

Systematic study of the rhacophorid  
frogs in Vietnam

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**DISCLAIMER.** This thesis is not issued for purposes of zoological nomenclature.

## ABSTRACT

### Background

Rhacophorids (Anura: Rhacophoridae) are a large group of arboreal frogs and represent one of the most diverse anuran families in the Old World, containing over 360 species in 16 genera, and distributed throughout subsaharan Africa, China, India, Southeast Asia, Japan, Taiwan, the Philippines, and the Greater Sunda Islands (Frost, 2014). With more than 70 species in 11 genera recorded in Vietnam, rhacophorid frogs form a large group in the amphibian fauna of the country, but, like many other amphibian groups, systematic study of this family is still incomplete, and taxonomic assignment for many species is still doubtful. On the other hand, numerous new species have been discovered during the past decades, mainly based on the results of molecular phylogenetic studies. This resulted in the change in generic allocation in many species (Delorme et al., 2005, Li et al., 2008, 2009, Wilkinson et al., 2002; Yu et al., 2008, 2009, 2010, Rowley et al., 2011a, b), but the study of morphological synapomorphies to define new groups has been rarely done mainly due to their high level of diversification.

### Materials and Methods

I studied Vietnamese rhacophorid frogs, with special attention to four genera: *Liuixalus*; *Kurixalus*; *Rhacophorus*, and *Theloderma*. Many species of these genera have restricted geographical distribution, hitherto known only from a single or very few specimens deposited in the museum collections. Thus, I tried in the present study to make new collections of specimens and relevant data from intensive fieldwork, so as to better and comprehensively investigate intra and interspecific variations of these rhacophorid frogs, and to fix their taxonomy. This approach could also elucidate the presence of possible undescribed and/or cryptic taxa. For these purposes, I first tried to elucidate phylogenetic relationships of rhacophorid frogs using molecular methods, and then made detailed examination of their morphology.

Field surveys were conducted between 2008 and 2014 in 28 provinces of Vietnam. As a result, I accumulated a total 320 specimens, many of which were examined for partial DNA sequences of the mitochondrial 12S rRNA, tRNA<sup>Val</sup>, and 16S rRNA genes. Using these newly obtained data and relevant sequence data available from GenBank, I tried to establish robust phylogenetic trees

## Results

I obtained the following results: (1) Finding of a new species of the genus *Liuixalus* in Vietnam based on morphological and molecular data. (2) Updates of molecular and morphological data for the genus *Kurixalus*, which resulted in the descriptions of two new cryptic species, *K. viridescens* and *K. motokawai*, from central highland in the country. (3) Based on molecular analyses, *Rhacophorus duboisi* was considered a member of the *R. dugritei* group and *R. pingbianensis* was strongly suggested to have close relationship with *R. omeimontis*. The relationship between *R. robertingeri* and *R. calcaneus* agreed with a phylogenetic hypothesis previously suggested. Two genetic groups were recognized within *R. rhodopus* from Vietnam, although they were similar in their morphological characters. (4) I discovered and recorded the first occurrence of *Philautus petilus* in Vietnam based on comparative studies on morphological similarities with the holotype from Laos, and confirmed its placement in the genus *Theلودerma* based on molecular data.

## Discussion

(1) The record of a new *Liuixalus* is biogeographically significant, because it is new to the territory of Vietnam, and considerably expanded the range of the genus, which had been known only from China. (2) Descriptions of two new cryptic *Kurixalus* species made Vietnam to encompass eight species of the genus now, confirming the country as the center of speciation of the genus *Kurixalus*. (3) Clarifying phylogenetic relationships and taxonomic problems of Vietnam's *Rhacophorus* is thought to be very important in understanding evolution of this genus, because Vietnam is the hotspot for the genus. For example, my study on *R. robertingeri* and *R. calcaneus* resulted in a robust taxonomic idea in this previously disputed problem. (4) My discovery and subsequent molecular analyses of *P. petilus* elucidated proper generic position of the species, and increased the species of the genus *Theلودerma* in Vietnam to 15, making the country the center of speciation of this genus.

## Conclusion

Due to the time limitation and difficulty in the collection of some species, the present study is still incomplete to clarify total diversity of Vietnamese rhacophorid frogs, and further researches on the following topics are now under study and left for future publications: (1) Further clarification of phylogenetic relationships using longer

sequences among all species of the rhacophorid frogs, especially of the genus *Rhacophorus* in Vietnam; (2) Analyses of breeding behavior and advertisement calls that allow more rapid identification of species in the field, especially for species that are very similar in morphology (e.g., *Kurixalus banaensis*; *K. viridescens* and *K. motokawai*); and (3) Further clarification of diversity and biogeography of the rhacophorid frogs in Asia so as to determine biogeographic uniqueness of the Vietnamese fauna.

