Indonesia, West Java, Bogor, Cisarua Safari Park	AB530422	Matsui *et al*. 2010
11	*L. hasseltii*	KUHE 42818	Indonesia, Southern Central Java, Purworejo, Kaligesing	AB530423	Matsui *et al*. 2010
12	*L. hasseltii*	KUHE 42820	Indonesia, Southern Central Java, Kulon Progo, Kiskendo	AB530424	Matsui *et al*. 2010
13	*L. hasseltii*	MZB Amph 14517	Indonesia, Southern Central Java, Kulon Progo, Kiskendo	LC215912	This study
14	*L. hasseltii*	KUHE 44535	Indonesia, Northern Central Java, Mt. Ungaran	AB646408	Matsui *et al*. 2010
15	*L. hasseltii*	MZB Amph 26904	Indonesia, Northern Central Java, Mt. Ungaran	LC215913	This study
16	*L. hasseltii*	MZB Amph 26905	Indonesia, Northern Central Java, Mt. Ungaran	LC215914	This study
17	*L. hasseltii*	MZB UN L01	Indonesia, Bali, Batu Karu	LC215915	This study
18	*L. hasseltii*	MZB UN L02	Indonesia, Bali, Batu Karu	LC215916	This study

Preparation of DNA, PCR and DNA sequencing

We obtained tissues from ethanol (95–99%) preserved specimens and extracted total genomic DNA using standard Phenol-Chloroform extraction procedure (Hillis *et al.* 1996). We homogenised tissues in 0.6 ml STE buffer containing 10 mM Tris/HCl, pH 8.0, 100 m MNaCl and 1 mM EDTA, pH 8.0. We added Proteinase K (0.1 mg/ml) to the homogenate solutions and digested proteins for 4 to 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 mMTris/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 sec at 94°C, primer annealing for 30 sec at 48–50 °C, and extension for 1 min 30 sec 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 were then run on an ABI PRISM 3130 genetic analyser. All samples were sequenced in both directions using the same primers as for PCR.

Phylogenetic analysis

Aligned, concatenated sequences of 12S rRNA, $tRNA^{val}$, and 16S rRNA yielded a total 2424 nucleotide sites. We used Chromas Prosoftware (Technelysium Pty Ltd., Tewntin, Australia) to edit the sequences, and align them using the ClustalX option of Bioedit (Hall 1999). The initial alignments were then checked by eye and adjusted slightly. Phylogenetic trees were constructed using neighbour joining (NJ), maximum likelihood (ML), and Bayesian inference (BI). ML analysis were performed by MEGA 7 (Kumar *et al.* 2011), with the general time-reversible (GTR) model of DNA evolution with a gamma shape parameter (G), which were identified as the best-fitting model under the Akaike information criterion implemented in MEGA 7 (Kumar *et al*. 2011). BI and Bayesian posterior probabilities (BPP) were estimated using MrBayes 3.2.6 (Huelsenbeck & Ronquist 2001). The best evolution model was selected by Kakusan 3 (Tanabe 2007). The best evolution models for 12S and 16S rRNA genes were the GTR model with G, and SYM Gamma for $tRNA^{val}$ gene. BI used four simultaneous Metropolis coupled Monte Carlo Markov chains for 6,000,000 generations. We sampled a tree every 1000 generations and calculated a consensus topology for 30,001 trees after discarding the first 30,000 trees (burn-in=3,000,000).

Table 2. Primers used in this study

Target	Primer	Sequence (5'-3')	Reference
12S rRNA and tRNA^{val}	12Sh	AAAGGTTTGGTCCTAGCCTT	Canatella *et al.* (1998)
	12SA-L	AAACTGGGATTAGATACCCCACTAT	Palumbi *et al.* (1991)
	L1507	TACACACCGCCCGTCACCCTCTT	Matsui *et al.* (2010b)
	12SFLeptobrachium	CCGCCAAGTCCTTTGGGTTT	Modified from Goebel *et al.* (1999)
	H1548	TACCATGTTACGACTTTCCTCTTCT	Matsui *et al.* (2005)
16S rRNA	L1879	CGTACCTTTTGCATCATGGTC	Matsui *et al.* (2010b)
	16sl2021	CCTACCGAGCTTAGTAATAGCTGGTT	Tominaga *et al.* (2006)
	16L-1	CTGACCGTGCAAAGGTAGCGTAATCACT	Hedges (1994)
	H1923	AAGTAGCTCGCTTAGTTTCGG	Matsui *et al.* (2010b)
	H2315Leptobrachium	TCGTTGTTACTAGTYCTAACAT	Matsui *et al.* (2010b)
	16sh2715	AAGCTCCATAGGGTCTTCTCGTC	Tominaga *et al.* (2006)
	16H1	CTCCGGTCTGAACTCAGATCACGTAGG	Hedges (1994)

