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Distribution extension of *Leptobrachella eos* (Ohler, Wollenberg, Grosjean, Hendrix, Vences, Ziegler & Dubois, 2011): first record from Thailand

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http://zoobank.org/0BC99BE4-0F4A-48B9-98E7-2ED606C04C8F

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Abstract

We report the first country record of *Leptobrachella eos* (Ohler, Wollenberg, Grosjean, Hendrix, Vences, Ziegler & Dubois, 2011) from Thailand, based on one specimen collected from Chom poo Phuka nature trail, Bo Kluea District. Morphologically, the specimen displayed good agreement with the original descriptions provided for *L. eos*. Phylogenetically, the specimens clustered according to the sequences of type locality of *L. eos*. Notably, our discovery increases the number of *Leptobrachella* species known to occur in Thailand to nine.

Key Words

Amphibia, *Leptobrachella eos*, new record, Thailand

Introduction

Thailand is an important component of the Indo-Burma biodiversity hotspot and its northern region is located in the same zoogeographic region where the faunas of China, Indochina, India and Himalaya converge (Myers et al. 2000). The amphibian fauna in Thailand is rich in terms of species count and endemism (Frost 2021). In recent years, several new and new record species of amphibians have been described (e.g. Matsui et al. 2018; Wu et al. 2019; Suwannapoom et al. 2021). These results suggest that the rich amphibian diversity in the region remains underestimated.

The genus *Leptobrachella* was originally described by Smith (1925) and *Leptobrachella mjöbergi* Smith from Sarawak (Malaysia) was designated as the type species. Dubois (1980) erected a new genus *Leptolalax* with the type species *Leptobrachium gracile* Günther. However, Chen et al. (2018) suggested that *Leptolalax* was a junior synonym of the genus *Leptobrachella* based on a large-scale molecular analysis. Currently, frogs of the genus *Leptobrachella* are renowned for their extraordinary diversity in small-sized, forest-floor specialists that inhabit montane evergreen forest throughout Oriental Zoogeographic Region (Chen et al. 2018; Frost 2021). This

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genus is comprised of 89 currently recognized species (Frost 2021). Among these, more than 70% of recognized species were described in the last two decades (Rowley et al. 2016; Yuan et al. 2017; Chen et al. 2020; Nguyen et al. 2021), which is attributed to intensified survey efforts and use of more integrative taxonomic approaches.

Rosy Litter Frog *L. eos* was recently described by Ohler et al. (2011) from Long Nai, Phongsaly Province, Laos. The species has since been reported from Dien Bien, Thanh Hoa, and Son La Provinces, northwestern Vietnam and in adjacent Yunnan, China (Pham et al. 2014; Chen et al. 2018; Nguyen et al. 2020).

During fieldwork in 2018, we collected a specimen in Nan Province of northern Thailand that can morphologically be assigned to the genus *Leptobrachella*. Subsequent studies based on morphological and molecular data indicate that the specimen should be classified as *L. eos*. Herein, we have reported on one Asian leaf-litter frogs, namely *L. eos*, for the first time from Thailand.

Materials and methods

Field surveys were conducted in Chom poo Phuka nature trail, Nan Province, Bo Kluea District, Thailand (Fig. 1). The specimen was collected and euthanized with ethyl acetate and then fixed in 75% ethanol for storage after taking photographs. Liver tissue sample was preserved in absolute ethyl alcohol for molecular analysis. The specimen and tissue sample were deposited in the herpetological collections of the School of Agriculture and Natural Resources, University of Phayao (AUP), Phayao, Thailand.