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## **CHAPTER 1**

### **Introduction**

Vietnam, with a total area of about 330,000 km<sup>2</sup>, and situated in the Indo-Burma region, is the country of monsoon tropical climate, and is known as one of the biodiversity hotspots in the world. The country is divided into eight geographical divisions: the Northwest Region, the Northeast Region, the Red River Delta, the North Central Region, the South Central Region, the Central Highlands, the Southeast Region, and the Mekong Delta (Nguyen et al., 2009). This country is represented by a wide diversity of ecosystems, including lowland evergreen, semi-evergreen, deciduous, and montane forests. There are also patches of shrublands and woodlands on karst limestone outcrops and, in some coastal areas, scattered heath forests are seen. In addition to these, the lowland floodplain swamps, mangroves, and seasonally inundated savanas and grasslands can be found in lowland and coastal areas (Sterling et al., 2006). The wide range of latitudes and elevations, as well as a wide variety of landforms, from swampy deltas to limestone karst and high mountains, has given the country a great diversity of natural environments and a high level of biodiversity (Biodiversity Action Plan for Vietnam in Anonymous [1995]).

### **Topography**

The topography of Vietnam, generally, consists of mountainous and lowland patterns. North of Vietnam includes the Northeast Region, the Northwest Region and the Red River Delta. The Northeast Region stretches from the Red River Valley to the Gulf of Tonkin with mountainous area scattered of elevations between 300–1600 m asl; and Tay Con Linh, the highest mountain peak in the region reaching 2,341 meters asl. The Northwest Region is comprised of mountains that run from the north of the Sino-Vietnamese border to the west of Thanh Hoa Province, Fansipan Mountain, which measures 3,143 m asl at the peak. The southern part of the country is well known for the lowland of Mekong Delta, approximately 40,000 sq. km with an average elevation at under 10 m asl (Averyanov et al., 2003; Sterling et al., 2006).

Central Vietnam is featured by Truong Son Range and coastal lowlands. Truong Son Mountains are the Vietnamese part of Annamite Range, which is the main uplands in Indochina, and spreading over Laos, Vietnam, and Cambodia, from northwest to southeast (Bain and Hurley, 2011). The Truong Son Mountains run from Nghe An Province to Da Nang City, along the boundary between Vietnam and Laos, and have a few peaks reaching 1300 m asl. This Chain is continued by Kon Tum Massif, with elevations over 500 m asl, with the highest peak at Ngoc Linh Mount (2598 m asl), and joins the lower uplift of Pleiku Plateau, at about 800–1400 m asl, in Gia Lai Province (Averyanov et al., 2003; Sterling et al., 2006).

## **Climates**

The climate of Vietnam can be divided into seven types: (1) Monsoon tropical climate with cold winter and summer rains; (2) Monsoon tropical climate with cold winter and summer-autumn rains; (3) Monsoon tropical climate with warm winter-autumn-winter rains; (4) Monsoon tropical climate with warm winter and autumn-winter rains; (5) Monsoon tropical climate with warm winter and summer rains; (6) Monsoon subequatorial climate with summer rains; and (7) Monsoon tropical climate associated with mountains (Averyanov et al., 2003).

## **Vegetation types**

Vegetation of Vietnam is belonging to the Indochinese Floristic Region, including six zones: Sikang-Yunnan, South Chinese, North Indochinese, Central Annamese, South Annamese, and South Indochinese (Averyanov et al., 2003).

## **Biodiversity**

Vietnam's biodiversity draws scientists for several reasons. First, the country harbors a globally significant diversity of species, second, scientists have described an

unexpectedly large number of new species since 1992, and third, a high proportion of known species are endemic to the country (Sterling et al., 2006).

Regarding the Vietnamese amphibian fauna, Bourret (1942) long ago documented 78 species, and Nguyen and Ho (1996) listed a total of 82 species. However, Inger et al. (1999) recorded 100 species from the country, which is approximately 20% higher than the Bourret's (1942) count. Soon after Inger et al. (1999), Orlov et al., (2002) summarized the diversity of amphibians in Vietnam with 147 species, and Nguyen et al. (2005) recorded a total of 162 species.

