

The Australasian frog family Ceratobatrachidae in China, Myanmar and Thailand: discovery of a new Himalayan forest frog clade

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ABSTRACT

In an effort to study the systematic affinities and species-level phylogenetic relationships of the enigmatic anurans variably assigned to the genera *Ingerana* or *Limnonectes* (family Dicroglossidae), we collected new molecular sequence data for five species including four Himalayan taxa, *Limnonectes xizangensis*, *Lim. medogensis*, *Lim. alpine*, *Ingerana borealis* and one southeast Asian species, *I. tasanae*, and analyzed these together with data from previous studies involving other ostensibly related taxa. Our surprising results demonstrate unequivocally that *Lim. xizangensis*, *Lim. medogensis* and *Lim. alpine* form a strongly supported clade, the sister-group of the family Australasian forest frog family Ceratobatrachidae. This discovery requires an expansion of the definition of Ceratobatrachidae and represents the first record of this family in China. These three species are distinguished from the species of *Ingerana* and *Limnonectes* by the: (1) absence of interdigital webbing of the foot, (2) absence of terminal discs on fingers and toes, (3) absence of circumarginal grooves on the fingers and toes, and (4) absence of tarsal folds. Given their phylogenetic and morphological distinctiveness, we assign them to the oldest available generic name for this clade, *Liurana* Dubois 1987, and transfer *Liurana* from Dicroglossidae to the family Ceratobatrachidae. In contrast, *Ingerana tasanae* was found to be clustered with strong support with the recently described genus *Alcalus* (Ceratobatrachidae), a

small clade of otherwise Sundaic species; this constitutes a new record of the family Ceratobatrachidae for Myanmar and Thailand. Finally, *Ingerana borealis* clustered with the “true” *Ingerana* (family Dicroglossidae), for which the type species is *I. tenasserimensis*.

Keywords: Dicroglossidae; Himalaya; *Liurana*

INTRODUCTION

The frogs of family Ceratobatrachidae (Boulenger, 2009) comprise a morphologically, developmentally, ecologically, and biogeographically greatly variable and, thus, unique clade (Brown et al., 2015). This family is notable for highly variable body size, direct larval development, and the ability to inhabit a wide variety of environments that lack

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standing water-from small oceanic islands, to high-elevation mossy montane forests (Brown & Alcala, 1982; Brown et al., 2013; Günther, 2015). Currently, 91 species are assigned to three genera: *Platymantis* Günther, 1858, *Cornufer* Tschudi, 1838, and *Alcalus* Brown, Siler, Richards, Diesmos, and Cannatella, 2015 (AmphibiaWeb, 2015; Brown et al., 2015; Frost, 2015). These species are distributed broadly from the South-West Pacific to the island archipelagos of South Asia, with primary centers of species diversity in Philippines and Solomon-Bismarck Archipelago (Brown, 2009; Brown et al., 2013, 2015).

Four species, formerly referred to Southeast Asian frogs *Ingerana* (Dubois, 1987), were recently assigned to the family Ceratobatrachidae based on molecular data (Brown et al., 2015). The four taxa (*I. baluensis*, *I. mariae*, *I. rajae*, *I. sariba*) comprise a monophyletic group now shown to be the sister group of Ceratobatrachinae (genera *Platymantis* and *Cornufer*). However, “true” *Ingerana* (based on the phylogenetic position of the type species, *Ingerana tenasserimensis* [Sclater, 1892]) has been shown in multiple studies to be more closely related to Dicroglossidae (Bossuyt et al., 2006; Wiens et al., 2009). Thus, these four species were just recently assigned to the new genus *Alcalus* in the family Ceratobatrachidae (Brown et al., 2015).