Strength of nodal support in the NJ and ML analyses used non-parametric bootstrapping (MPBS; Felsenstein 1985) with 1,000 replicates (NJBS and MLBS). A priori, we regarded tree nodes with bootstrap value 70% or greater as sufficiently resolved (Huelsenbeck & Hillis 1993), and those between 50 to 70% as tendencies. In the BI analysis, nodes with a BPP of 95% or greater were considered significant (Leaché & Reeder 2002).

We also estimated the genetic distance (uncorrected p-distance) among population of *L. hasseltii* from about 1.4 kbp of mitochondrial 16S rRNA gene using MEGA 7 (Kumar *et al.* 2011).

RESULTS

Sequence and statistics

Sequence statistics for the three gene fragments and for the combined alignment including all nucleotide positions are provided in Table 3. The aligned 12S rRNA, $tRNA^{val}$, and 16S rRNA data set consisted of 2,424 characters, in which 925 sites were variable and 800 potentially phylogenetically informative. The ML analysis produced a topology with l nL -8564.271 (gamma shape parameter=0.290; nucleotide frequencies: A=0.345, C=0.216, G=0.169, and T=0.271). BI calculated average parameter estimates for nucleotide frequencies in each gene: for 12S rRNA, A=0.321, C=0.233, G=0.183, T=0.263, and a gamma shape parameter 0.316; for 16S rRNA, A=0.361, C=0.203, G=0.159, T=0.277, and a gamma shape parameter 0.285; and for $tRNA^{val}$, A=0.324, C=0.258, G=0.176, T=0.241, and a gamma shape parameter 0.764.

Table 3. The Genetic distance (% p-uncorrected) among five population of *L. hasseltii* and outgroup members

No	Species	1	2	3	4	5	6	7	8	9
1	*L. chapaense*									
2	*L. smithi*	18.7								
3	*L. nigrops*	19.5	17.6							
4	*L. hendricksoni*	17.6	14.8	14.7						
5	Southern Central Java	15.9–16.0	14.2–14.3	13.5–13.7	10.2–10.4	0.1–0.4				
6	Northern Central Java	15.7–15.8	14.2–14.5	13.9–14.0	10.9–11.1	1.3–1.8	0.1			
7	Southern Sumatra	16.1–16.4	14.0–14.3	13.7–13.9	10.7–11.0	1.8–2.3	2.6–3.1	0.3–0.6		
8	West Java	16.0–16.2	14.0–14.3	13.3–13.4	10.4–10.4	1.9–2.5	2.4–2.8	1.8–2.6	0.6–0.9	
9	Bali	16.7–16.7	14.2–14.2	13.5–13.5	10.6–10.6	3.2–3.4	3.8–4.0	3.2–3.6	3.0–3.3	0.1

Phylogenetic relationships

All analyses resulted in essentially the same topologies, which differed only in associations at poorly supported nodes. The Bayesian tree (Fig. 2) infers the following sets of relationships:

(i) Monophyly of *L. hasseltii* with respect to *L. hendricksoni, L. nigrops, L. smithi* and *L. chapaense* was supported in all trees (MPBS=71%, MLBS=78%, BPP=0.98).

(ii) The clade of *L.hasseltii* was divided into two basal subclades, Javan-Sumatran subclade (MPBS=78%, MLBS=93%, BPP=1.00) and Bali subclade (all support values = 100%).

(iii) Javan-Sumatran subclade contained two monophyletic groups: one group consisted of population from central Java (all values 100%) and another encompassing populations from West Java and southern Sumatra (MPBS=76%, MLBS=100%, BPP=100%).

(iv) The uncorrected p-distances among groups and subclades are shown in Table 2. The Bali subclade diverged from the other subclade (Java and Sumatra) with large genetic distances of 3.0–4%. Intra population genetic distance was low within the Bali subclade (0.1%) and the Sumatra population (0.3–0.4%). In contrast, slightly larger genetic distances, ranging from 0.1% to 2.8% were observed within Java, and samples from West Java was genetically closer to Sumatran samples (1.8–2.6%) than to samples from Central Java (1.9–2.8%).