Although Nguyen et al. (2009) reported 177 species of amphibians for Vietnam in the most recent checklist, the species number of amphibians from this country has even raised to 181 in 2010 (see Ziegler and Nguyen, 2010), and new discoveries have been continued in the recent four years (Milto et al., 2013; Nguyen et al., 2013a, b; Nguyen et al., 2014a, b, c, d; Nishikawa et al., 2012, 2013; Ohler et al., 2011; Orlov et al., 2012; Ostroshabov et al., 2013; Rowley et al., 2011a, b, 2012a, b, 2014; Stuart et al., 2011). Molecular analyses combined with other traditional methods such as morphology, and bioacoustics, could help scientists to understand the taxonomy and evolution of amphibians (Cocroft and Ryan, 1995; Brown and Stuart, 2012), and many species have been newly discovered or revised based on molecular phylogenetic evidence (e.g., Biju et al., 2010; Li et al., 2011; Yu et al., 2013).

Recent enormous increase in Vietnamese amphibian diversity is the consequence of such works, Amphibian fauna of Vietnam comprises of many complex and taxonomically uncertain species (Nguyen et al., 2009; Orlov et al., 2012). In addition, researches on natural history of Vietnamese amphibians are still limited (Ziegler et al., 2008; Ziegler and Nguyen, 2008). Although many past researches focused on the herpetofauna of this country, the knowledge about the actual herpetofauna diversity of Vietnam is incompletely studied and still imperfect, particularly in the case of tree frogs of the family Rhacophoridae.

### **Vietnamese rhacophorid tree frogs**

In Vietnam, Rhacophoridae (Amphibia: Anura) represents one of the most diverse families, and approximately 70 species in 11 genera have been recorded so far, among a

total of more than 360 currently known confamilial species in the world (Frost, 2014). Of these, 16 species have been described as new during recent five years (Milto et al., 2013; Nguyen et al., 2013; Nguyen et al., 2014 a, b, c; Orlov et al., 2012; Ostroshabov et al., 2013; Rowley et al., 2010, 2011a, b, 2012, 2014). Vietnamese rhacophorids occupy various ecological niches in mountain tropical rainforests on various altitudes from the sea level to more than 2500 m asl (Orlov and Ananjeva, 2007). Therefore, field survey on these frogs is not easy, and like many other amphibian groups, systematic study of the family Rhacophoridae is still incomplete. Taxonomic assignment for many species is still doubtful, particularly in the small-sized and hidden life-mode groups. Identification of numerous new species discovered during the past decades, has been made mainly based on the results of molecular phylogenetic studies. These studies also resulted in the change in generic allocation in many species (Delorme et al., 2005; Li et al., 2008, 2009; Wilkinson et al., 2002; Yu et al., 2008, 2009, 2010; Rowley et al., 2011a), while the study of morphological synapomorphies to define new taxonomic groups has been rarely done mainly due to their high level of diversification, and limited number of diagnostic characters.

## **Outlines of the thesis**

In this thesis, I study Vietnamese rhacophorid frogs, with special attention to four representative genera: *Liuixalus* Li, Che, Bain, Zhao, and Zhang, 2008; *Kurixalus* Ye, Fei, and Dubois, 1999; *Rhacophorus* Kuhl and Van Hasselt, 1822, and *Theloderma* Tschudi, 1838. Many species in these genera have restricted geographical distribution range, and some are hitherto known only from a single or very few specimens deposited in the museum collections. Thus, I tried in the present study to make new collections of specimens and relevant data of rhacophorid frogs from intensive fieldwork, so as to better and comprehensively investigate intra- and interspecific variations of these frogs, so as to fix their taxonomy. This approach could also elucidate presence of possible undescribed and/or cryptic taxa. For these purposes, I first tried to elucidate phylogenetic relationships of rhacophorid frogs using molecular methods, and then made detailed examination of their morphology. Specific objectives of this study are: (1) discovery the new rhacophorid taxa and new record for Vietnam; (2) updates of molecular and morphological data for the

Vietnamese rhacophorid; (3) estimation of phylogenetic and systematic relationships for the Vietnamese species; and (4) study on generic placement and taxonomic assignment for Vietnamese rhacophorids.