Total genomic DNA was extracted from tissue sample using a standard phenol-chloroform extraction protocol (Sambrook et al. 1989). The mitochondrial gene 16S ribosomal RNA gene (16S rRNA) was amplified and sequenced using the primer pairs 16S rRNA-F (CGCCTGTTTAYCAAAAACAT) and 16S rRNAR (CCGGTYTGAACTCAGATCAYGT) (Kocher et al. 1989). PCR amplification was performed in a 25 µl reaction volume with the following cycling conditions: an initial denaturing step at 95 °C for 5 min; 35 cycles of denaturing at 95 °C for 40 s, annealing at 55 °C for 40 s and extending at 72 °C for 1 min, followed by a final extension step of 72 °C for 10 min. The amplified PCR product was purified using Qiagen PCR purification kit and sequences were obtained from an ABI 3100 automated sequencer. The sequence was deposited in GenBank under accession number (GenBank accession number is available in Table 1).

The newly obtained nucleotide sequence was first assembled and edited using AutoSeqMan (Sun 2018). New sequences incorporated with homologous data downloaded from GenBank were aligned using MUSCLE 3.8 with default settings (Edgar 2004), and then visually checked by eye for accuracy and trimmed to minimize missing characters in MEGA6 (Tamura et al. 2013).

Figure 1. Map showing the new record in Thailand (red circle) and the type locality of *L. eos* (red star) in Laos.

Table 1. Localities, voucher ID, and GenBank numbers for all samples used in this study.