DISCUSSION

Leptobrachium hasseltii, the type species of the genus, was named after Johan Conrad van Hasselt (1797–1823), a Dutch naturalist who greatly contributed to scientific collections on Java. The type specimens of *L. hasseltii* (RMNH 2015, lectotype & RMNH 2014, paralectotype; Fig. 3) were collected by H. Boie and H. Macklot from Java, without any specific locality data. Some localities in western Java such as southern Buitenzorg (now known as Bogor), Mt. Gede, and Mt. Salak were important collection sites during Dutch period (Cluver 2007). One of our samples from Mt. Gede (Fig. 4) possibly represents atopotype of the species.

The level of genetic divergence as shown by genetic distances was suggested to be useful in detecting cryptic species within the genus *Leptobrachium* from Sundaland (*e.g.* Matsui *et al.* 2010, Hamidy *et al.* 2011) and the Philippines (*e.g.* Brown *et al.* 2009). Fouquet *et al.* (2007) suggested the uncorrected p-distance in 16S rRNA of 3% could indicate different species status of two compared taxa. This idea was roughly concurred with Matsui *et al.* (2010) and Hamidy *et al.* (2011), where two Bornean species (*L. montanum* and *L. abbotii*), which distinctly could be differentiated in morphology and ecology, exhibited p-distances ranging from 2.4 to 3.2%. Given uncorrected p-distance of 3% in 16S rRNA as a measure of species differentiation, the Bali

population with the distances of 3.0–4.0% can be considered a different species from *L. hasseltii* from Java and Sumatra. In contrast, our results strongly indicated that the Java and Sumatra populations are conspecific with genetic distances smaller than the threshold. This also indicated that the possibility proposed by Ohler & Dubois (1998) of the occurrence of more than one species of *L. hasseltii* in Java was small, although further sampling from wider areas including East Java would still be necessary to settle down this problem.

Matsui *et al.* (2010) estimated that the sister species of *L. hasseltii* was *L. hendricksoni* which diverged at 18.8 (CI=11.0-27.2) MYBP, and that diversification within *L. hasseltii* began at 2.8 (1.4-4.5) MYBP. Unfortunately, the Bali population was not included in that study, but our present result suggested that the common ancestor of *L. hasseltii* first diverged into the ancestor of the present Bali population and the ancestral Javan-Sumatran population. Then, the ancestral East Javan population diverged from the ancestor of Sumatran-West Javan populations, which are now split by the Sunda Strait.

According to Rutherford *et al.* (2001), Bali emerged since ~11 MYBP, while the West and East Java were still separated islands, but re-emerged from the Java Sea between 10 to 5 Ma, and coalesced to form the current island shape only quite recently (Hall 2012, 2013). Furthermore, the Sunda Strait started opening before 2 Ma (Nishimura *et al*. 1992) which acted as an effective barrier for dispersal between West Java and Sumatra. Although these regions were submerged and below sea level, the ancestral *L. hasseltii* should have expanded its range from southern Sumatra eastward to Bali not much later than its appearance, then isolated to three regions, i.e., Bali, Central-East Java, and West Java and Sumatra, probably strongly affected by Javan volcanic activities in early period.

In addition to historical vicariance events, artificial environmental changes seemed to be also responsible for regional isolation of *L. hasseltii* within Java. *Leptobrachium hasseltii* occuring at altitudes ranging from 300 to 1500 m a.s.l. In Sumatra, this species was found in 300 m a.s.l., whereas in Java it was mostly found in the mountain regions up to 1500 m a.s.l. This altitudinal distribution might have been secondarily acquired. Within Java, most lowland forests have disappeared through human activities. Only a few mountain forests remain today forming isolated habitat islands without corridors among populations of *L. hasseltii*. This condition probably prevented the gene flow among populations of *L. hasseltii* within Java. This held for many animal taxa, not only amphibians but also reptiles and mammals in Java, recalling the species conservation by conserving the habitat that provide corridor among isolated populations.