For these purposes, field survey was conducted during a period of more than six years (2008–2014) in 28 provinces of Vietnam (Fig. 1-1). Special field surveys were conducted from 2012 to 2014 with the priority in poorly studied areas such as the border between Vietnam and China [the study site of number 9 in Fig. 1-1: Lao Cao province (Y Ty Mountain); number 10: Dien Bien province (Muong Nhe Nature Reserve)], the border between Vietnam and Lao [number 11: Son La province (Copia and Sop Cop Nature Reserves), number 13: Thanh Hoa province (Xuan Lien Nature Reserve)], the border between Vietnam and Cambodia [number 22: Kon Tum province (Dak Gle); number 23: Gia Lai province (K'Bang)] and Cat Ba Island (number 3: Hai Phong City). As a result, I could accumulate a total of 320 specimens, most of which I examined morphologically and molecularly. In the Chapter 2, I report on the first discovery of *Liuixalus* from Vietnam, in the Chapters 3 and 4, I introduce two new cryptic species of *Kurixalus*, in the Chapter 5, I summarize relationships of poorly known or disputed species of *Rhacophorus*, and in the Chapter 6, I summarize relationships of *Theloderma* based on a species new to Vietnamese fauna. Finally, I summarize my findings on Vietnamese rhacophorids and discuss problems clarified and left unresolved.

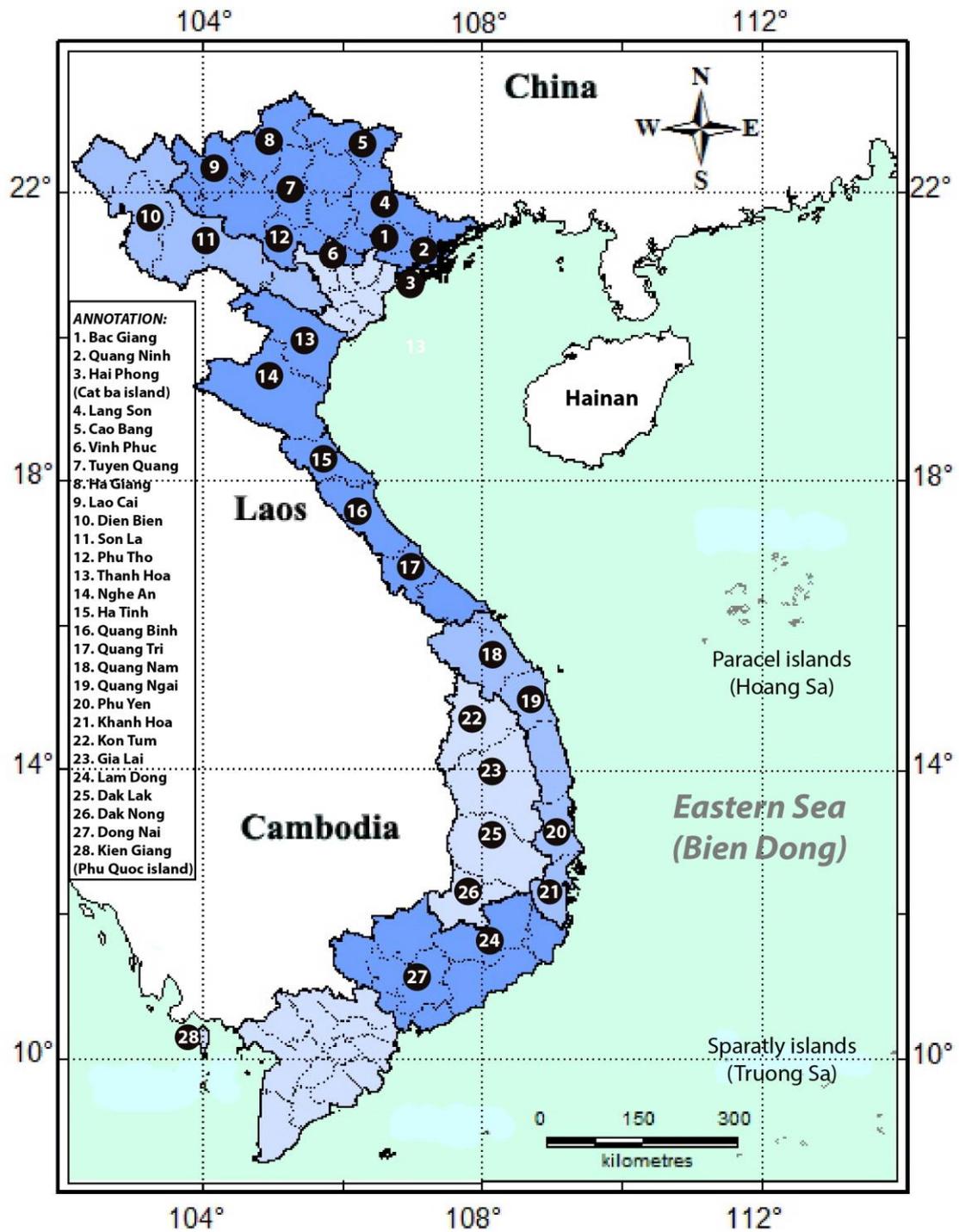


Figure 1-1. Map showing the study site in Vietnam.