The species in genus *Ingerana* are small, plump frogs with flattened and expanded toe and finger tips (Dubois, 1987). Thirteen species previously have been referred to this genus on the basis of morphological characters and life history traits. However, recently its members have been placed in different genera, and even different families, based on phylogenetic analysis of molecular data analysis, i.e., *A. baluensis*, *A. mariae*, *A. rajae* and *I. tenasserimensis* (Bossuyt et al., 2006; Frost et al., 2006; Wiens et al., 2009; Brown et al., 2015). The placement of other *Ingerana* species was controversial, and some species were tentatively placed in different genera, in the absence of accompanying molecular data. For example, *Limnonectes xizangensis* was variably assigned to the genera *Cornufer* (Hu, 1977), *Ingerana* (subgenus *Liurana*) (Dubois, 1987), *Platymantis* (Fei et al., 1990), *Micrixalus* (Zhao & Adler, 1993), and finally to *Limnonectes* (subgenus *Taylorana*) (Borah et al., 2013; Frost, 2015). The complex and convoluted taxonomic placement of several of these species has based on morphological or reproductive characters. Because the few key diagnostic characters emphasized by previous worker are variable, and subject to individual interpretation they may have mislead previous attempts to determine systematic affinities of these poorly known frog species.

Here we report the results of a systematic study of five species variably referred to *Limnonectes* or *Ingerana*, including *Lim. xizangensis*, *Lim. medogensis*, *Lim. alpine*, *I. borealis* and *I. tasanae*. We redistribute them among two families, according to their phylogenetic affinities, as *Liurana xizangensis*, *Liu. medogensis*, *Liu. alpine*, and *Alcalus tasanae* (family Ceratobatrachidae) and *Ingerana borealis* (family Dicroglossidae). These discoveries greatly extend the westernmost geographic distribution of the primarily Australasian archipelago family Ceratobatrachidae into

Indochina and China and assign early mainland branching events in this family to lineages now exclusively represented by species with restricted ranges in the high-elevation Himalayan mountains of Tibet.

MATERIALS AND METHODS

Sampling

Four species, *Limnonectes xizangensis*, *Lim. medogensis*, *Lim. alpine* and *Ingerana borealis*, were sampled from Medog (=Motuo), Tibet (=Xizang), PR China (locality 1 in Figure 1, Table 1). Following the collection of liver tissue samples (preserved in 95% ethanol), the voucher specimens were fixed with 10% formalin and then stored in 70% ethanol. Collection of specimens followed animal-use protocols approved by the Kunming Institute of Zoology Animal Use and Ethics Committee. Two more species, *I. tasanae* and *Occidozyga martensii* distributed in Myanmar and Thailand, were also included. We borrowed their tissue samples from the collections of the California Academy of Sciences (CAS), Thailand National History Museum (THNHM), and Field Museum of Natural History (FMNH) (Figure 1, Table 1).

DNA extraction and sequencing

Total DNA was extracted using standard phenol-chloroform protocols (Sambrook et al., 1989). One fragment of mitochondrial DNA of 12S *rRNA*, *tRNA-Val*, and 16S *rRNA* (12S-16S) was sequenced for all samples using primers L2519 and 16Sbr (Table 2). Three partial nuclear DNA sequences of recombination activating gene 1 (*Rag1*), tyrosinase (*Tyr*) and rhodopsin (*Rhod*) were sequenced for all samples using primers included in Table 2. Amplifications were conducted in a 25 μ L volume reaction, involved initial denaturing step at 94 °C for 5 min; then 35 cycles of denaturing at 94 °C for 45 sec, annealing at 50 °C or 55 °C for 45 sec, and extending at 72 °C for 45 sec; and a final extending step of 72 °C for 7 min. The products were purified with Gel Extraction Mini Kit (Watson BioTechnologies, Shanghai, China), then sequenced on an ABI 3730x1 DNA automated sequencer (Applied Biosystems, UK).

For species not sampled by us, the sequences of 12S-16S, *Rag1*, *Tyr* and *Rhod* were downloaded from GenBank (Table 1). All data were aligned with MUSCLE (Edgar, 2004) and edited using MEGA 5.05 (Tamura et al., 2011).