Species	Voucher ID	Locality	GenBank Accession	Reference
Leptobrachella eos	ROM 14406	Con Cuong, Nghe An, Vietnam	MH055883	Chen et al. 2018
Leptobrachella eos	ZMMU-NAP-04873	Con Cuong, Nghe An, Vietnam	MH055884	Chen et al. 2018
Leptobrachella eos	ZMMU-NAP-05037	Kim Son, Nghe An, Vietnam	MH055885	Chen et al. 2018
Leptobrachella eos	ZMMU-NAP-02279	Thuong Xuan, Thanh Hoa, Vietnam	MH055886	Chen et al. 2018
Leptobrachella eos	IEBR ADPH067	Pu Hu Nature Reserve, Thanh Hoa, Vietnam	MH055882	Chen et al. 2018
Leptobrachella eos	NCSM 77714	Phoukhoune, Luang Prabang, Laos	MH055879	Chen et al. 2018
Leptobrachella eos	ZMMU-NAP-02278	Vientiane, Laos	MH055880	Chen et al. 2018
Leptobrachella eos	NCSM 79810	Xaysomboun, Vientiane, Laos	MH055881	Chen et al. 2018
Leptobrachella eos	NCSM 80551	Boun Tay, Phongsaly, Laos	MH055887	Chen et al. 2018
Leptobrachella eos	MNHN:2004.0277	Long Nai, Phongsaly, Laos	JN848448	Ohler et al. 2011
Leptobrachella eos	SYS a003959	Zhushihe, Yunnan, China	MH055888	Chen et al. 2018
Leptobrachella eos	AUP 00377	Chom poo Phuka nature trail, Nan, Bo Kluea, Thailand	OM258178	In this study
Leptobrachella eos	2004.0276	Long Nai Khao, Phongsali, Laos	KR827862	Grosjean et al. 2015
Leptobrachella eos	2004.0275	Long Nai Khao, Phongsali, Laos	KR827861	Grosjean et al. 2015
Leptobrachella eos	K1684	Long Nai, Laos	JN848452	Ohler et al. 2011
Leptobrachella eos	K1685	Long Nai, Laos	JN848451	Ohler et al. 2011
Leptobrachella eos	K1730	Long Nai, Laos	JN848450	Ohler et al. 2011
Leptobrachella eos	K1976	Phongsaly, Laos	JN848449	Ohler et al. 2011
Leptobrachella eos	K1731	Long Nai, Laos	JN848447	Ohler et al. 2011
Leptobrachella eos	K1728	Long Nai, Laos	JN848446	Ohler et al. 2011
Leptobrachella bourreti	ZMMU-A5636-02280	Bat Xat, Lao Cai, Vietnam	MH055872	Chen et al. 2018
Leptobrachella alpina	KIZ046816	Huangcaoling, Yunnan, China	MH055866	Chen et al. 2018
Leptobrachella alpina	KIZ049024	Caiyanghe, Yunnan, China	MH055867	Chen et al. 2018
Leptobrachella oshanensis	KIZ025776	Emei Shan, Sichuan, China	MH055895	Chen et al. 2018
Leptobrachella tengchongensis	SYS a004598	Gaoligong Shan, Yunnan, China	KU589209	Yang et al. 2016
Leptobrachella khasiorum	SDBDU 2009.329	Khasi Hills, Meghalaya, India	KY022303	Mahony et al. 2017
Leptobrachella petrops	ROM 13483	Ba Vi National Park, Ha Tay, Vietnam	MH055901	Chen et al. 2018
Leptobrachella petrops	ZMMU-NAP-06537	Xuan Son National Park, Phu Tho, Vietnam	MH055902	Chen et al. 2018
Leptobrachella puhoatensis	IEBR ADPH049	Pu Hu Nature Reserve, Thanh Hoa, Vietnam	MH055898	Chen et al. 2018
Leptobrachella puhoatensis	IEBR ADPH101	Pu Hu Nature Reserve, Thanh Hoa, Vietnam	MH055899	Chen et al. 2018
Leptobrachella liui	SYS a004035	Wugong Shan, Jiangxi, China	MH055916	Chen et al. 2018
Leptobrachella liui	SYS a004051	Jinggang Shan, Jiangxi, China	MH055917	Chen et al. 2018
Leptobrachella laui	SYS a002444	Shenzhen, Guangdong, China	MH055905	Chen et al. 2018
Leptobrachella laui	SYS a003601	Yinping Shan, Guangdong, China	MH055906	Chen et al. 2018
Leptobrachella maoershanensis	KIZ07614	Mao'er Shan, Guangxi, China	MH055927	Chen et al. 2018
Leptobrachella maoershanensis	KIZ027236	Mao'er Shan, Guangxi, China	MH055928	Chen et al. 2018
Leptobrachella minima	NCSM 79245	Xayabury, Sainyabuli, Laos	MH055846	Chen et al. 2018
Leptobrachella minima	NCSM 79277	Parklai, Sainyabuli, Laos	MH055847	Chen et al. 2018
Leptobrachella aerea	ZMMU-A-5605-05608	Tuyen Hoa, Quang Binh, Vietnam	MH055808	Chen et al. 2018
Leptobrachella aerea	NCSM 80856	Viengthong, Bolikhamsai, Laos	MH055810	Chen et al. 2018
Leptobrachella ventripunctata	KIZ013621	Wenlong, Yunnan, China	MH055824	Chen et al. 2018
Leptobrachella nyx	ROM 35606	Malipo, Yunnan, China	MH055814	Chen et al. 2018
Leptobrachella pluvialis	ROM 30685	Fansipan, Lao Cai, Vietnam	MH055843	Chen et al. 2018
Leptobrachella nahangensis	ROM 7035	Na Hang Nature Reserve, Tuyen Quang, Vietnam	MH055853	Chen et al. 2018
Leptobrachella sungi	ROM 21805	Van Ban National Park, Lao Cai, Vietnam	MH055861	Chen et al. 2018
Leptobrachella zhangyapingi	KIZ07258	Chiang Mai, Thailand	MH055864	Chen et al. 2018
Leptobrachella zhangyapingi	KIZ07460	Chiang Mai, Thailand	MH055865	Chen et al. 2018
Outgroup				
Leptobrachium boringii	Tissue ID: YPX37539	Sichuan, China	KX811930	Chen et al. 2017
Xenophrys glandulosa	KIZ048439	Yunnan, China	KX811762	Chen et al. 2017

Leptobrachium boringii and *Xenophrys glandulosa* were selected as the outgroups (Chen et al. 2018).