## CHAPTER 2

### First record of the tree frog genus *Liuixalus* from Vietnam

#### 2.1 Introduction

The Old-World tree-frog genus *Liuixalus* Li, Che, Bain, Zhao, and Zhang, 2008 has been known to contain three species *L. romeri* (Smith, 1953), *L. ocellatus* (Liu and Hu in Liu, Hu, Fei, and Huang, 1973), and *L. hainanus* (Liu and Wu, 2004) occurring in southeastern China: Guangdong-Guangxi border area, Hongkong, and Hainan island, (e.g., Fei et al., 2009). These species were formerly placed in various genera, *Philautus* Gistel, 1848, *Chirixalus* Boulenger, 1893, *Chiromantis* Peters, 1854, and *Aquixalus* Delorme, Dubois, Grosjean and Ohler, 2005 (Smith, 1953; Liu et al., 1973; Bossuyt and Dubois, 2001; Frost et al., 2006; Fei et al., 2010). However, based on results of recent molecular studies on rhacophorids (Li et al., 2008), they were moved to a distinct genus *Liuixalus* with *L. romeri* as the type species.

Although Frost (2013) listed Vietnam in the distribution range of the genus *Liuixalus*, no formal report of occurrence of the genus has been given outside Southeast China. However, the collection of small rhacophorid species in our recent field survey in northern Vietnam proved to include a member of the genus *Liuixalus* through subsequent morphological and molecular analysis. In the latter analysis, a juvenile specimen formed a clade on mtDNA trees with members of the genus, but was placed in a unique position different from available sequences of the congeners. Because the specimens also differed morphologically from all the known members of *Liuixalus*, we described them as a new member of the genus. This is also the first record of the genus outside China.

#### 2.2 Materials and methods

Specimens of a rhacophorid sp. were collected on Cat Ba Island (Fig. 2-1) from 29 to 30 April 2008 by Nikolai L. Orlov, Konstantin D. Milto, and Roman A. Nazarov; 10 – 22 October 2011 by Nikolay A. Poyarkov and Eduard A. Galoyan; 18 September 2013 by An Thi Hang, and were deposited in the collections of: Vietnam National Museum of

Natural, Vietnamese Academy of Science and Technology Hanoi (VNMN), Vietnam; Zoological Institute, Russian Academy of Sciences (ZISP) and Department of Vertebrate Zoology, Lomonosov Moscow State University (ZMMU), Russia. For comparisons, continental *Liuixalus* specimens deposited in the collections of Chengdu Institute of Biology (CIB), China were studied.

We obtained DNA sequence data from tissues preserved in 99% ethanol for a juvenile specimen of rhacophorid species from Vietnam (VNMN 3684 = TH 2155 [Tsutomu Hikida's field number]; GenBank accession number AB871420). Methods for DNA extraction, and amplification and sequencing of the mtDNA fragments are the same as those reported by Kuraishi et al. (2013). We deposited the resultant sequence (858 base pairs [bp] of partial sequences of mitochondrial 16S rRNA gene) in GenBank (Accession numbers as shown above).

In order to generically assign the rhacophorid specimen from Vietnam, we first compared partial sequence of 16S rRNA (ca. 800bp) with GenBank data of rhacophorid species whose congeners are recorded from Vietnam (Orlov 2012), i.e., *Chiromantis hansenae* (Cochran, 1927) (AB813161), *Feihyla kajau* (Dring, 1984) (AB847122), *Gracixalus gracilipes* (Bourret, 1937) (DQ283051), *Kurixalus appendiculatus* (Günther, 1859) (AB847125), *Liuixalus romeri* (EU215528), *Nyctixalus pictus* (Peters, 1871) (DQ283133), *Philautus abditus* Inger, Orlov, and Darevsky, 1999 (GQ285673), *Polypedates leucomystax* (Gravenhorst, 1829) (AB728138), *Rhacophorus kio* Ohler and Delorme, 2006 (AB781695), *Rhaorchestes parvulus* (Boulenger, 1893)(AB871421), *Theloderma leprosum* Tschudi, 1838 (AB847128), and *Buergeria buergeri* (Temminck and Schlegel, 1838) (AB127977). Following the result obtained (see results), we then determined the relationships of the specimen from Cat Ba Island, Vietnam, by comparing the partial sequence of 16S rRNA (ca. 500bp) with those of *Liuixalus romeri* from Hong Kong (CIB [Chengdu Institute of Biology] 20080048; AB871412) and *L. ocellatus* from Wuzhishan, Shuiman, and Diaoluoshan, Hainan Island, China (CIB 20081062, 63, 65–69; AB871413–871419), and additional GenBank data (*L. hainanus* [GQ285671, KC465826], *L. ocellatus* [GQ285672, GU120328, KC465829] and *L. romeri* [EF564535, EF564536]), as well as *Chiromantis hansenae* (AB813161) and *Buergeria buergeri* (AB127977) as out group. For tree construction and calculation of genetic distances (uncorrected p-distance), we followed Kuraishi et al. (2013).