Phylogenetic analysis

We estimated phylogenetic relationships using Bayesian inference (BI) and maximum parsimony (MP) using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) and PAUP* 4.0b10a (Swofford, 2003). Mitochondrial and nuclear sequence data were analyzed separately. Then a phylogenetic tree was conducted using the concatenated sequence of all genes. For BI analysis, the best-fitting nucleotide substitution models were selected for 12S-16S and each codon of *Rag1*, *Tyr* and *Rhod* using the Akaike information criterion in MRMODELTEST v2.3 (Nylander, 2004). The BI analysis used four Markov chains, with default heating

Table 1 Samples information used in molecular analysis

Family	Genus	Species	Specimen voucher No.	Locality (site number)	GenBank No.			
					12S-16S	Rhod	Rag1	Tyr
Brevicipitidae	<i>Callulina</i>	<i>krefftii</i>	VUB1068 (ES700)	Tanzania	DQ347056	DQ347400	DQ347281	DQ347189
Microhylidae	<i>Elachistocleis</i>	<i>ovalis</i>	TNHC-DCC 3301	South America	DQ347057	DQ347401	DQ347282	DQ347190
Leptodactylidae	<i>Leptodactylus</i>	<i>melanonotus</i>	MVZ 207294 (FC14298)	Costa Rica	DQ347060	-	-	DQ347193
Ceratobatrachidae	<i>Alcalus</i>	<i>baluensis</i>	FMNH 44690	Mt. Kinabalu, Borneo, Malaysia	DQ347044	DQ347389	DQ347270	DQ347177
	<i>Alcalus</i>	<i>mariae</i>	KU 309518	Palawan PAIC, Palawan Province, Philippines	KP298038	KP298263	-	-
	<i>Alcalus</i>	<i>tasanae</i>	CAS 232349	Kachin State, Myanmar (2)	KU243087	KU243106	KU243096	KU243116
	<i>Alcalus</i>	<i>tasanae</i>	CAS 247243	Tanintharyi Division, Myanmar (3)	KU243088	KU243107	KU243097	KU243117
	<i>Alcalus</i>	<i>tasanae</i>	THNHM20534	Ranong Province, Thailand (4)	KU243089	KU243108	KU243098	KU243118
	<i>Platymanthis</i>	<i>hazellae</i>	CMNH-RSK3918	Negros Isl, Philippines	DQ347019	DQ347369	DQ347248	DQ347153
	<i>Platymanthis</i>	sp.	FMNH 259000	Luzon PAIC, Kalinga Province, Philippines	KP298055	-	KP298265	KP298332
	"Batrachylodes"	sp.	VUB0799	Solomon Islands	DQ346991	DQ347352	DQ347228	DQ347136
	<i>Cornufer</i>	<i>guppyi</i>	UW-JF189	New Britain Island	DQ347043	DQ347388	DQ347269	DQ347176
	<i>Cornufer</i>	<i>guentheri</i>	VUB1017 (SR5543)	Solomon Islands	DQ347046	DQ347391	DQ347272	DQ347179
	<i>Liurana</i>	<i>xizangensis</i>	KIZ06707	Medog Xian, Xizang, China (1)	KU243083	KU243101	KU243092	KU243111
	<i>Liurana</i>	<i>xizangensis</i>	KIZ011107	Medog Xian, Xizang, China (1)	KU243084	KU243102	KU243093	KU243112
	<i>Liurana</i>	<i>medogensis</i>	KIZ010955	Medog Xian, Xizang, China (1)	-	KU243103	-	KU243113
	<i>Liurana</i>	<i>alpine</i>	KIZ011140	Medog Xian, Xizang, China (1)	KU243085	KU243104	KU243094	KU243114
<i>Liurana</i>	<i>alpine</i>	KIZ011141	Medog Xian, Xizang, China (1)	KU243086	KU243105	KU243095	KU243115	
Conrauidae	<i>Conraua</i>	<i>crassipes</i>	ZFMK 75446	Cameroon	DQ347015	DQ347364	DQ347244	DQ347148
Petropedetidae	<i>Arthroleptides</i>	<i>martiensseni</i>	CR 10898	Africa	DQ347064	DQ347410	DQ347289	DQ347197
	<i>Petropedetes</i>	<i>parkeri</i>	VUB0955 (MV)	Africa	DQ347014	-	-	DQ347147
Pyxicephalidae	<i>Tomopterna</i>	<i>tandyi</i>	-	-	DQ347009	DQ347360	DQ347240	DQ347143
	<i>Arthroleptella</i>	<i>villiersi</i>	CR 1070	Africa	DQ347062	DQ347408	DQ347287	DQ347195
	<i>Amietia</i>	<i>fuscigula</i>	CR 1073	Africa	DQ347065	DQ347411	DQ347290	DQ347198
Dicroglossidae	<i>Limnonectes</i>	<i>limborgi</i>	VUB1218	Laos	DQ347061	DQ347407	DQ347286	DQ347194
	<i>Limnonectes</i>	sp.	TNHC 59014	Sulawesi Isl., Indonesia	DQ347045	DQ347390	DQ347271	DQ347178
	<i>Hoplobatrachus</i>	<i>occipitalis</i>	VUB0537	unknown (pet shop)-Africa	DQ346979	DQ347349	DQ347217	DQ347132
	<i>Occidozyga</i>	<i>lima</i>	TNHC 59864-RMB 2134	Java Isl., Indonesia	DQ347025	DQ347375	DQ347255	DQ347159
	<i>Occidozyga</i>	<i>martensi</i>	FMNH 268805	Krabi, Thailand (5)	KU243090	KU243109	KU243099	KU243119
	<i>Ingerana</i>	<i>tenasserimensis</i>	CAS 205064	Myanmar	DQ347030	AY322236	DQ347258	AY322344
	<i>Ingerana</i>	<i>borealis</i>	KIZ020455	Medog Xian, Xizang, China (1)	KU243091	KU243110	KU243100	KU243120
Phrynobatrachidae	<i>Phrynobatrachus</i>	<i>krefftii</i>	VUB1068 (ES700)	Tanzania	DQ347059	DQ347403	DQ347284	DQ347192
	<i>Phrynobatrachus</i>	<i>africanus</i>	CAS 207779	Equatorial Guinea	DQ347031	DQ347378	DQ347259	DQ347164
Ranidae	<i>Amolops</i>	<i>lanutensis</i>	TNHC 57944-JAM 1371	Thailand	DQ347053	DQ347398	DQ347279	DQ347186
	<i>Staurois</i>	<i>natator</i>	CMNH-H1626	Mindanao Isl., Philippines	DQ347020	DQ347371	DQ347250	DQ347155
	<i>Rana</i>	<i>sylvatica</i>	MVZ 137426	New York, USA	DQ347052	DQ347397	DQ347278	DQ347185