Phylogenetic reconstructions using Bayesian inference (BI) and maximum likelihood (ML) were executed in the CIPRES web server (Miller et al. 2010). ML analysis was conducted with the rapid bootstrapping algorithm using the program RAxML v8.00 (Stamatakis 2014). Nodal support for ML was assessed with 1000 rapid bootstrap replicates (BS). The best-fit substitution model was selected under the Bayesian Information Criterion by the program jModeltest 2.1.4 (Darriba et al. 2012). The best fit substitution model for the 16S dataset was GTR + I + G. For BI analysis, two independent runs were initiated each with four simultaneous Markov Chain Monte Carlo (MCMC) chains for 10 million generations and sampled every 1000 generations. The first 25% generations were discarded as burn-in, and the last remaining trees were used to create a 50% majority-rule consensus tree and estimate Bayesian posterior probabilities (BPP). Pairwise divergences (uncorrected p-distance) between species on 16S dataset were calculated using MEGA6 (Tamura et al. 2013).

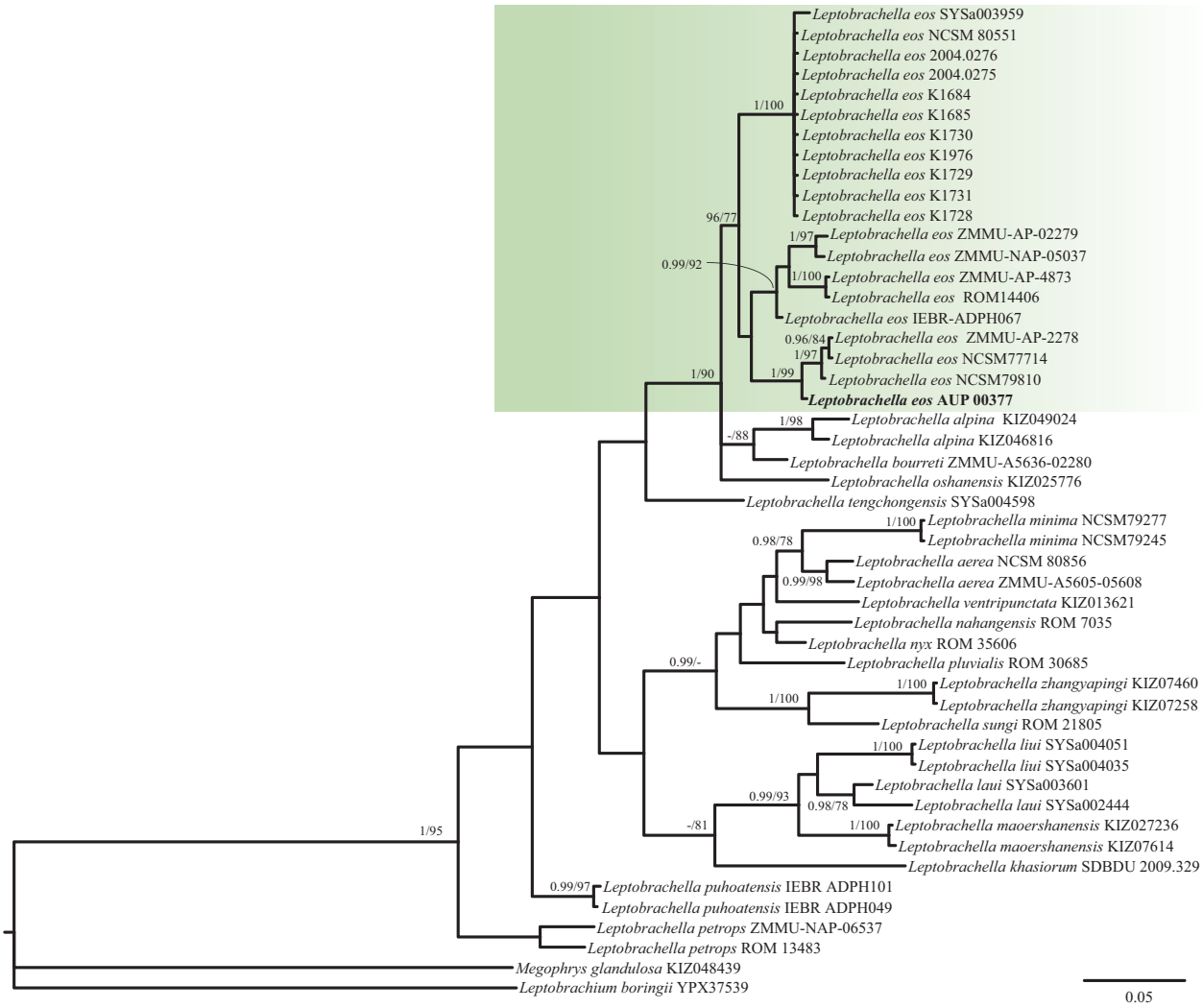

Figure 2. Phylogram of *Leptobrachella* resulting from the analyses of one fragment of the mitochondrial 16S gene. Nodal support values with Bayesian posterior probabilities (BPP) > 95%/bootstrap support (BS) > 70 are shown near the node. A "–" denotes Bayesian posterior probabilities (BPP) < 95% and bootstrap support (BS) < 70. Node values with Bayesian posterior probabilities (BPP) < 95%/bootstrap support (BS) < 70 are not shown.

Measurements were taken using a digital caliper to the nearest 0.1 mm (Table 1). Abbreviations are presented following the method employed by Matsui (1984) for 25 morphological characteristics: (1) Snout-vent length (**SVL**); (2) Head length (**HL**); (3) Head width (**HW**); (4) Snout length (**SL**); (5) Distance from the center