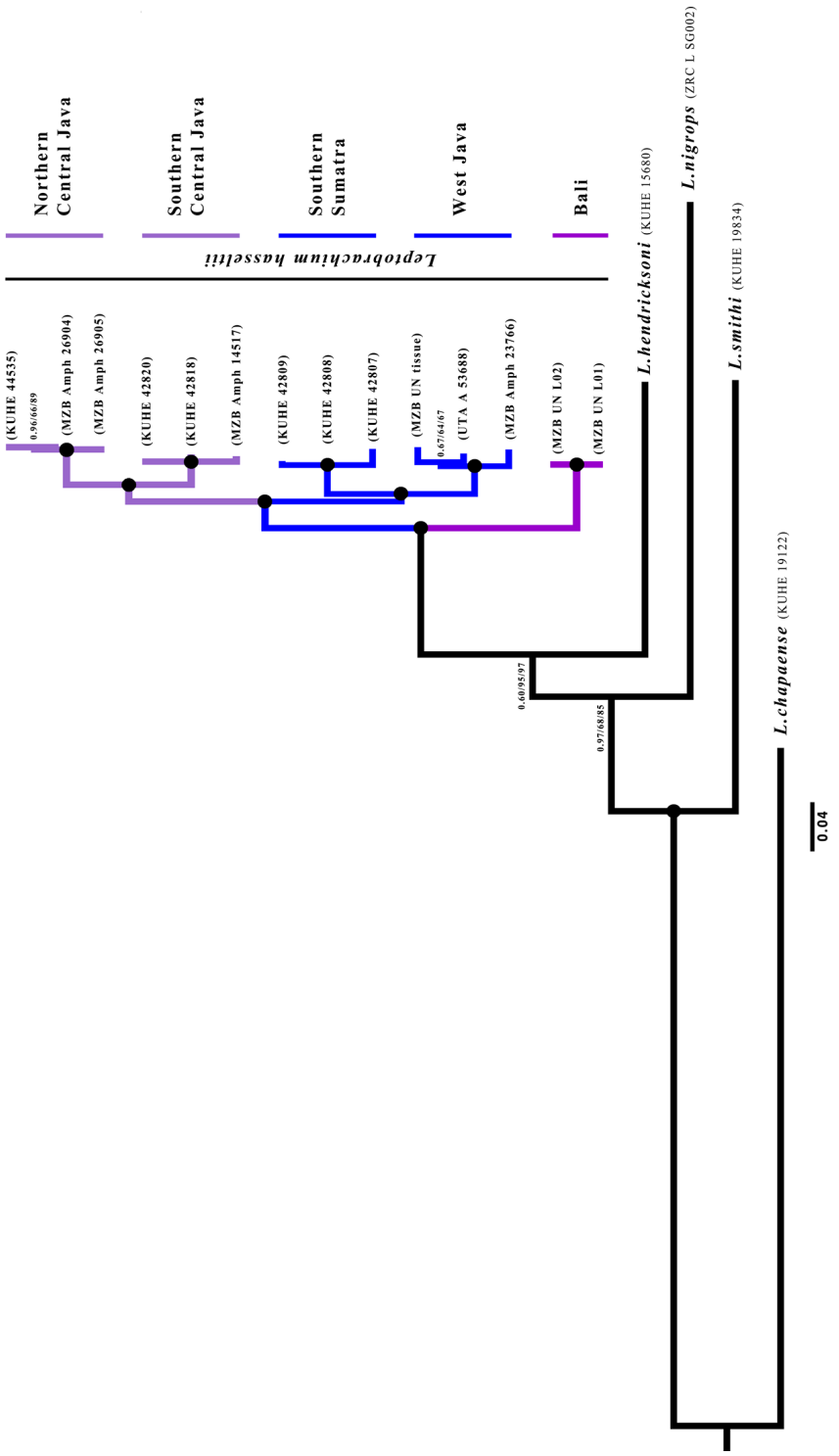

Figure 2. The Bayesian phylogram of 2424 bp of 12S rRNA, tRNA^{Val}, and 16S rRNA mitochondrial genes for samples of *L. hasseltii* and outgroup species. Numbers above branches represent bootstrap supports for NJ/ML/and Bayesian Inferences (BI). Closed circles indicate nodes with significant bootstrap supports for NJ and ML (>70%) inferences and Bayesian Inference (BI>95%).