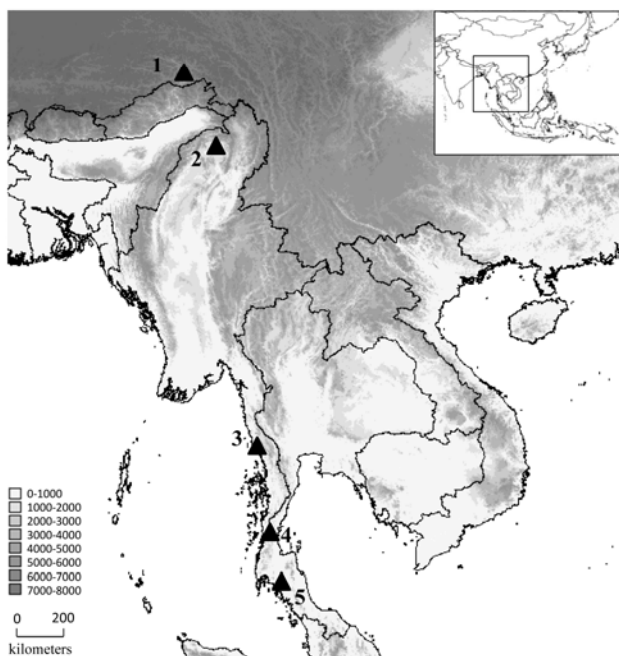


Figure 1 Map of sampling sites

Numbers correspond to localities in Table 1.