of the nostril to the tip of the snout (**SN**); (6) Nostril-eye distance (**N-EL**); (7) Eye diameter (**ED**); (8) Tympanum diameter (**TD**); (9) Internarial distance (**IND**); (10) Interorbital distance (**IOD**); (11) Upper eyelid width (**UEW**); (12) Forelimb length (**FLL**); (13) Lower arm length (**LAL**); (14) Hand length (**HAL**); (15) First finger length (**1FL**); (16) Third finger disc diameter (**3FDD**); (17) Outer palmar tubercle length (**OPTL**); (18) Inner palmar tubercle length (**IPTL**); (19) Tibia length (**TL**); (20) Foot length (**FL**); (21) Hindlimb length (**HLL**); (22) Fourth toe disc diameter (**4TDD**); (23) Inner metatarsal tubercle length (**IMTL**); (24) Outer metatarsal tubercle length (**OMTL**), and (25) First toe length (**1TOEL**).

Results

Aligned sequence matrix of 16S gene contained 508 bp, among which, 195 sites were variable and 148 were parsimony-informative (include outgroups). Phylogenetic trees from ML and BI present identical topologies and the node supports are very high except for some internal nodes (Fig. 2). The results indicated that the monophyly of the genus *Leptobrachella* was strongly supported, and in agreement with results of Chen et al. (2018). The phylogenetic analysis suggested that this specimen was nested in the genus *Leptobrachella* and formed a monophyletic clade with *L. eos* obtained from China, Laos, and Vietnam.

Genetic distance on 16S between the specimen of the *L. eos* collected from Nan province and *L. eos* from Laos, Vietnam, and China was 3.5%, lower than interspecific genetic distances of other species, varying from 3.9% (between *L. bourreti*) to 12.8% (between *L. minima*, *L. zhangyapingi*, and *L. ventripunctata*) (Table 2).

Table 2. Average uncorrected p-distances among the *Leptobrachella* species calculated from 16S rRNA gene sequences.