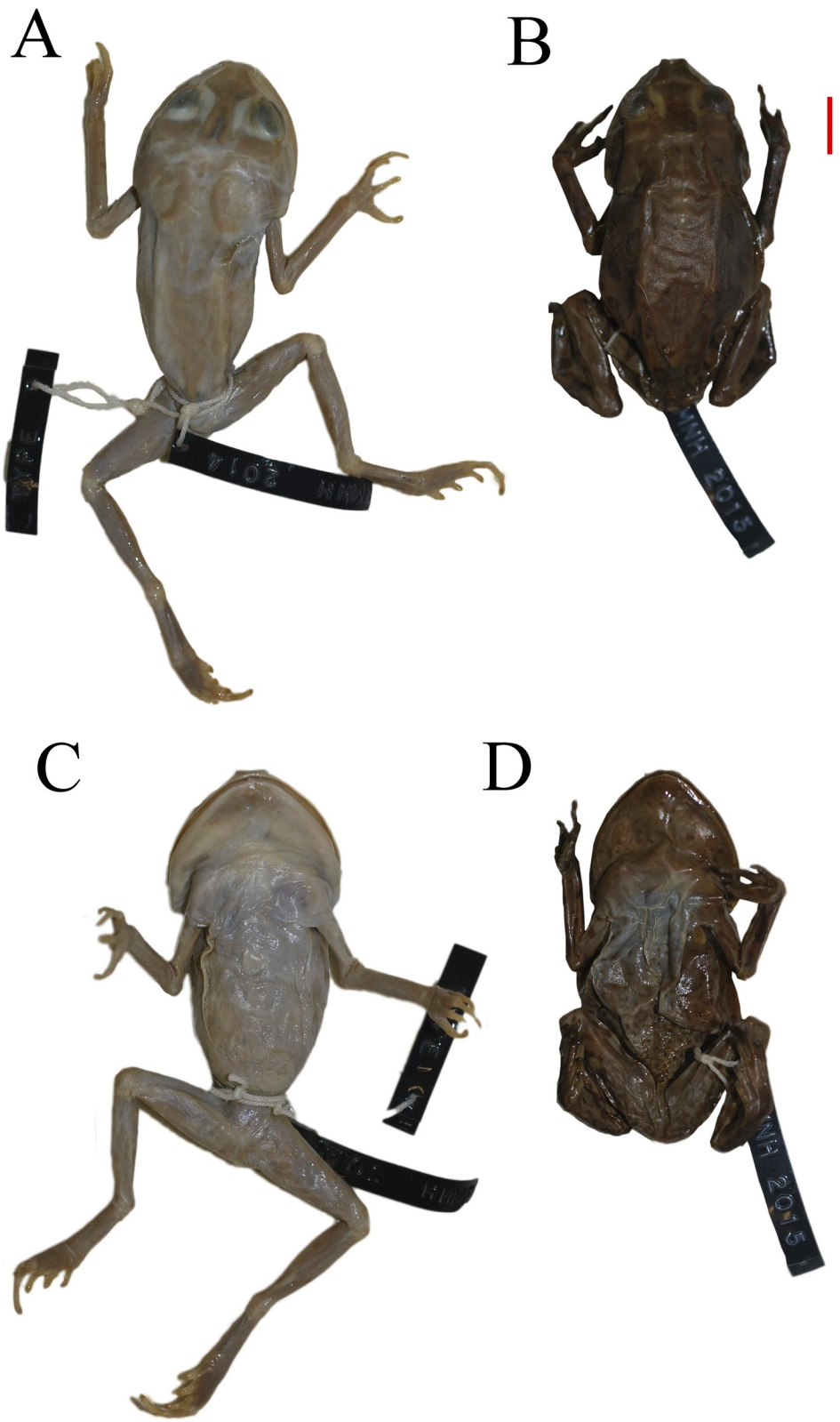

Figure 3. Dorsal (A, B) and ventral (C, D) views of the type specimens of *L. hasseltii* (A, C: RMNH 2014, paralectotype; B, D: RMNH 2015, lectotype).

Figure 4. *Leptobrachium hasseltii* from Mt. Gede (MZB Amph 23766) in life.

In Bali, *L. hasseltii* has been found on Batu Karu at the elevation about 800 m a.s.l. The tadpoles were collected in a rocky stream covered by trees in its surrounding forest. This type of habitat would be suitable both for adults and tadpoles. Further morphological and acoustic studies of Bali population are needed to determine its taxonomic status.

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