Table 2 Primers information used for four DNA fragments sequencing

Locus	Primer name	Sequence (5'-3')	<i>T_m</i>	Citation	
12S-16S	L2519	AAACTGGGATTAGATACCCCACTAT	55	Richards & Moore, 1996	
	H3296	GCTAGACCATKATGCAAAAAGTA			
	16Sbr	CCGGTYTGAACCTCAGATCAYGT		Palumbi et al., 1991	
	16Sbr	CCGGTYTGAACCTCAGATCAYGT			
	12/16S-696F	TATARCAATAGTACCGCAAG			This study
	STW-NA1	GGGTGACGGGCGGTTTGT			
<i>Rag1</i>	L-RAG1RAn	CTGGTCGTCAGATCTTTTCAGC	50	Stuart, 2008	
	H-RAG1RAn	GCAAAACGTTGAGAGTGATAAC			
	L-RAG1RAninT	GGAAATTGGTGGAAATCCTCAG			
	H-RAG1RAninT	ATATAGATAGAGCCTGAGGC			
<i>Tyr</i>	TYR 1 G	TGCTGGGCRTCTCTCCARTCCCA	50	Bossuyt & Milinkovitch, 2000	
	TYR 1 B	AGGTCTCYTRAGGAAGGAATG			
<i>Rhod</i>	RhoG 1 A	ACCATGAACGGAACAGAAGGYCC	50		
	RhoG 1 G	GTAGCGAAGAARCCCTTCAAMGTA			

Phylogenetic relationships

The best-fitting model were TVM+I+G for mitochondrial 12S-16S, K80+I, TIMef+I and TIMef+I for three codon positions of *Rag1*, TVM+I+G, K81+I and GTR+G for three codon positions of *Tyr*, SYM+G, TVM+I+G and TIM+G for three codon positions of *Rhod*. The phylogenetic analyses based on nuclear DNA and mtDNA showed similar topologies. Most recognized families formed monophyletic groups; however, the monophyly of Dicroglossidae was not recovered using mtDNA, but highly supported by nuclear DNA. This possibly is due to the inability of mtDNA

values, and run for 5 million generations while sampling trees every 1 000 generations. The first 25% sampled trees were discarded as burn-in, and log-likelihood scores were examined using Tracer v 1.4 (Rambaut & Drummond 2007) to assure convergence (effective sample size [ESS] values >200). For the MP analysis, full heuristic tree searches were used, with 1 000 replications, random addition of sequences and tree-bisection-reconnection (TBR) branch swapping. Non-parametric bootstrap support was estimated using 1 000 replicates of full heuristic searches.

RESULTS

Sequence information

Sequencing generated a total of 1 371 base pairs (bp) of 12S-16S data for *Limnonectes alpine* and *Ingerana tasanae*. Additionally, a part of fragment of 12S-16S was successfully sequenced for *Occidozyga martensii*, *Lim. xizangensis* and *I. borealis*. We were unable to collect 12S-16S for *Lim. medogensis*. For nuclear sequences of *Rag1*, 1 100 bp was successfully sequenced for all samples except for *Lim. medogensis*, but we only included 553 bp in subsequent analyses so as to match *Rag1* data sequences available on GenBank. Sequences of 553 bp *Tyr* and 316 bp *Rhod* were successfully sequenced for all samples. All new generated sequences were submitted to GenBank (Accession numbers KU243083-KU243120, Table 1).

sequence to resolve phylogenetic relationship at deeper levels (i.e., Kingston et al., 2009), or sparse taxon sampling in our analysis. The five focal species were yielded the same topology in both phylogenetic analyses, so the difference between mtDNA and nuclear DNA topologies do not affect our taxonomy. The Bayesian tree resulting from based on concatenated sequence of all genes is shown in Figure 2. *Limnonectes xizangensis*, *Lim. medogensis*, *Lim. alpine* and *Ingerana tasanae* clustered with species of family Ceratobatrachidae. Three primary lineages were identified in this family, corresponding to two

known subfamilies Alcalinae (Clade A) and Ceratobatrachinae (Clade B), and a new lineage (Clade C), unsampled in previous phylogenetic estimates (Brown et al., 2015). Samples of *Ingerana tasanae* from Thailand and Myanmar grouped together, and this clade formed a strongly supported group with *Alcalus baluensis* and *A. mariae* (Clade A). *Limnonectes xizangensis*, *Lim.*

medogensis and *Lim. alpine* formed a monophyletic group (Clade C), which is strongly supported as related to the family Ceratobatrachidae. Finally, *Ingerana borealis* samples clustered with species in the subfamily Occidozyginae (Dicroglossidae). This species formed a clade with *I. tenasserimensis* (type species of *Ingerana*), as the sister group to *Occidozyga*.