ID	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	**AUP 00377**		1.6	1.7	1.3	1.7	1.5	1.4	0.8	1.0	1.3	1.9	1.6	1.5	1.7	1.7	1.6	1.6	1.6	1.5
2	***L. maoershanensis***	10.6		1.4	1.3	1.7	1.7	1.6	1.4	1.7	1.7	2.0	1.8	1.5	1.7	1.5	1.5	1.5	1.4	1.5
3	***L. liui***	10.7	7.8		1.4	1.9	1.9	1.7	1.8	1.9	1.8	2.0	2.0	1.6	1.7	1.6	1.7	1.5	1.6	1.6
4	***L. laui***	8.7	7.2	6.9		1.7	1.5	1.7	1.4	1.6	1.5	2.0	1.9	1.7	1.6	1.8	1.7	1.6	1.6	1.6
5	***L. petrops***	11.9	11.0	13.1	11.9		1.5	1.8	1.8	1.9	1.8	2.0	1.7	1.6	1.8	1.7	1.7	1.6	1.6	1.7
6	***L. puhoatensis***	9.9	10.7	11.6	10.1	9.1		1.6	1.5	1.7	1.7	1.9	1.7	1.2	1.4	1.5	1.4	1.3	1.3	1.7
7	***L. oshanensis***	7.5	10.0	10.1	10.3	13.1	11.0		1.2	1.3	1.3	2.0	1.8	1.5	1.7	1.6	1.7	1.8	1.6	1.7
8	***L. eos***	3.5	9.8	11.1	9.4	12.2	9.3	6.7		1.0	1.1	1.9	1.6	1.5	1.7	1.7	1.7	1.5	1.6	1.6
9	***L. bourreti***	3.9	10.9	11.3	9.6	12.8	10.7	6.9	4.2		1.1	2.1	1.9	1.5	1.7	1.7	1.7	1.7	1.6	1.7
10	***L. alpina***	6.0	10.9	11.0	9.9	12.8	10.3	7.5	5.7	4.6		1.9	1.9	1.6	1.8	1.8	1.6	1.7	1.7	1.6
11	***L. zhangyapingi***	12.8	13.3	12.5	12.4	14.8	12.5	13.4	12.6	12.2	11.9		1.6	1.9	1.9	2.0	2.0	1.7	1.9	2.2
12	***L. sungi***	10.7	11.2	12.5	12.2	11.5	9.9	12.5	11.3	11.0	12.2	7.2		1.5	1.6	1.7	1.6	1.5	1.5	1.9
13	***L. nahangensis***	9.9	9.7	10.1	10.3	11.8	8.7	10.7	11.0	10.4	10.9	11.3	8.4		1.3	1.2	1.2	1.1	1.2	1.6
14	***L. minima***	12.8	10.6	11.9	10.7	14.2	11.0	12.8	13.2	13.1	14.2	12.8	10.4	6.6		1.5	1.3	1.3	1.2	1.8
15	***L. pluvialis***	12.2	8.2	9.9	10.6	12.8	11.3	11.3	11.7	11.0	12.2	11.3	9.6	6.3	8.4		1.4	1.3	1.2	1.7
16	***L. ventripunctata***	12.8	10.3	11.3	10.9	13.6	9.9	12.2	12.7	12.2	12.5	11.3	9.9	6.6	7.2	7.5		1.2	1.2	1.7
17	***L. nyx***	10.1	9.1	9.6	9.7	12.4	9.9	11.0	10.8	10.7	11.3	10.1	8.1	4.2	6.0	7.2	5.4		1.0	1.6
18	***L. aerea***	11.2	9.4	11.5	11.0	11.9	9.9	11.3	11.5	11.0	11.8	11.5	8.2	5.1	6.1	6.7	5.5	4.6		1.7
19	***L. khasiorum***	11.0	11.5	12.5	12.2	13.9	12.5	13.1	11.7	13.7	13.6	15.5	13.4	11.9	14.3	13.4	14.3	12.8	13.9	
20	***L. tengchongensis***	8.4	10.3	11.3	8.8	9.6	8.4	9.3	8.3	8.4	8.8	11.9	10.1	9.9	10.1	11.6	10.1	9.0	10.0	11.3

Morphologically, the specimen from Nan province shows a similar appearance to the original description of *L. eos*. Therefore, we considered AUP 00377 to belong to *L. eos*.

Taxonomic account

Leptobrachella eos (Ohler, Wollenberg, Grosjean, Hendrix, Vences, Ziegler & Dubois, 2011)

Specimen examined. Adult female (AUP 00377) collected on 05 October 2018 by the Chatmongkon Suwannapoom and Parinya Pawangkhanant from Chom poo Phuka nature trail, Nan Province, Bo Kluea District, Thailand (19.0181°N, 100.9731°E, 1300 m elevation).

Morphological description (measurements in mm; provided in Table 3). Morphological characters of the specimen from Thailand agreed well with the original description of Ohler et al. (2011). Adult female with SVL 34.2 mm; head length (HL 13.6 mm, 39.7% of SVL) slightly longer than width (HW 12.1 mm, 35.5% of SVL); snout slightly protruding, its length (SL 5.3 mm, 15.5% of SVL) longer than horizontal diameter of eye (EL 4.4 mm, 12.8% of SVL); canthus rostralis rounded, loreal region concave; interorbital space flat, larger (IOD 4.0 mm, 11.6% of SVL) than width of upper eyelid (UEW 2.8 mm, 8.2% of SVL) and internarial distance (IN 3.6 mm, 10.6% of SVL); snout longer than eye diameter (SL/ED 120.5%); tympanum distinct (TD 4.0 mm), rounded, about half eye diameter (ED 4.4 mm); vomerine teeth absent; supratympanic fold distinct; pupil vertical (Fig. 3).

Forelimbs slender; lower arm length (LAL 22.0 mm, 47.3% of SVL) shorter than hand length (HAL 10.1 mm, 29.6% of SVL); relative finger lengths: I<II<IV<III; tips of all fingers slightly enlarged; no webbing between fingers; subarticular tubercles distinct, big; two metacarpal tubercles, inner metacarpal tubercle (IPTL 1.5 mm, 4.4% of SVL) almost equal to outer metacarpal tubercle relatively (OPTL 1.6 mm, 4.6% of SVL).

Hindlimbs long, tibia (TL 16.4 mm) about half SVL and shorter and foot (FL 23.5 mm); relative length of toes: I<II<III<V<IV; tibiotarsal articulation reaching the nostril when the leg is stretched forward; heels overlapping when thighs are positioned at right angles to the body; tips of toes rounded and not swollen; rudimentary webbing between toes; subarticular tubercles distinct, rounded; inner metatarsal tubercle distinct and oval (IMTL 2.0 mm, 5.7% of SVL), outer metatarsal tubercle distinct (OMTL 2.2 mm, 6.3% of SVL).

Table 3. Measurement (in mm) of and proportions of the *Leptobrachella eos* (see Materials and methods section for list of abbreviations).

Characters	AUP-00377	Ratio (-/SVL)
SEX	F	-
SVL	34.2	-
HL	13.6	39.7%
SL	5.3	15.5%
ED	4.4	12.8%
N-EL	3.2	9.4%
HW	12.1	35.5%
IND	3.6	10.6%
IOD	4.0	11.6%
UEW	2.8	8.2%
FLL	22.0	64.3%
LAL	16.2	47.3%
HAL	10.1	29.6%
1FL	4.8	13.9%
IPTL	1.5	4.4%
OPTL	1.6	4.6%
3FDD	0.8	2.2%
HLL	53.3	155.8%
TL	16.4	47.9%
FL	23.5	68.6%
IMTL	2.0	5.7%
1TOEL	4.5	13.0%
4TDD	0.8	2.4%
TD	4.0	11.7%
OMTL	2.2	6.3%

Figure 3. Dorsolateral view and lateral view of head of *L. eos* (photos taken by Parinya Pawangkhanant).