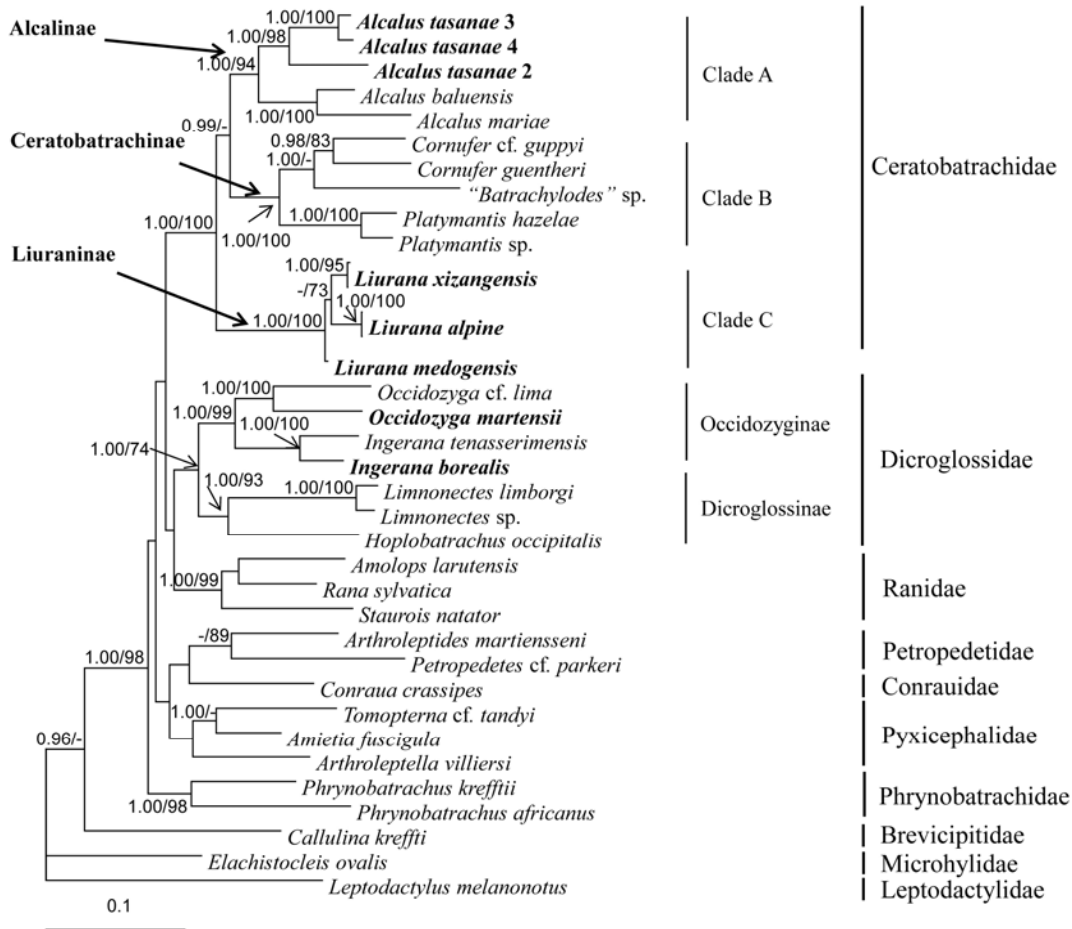


Figure 2 Bayesian inference tree based on concatenated analysis of all genes

Nodal support values are Bayesian posterior probabilities (only ≥ 90 are shown) and bootstrap proportions from maximum parsimony analysis (only ≥ 70 are shown). Newly sequenced samples are emphasized with bold text.