Dorsal skin relatively smooth, with small tubercles; side of head and dorsum shagreened; tiny warts scattered on flanks; supratympanic fold prominent, running from posterior corner of eye towards axilla; dorsal parts of limbs: forelimbs shagreened; thigh and shank with glandular warts; tarsus smooth; femoral glands and pectoral gland distinct, oval; axillary glands indistinct; ventrolateral glands forming continuous white line on flanks.

In life, dorsal surface brown, with reddish-brown W-shaped marking on scapular region; distinct reverse-triangle black marking between eyes; tympanic region brown gray; dorsal surfaces of elbow to upper arm with distinctive reddish-brown coloration; transverse dark-brown bars present on dorsal surface of the limbs; iris distinctly bicolored, bright orange-red in upper half and silvery-white in lower half (Fig. 3).

In preservation. Dorsum of the body and hindlimbs light brownish gray; transverse bars on the limbs distinct, and dark-brown patterns; marks and spots on the back are indistinct; ventral surface of the body is yellowish brown with brown marbling on the sides and chest; axillary glands, femoral, pectoral and ventrolateral glands fade to grayish white.

Ecological notes. *Leptobrachella eos* was found along a rocky stream in Montane Forest, with dense vegetation of Wild Banana (*Musa acuminata*) and Bamboo (*Cephalostachyum* sp.) (Fig. 4). The male was found calling mainly hidden under leaf litter.

Distribution. *Leptobrachella eos* is currently known in Phongsaly, Bolikhamxay, Oudomxai, and Xiasomboun Provinces, Laos; Dien Bien, Thanh Hoa, and Son La Provinces, northwestern Vietnam; Yunnan province, China and Nan province, Thailand.

Discussion

Currently, seven *Leptobrachella* species including *L. fuliginosa*, *L. melanoleuca*, *L. minima*, *L. pelodytoides*, *L. sola*, *L. ventripunctata*, *L. murphyi*, and *L. zhangyapingi* are recorded in Thailand (Chen et al. 2021; Frost 2021; Wu et al. 2021). The present study supports that this specimen from Chom poo Phuka nature trail, Nan Province, Thailand belongs to *L. eos*, representing the first record of this species in Thailand. The discovery of *L. eos* in this study increases the total number of known species in Thailand from 125 (Khonsue and Thirakhupt 2001) to 195 (Frost 2021), along with the known number of *Leptobrachella* species from eight to nine (Frost 2021). In addition, our results further confirm that amphibian diversity in Thailand has been underestimated. Therefore, investigation and research need to be strengthened in the future.

Although phylogenetic analyses based on 16S suggested that the *L. eos* is monophyletic it contains three genetically independent clades, and in agreement with results of Chen et al. (2018). In addition, intraspecific genetic distance of this species was over 3.0% , a value that was earlier proposed as a good indicator for candidate new species in frogs (Fouquet et al. 2007). But it is also common to the intraspecific genetic distance over 3% in amphibians and reptiles, such as *L. minima* (Chen et al. 2018) and *Amolops spinapectoralis* (Wu et al. 2020). Some taxa also exhibit very small interspecific genetic divergence, such as *Amolops mantzorum* group (Wu et al. 2020) and the genus *Nidirana* (Lyu et al. 2020). Therefore, there is no clear threshold for genetic distance to distinguish intraspecific and interspecific, but merely a reference.

There are two main reasons for high genetic differentiation within species. The first is the possible existence of cryptic species, and the second is geographical genetic variation in a low-dispersal group. To investigate these possibilities, further studies employing more comprehensive sampling, integrative taxonomy methods, and nuclear genetic data will be necessary to investigate the species diversity within *L. eos*.

Figure 4. Habitat at collection site of *L. eos* in Chiang Rai Province, Thailand (photos taken by Parinya Pawangkhanant)

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