DISCUSSION

Taxonomy of species of *Limnonectes* and *Ingerana*, and a record of a new family for China, Myanmar and Thailand

The three poorly understood species, formerly referred to *Limnonectes* and *Ingerana* from the largely unexplored area of Himalayan Tibet (*Lim. xizangensis*, *Lim. medogensis* and *Lim. alpine*), have had unstable taxonomic histories (Frost, 2015) and, until now, unclear systematic affinities. Dubois (1987) established the genus *Ingerana*, in which there are two

subgenera *Ingerana* (*Ingerana*) and *Ingerana* (*Liurana*). *Ingerana xizangensis* (formerly *Cornufer xizangensis*, Hu, 1977) was included in subgenus *Ingerana* (*Liurana*) by Dubois (1987). Fei et al. (1997) identified significant morphological differences between these two subgenera, including the presence of lingual papilla on the tongue, the absence of terminal discs on fingers and toes, and the absence of circumarginal grooves on fingers and toes in *Ingerana* (*Liurana*), (Figures 3-4). Thus, *Liurana* was elevated to the level of genus to include the species *Liu. xizangensis*, *Liu.*



Figure 3 Photos of *Liurana alpine* and *Liurana xizangensis* in life (Photos by Kai WANG)

A-D: dorsolateral view; ventral view; ventral view of hand; and ventral view of foot of *Liurana alpine*, respectively; E-H: dorsolateral view; ventral view; ventral view of hand, and ventral view of foot of *Liurana xizangensis*, respectively.

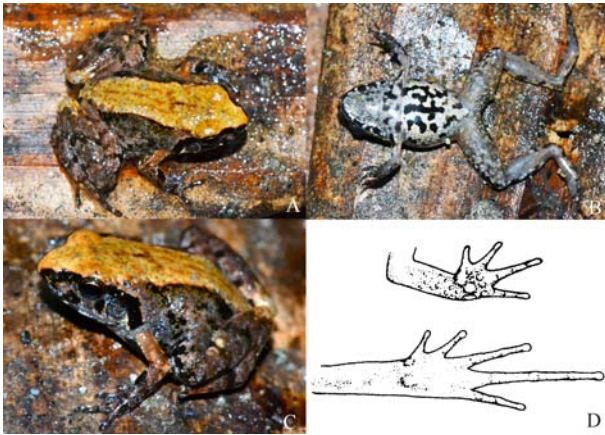


Figure 4 Photos of *Liurana medogensis* (Photos by Kai WANG)

A-C: dorsal view, dorsolateral view, and ventral view (C) in life, respectively; D: ventral view of hand (above) and foot (below) in drawing (from Fei et al., 2009).

medogensis, *Liurana alpine* and *Liurana liui* (Fei et al., 1997, 2009, 2012; Huang & Ye, 1997). Fei et al. (2009) considered *Liurana* to be part of the family Occidozygidae. Subsequently, Fei et al. (2010) established a new subfamily Liuraninae in the family Occidozygidae based on morphological data. Frost et al. (2006) considered *Liurana* to be a junior synonym of *Ingerana* on the basis of the original description and overlapping character states. Based on available morphological characters, Borah et al. (2013) placed *Liurana* in synonymy with *Taylorana* (now considered to be a subgenus of

Limnonectes, [Frost, 2015]). Thus, for the last several years, these species have resided in *Limnonectes* (Frost, 2015) pending appropriate phylogenetic analysis to determine of their systematic affinities.

Based on analysis of multilocus DNA sequence data, *Liurana xizangensis*, *Liurana medogensis* and *Liurana alpine* are herein assigned to the family Ceratobatrachidae and represent the first record of this family in China. In our analysis these species formed strongly supported monophyletic group, clustering with members of the Ceratobatrachidae (*sensu* Brown et al., 2015). In contrast, species of genus *Ingerana* (*I. tenasserimensis* and *I. borealis*) and *Limnonectes* (*Lim. limborgi*, *Lim. sp.*) formed the strongly supported clades in subfamily Occidozyginae, as showed in previous studies (i.e. Bossuyt et al., 2006; Wiens et al., 2009; Pyron & Wiens, 2011). Based on our observations of morphological variation, these three species likewise are distinguished from the species of *Ingerana* and *Limnonectes* by the: (1) absence of interdigital webbing of the feet, (2) absence of terminal discs on fingers and toes, (3) absence of circummarginal grooves on the fingers and toes, and (4) absence of tarsal folds. All available evidence supports the recognition of *Liurana xizangensis*, *Liurana medogensis* and *Liurana alpine* as single taxon, for which *Liurana* is the available generic name with priority. We assign *Liurana* to the family Ceratobatrachidae. Within Ceratobatrachidae, three lineages are recognized: Clade A and Clade B (Figure 2) correspond to previously recognized subfamilies Alcalinae and Ceratobatrachinae, respectively. The genus *Liurana* (Clade C) is equivalent in species content to the subfamily Liuraninae Fei, Ye and Jiang, 2010, now transferred to the family Ceratobatrachidae.

Ingerana tasanae is distributed in western and central peninsular Thailand, and its range possibly extends into adjacent Tenasserim and Myanmar (Stuart et al., 2008). Our molecular data clearly place all *Ingerana tasanae* samples in the same clade as other members of the genus *Alcalus* (Ceratobatrachidae). This constitutes a new record of family Ceratobatrachidae for Myanmar and Thailand. Our northern Myanmar samples of *A. tasanae* is highly divergent from individuals from southern Myanmar and southern Thailand. It remains possible that additional taxonomic diversity will be revealed in the genus *Alcalus* with accumulation of data and field studies of these populations.

Previous studies placed *I. borealis* in the genus *Phrynoglossus* (Fei et al., 2009, 2010, 2012), *Occidozyga* (Ahmed et al., 2009; Mathew & Sen, 2010), and *Ingerana* (Sailo et al., 2009). Based on our molecular data, *I. borealis* falls into a strongly supported clade with *I. tenasserimensis*, the type species of *Ingerana*. Thus, our molecular data support its systematic position within genus *Ingerana* based on morphological comparison by Sailo et al. (2009).

New insight from the phylogeny and distribution of Ceratobatrachidae

Brown et al. (2015) developed a stable taxonomy for the family Ceratobatrachidae. Two subfamilies were identified: Ceratobatrachinae and Alcalinae. Ceratobatrachinae includes

two large monophyletic radiations, *Cornufer* and *Platymantis*. The species belonging to the subfamily Ceratobatrachinae have a broad distribution in the south-west Pacific, including Philippines, Borneo, New Guinea, Admiralty and Bismarck archipelagos, Solomon Islands, and Fiji. Alcalinae includes only four species of *Alcalus*, which are distributed only on the island archipelagos of Southeast Asia (Sundaland).

Our research identified other four species which we now transfer to Ceratobatrachidae; this greatly increases the distribution of the family to the mainland of Southeast Asia and the Himalayan region (Figure 1). Given our experience with the unexpected phylogenetic affinities of the species studied here, we would not be surprised if additional phenotypically similar taxa are found to belong in Ceratobatrachidae in the near future. Of particular note, *Ingerana charlesdarwini* (Das, 1998), distributed in the Andaman Islands (India), could very well be the sister lineage to the remaining lineages in this large and spectacularly diverse anuran family.

The surprising discovery that the clade Ceratobatrachidae is broadly distributed from the Himalayas, mainland and peninsular southeastern Asia, to the southwest Pacific, will help us to understand the biogeography in this region. The sister-group relationship of Ceratobatrachinae and Alcalinae, although not unequivocally supported mirrors the geographic distribution of these clades. This relationship between mainland and archipelago species is also seen in the divergence between the mainland species *Alcalus tasanae* and the archipelago species *A. mariae* and *A. baluensis*. Additional, unexpected patterns between mainland and island taxa may be found with more complete taxon sampling, which emphasizes the need for additional fieldwork in mainland southeastern Asia.

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