For the specimen molecularly analyzed, we took the following 32 body measurements to the nearest 0.1 mm with a dial caliper under a binocular microscope, and to the nearest 0.01 mm using a micrometer, mainly following Matsui (1984, 1994): (1)

snout-vent length (SVL); (2) head length (HL) from tip of snout to hind border of angle of jaw, not measured parallel with the median line; (3) snout-nostril length (S-NL); (4) nostril-eyelid length (N-EL); (5) snout length (SL); (6) eye length (EL); (7) eye diameter (ED), diameter of the exposed portion of the eyeball; (8) tympanum-eye length (T-EL); (9) tympanum diameter (TD); (10) head width (HW); (11) internarial distance (IND); (12) interorbital distance (IOD); (13) upper eyelid width (UEW); (14) forelimb length (FLL); (15) lower arm and hand length (LAL); (16–19) first to fourth finger disk diameters (1–5FDW); (20) inner palmar tubercle length (IPTL); (21) outer palmar tubercle length (OPTL); (22) hindlimb length (HLL); (23) thigh length (THIGH); (24) tibia length (TL); (25) foot length (FL); (26) first toe length (1TOEL); (27) inner metatarsal tubercle length (IMTL); (28–32) first to fifth toe disk diameters (1–5TDW). We followed the system of description of toe-webbing states used by Savage (1975).

### 2.3 Systematics

In the preliminary analyses using all known 11 genera from Vietnam and an outgroup *Buergeria*, the rhacophorid sp. from northern Vietnam formed a clade with *Liuixalus romeri* with full support (Bayesian posterior probability [BPP]=1.00, bootstrap support [BS]=100%). Rhacophorid sp. from Vietnam substantially differed from species of the ingroup genera (16.3% from *Gracixalus* to 22.6% from *Kurixalus*) other than *Liuixalus* (2.4%). These results clearly suggest placement of the rhacophorid sp. from Vietnam in the genus *Liuixalus*.

After adding sequences both from our own data and from GenBank, support for the monophyly of *Liuixalus* including the rhacophorid sp. from Vietnam was sufficient (BPP=1.00, BS=99%: Fig. 2-2), but the relationships within the clade was not resolved and five groups (*L. hainanus*, *L. romeri* from Jinxiu, Gungxi, the rhacophorid sp. from Vietnam, *L. romeri* from Hong Kong and Shiwanshan, Guangxi, and *L. ocellatus*) were recognized only in ML tree. The rhacophorid sp. from Vietnam was close to *L. romeri* from Jinxiu, Guangx (EF564535, EF564536), but their monophyly was not supported (BPP=0.53, BS=35%).

The rhacophorid sp. from Vietnam differed from the species of *Liuixalus* by small genetic distances (2.2–3.0%). Because the distances between the three species of *Liuixalus*

were also small, ranging from 1.8–3.0%, the rhacophorid sp. from Vietnam can be genetically regarded as a good species. Furthermore, it is also clearly separated morphologically from all nominal species of *Liuixalus*. Thus, we conclude the rhacophorid sp. from Vietnam as a distinct species in the genus *Liuixalus*. Unfortunately, through wrong communications between the colleagues, the species was described as new in two different publications (Milto et al., 2013 and Nguyen et al., 2014a) in December 2013 and February 2014, respectively. According to the law of priority, the name in Milto et al. (2013) is valid, and that of Nguyen et al. (2014a) is relegated to its synonymy.

***Liuixalus calcarius* Milto, Poyarkov, Orlov, and Nguyen, 2013.**

**Synonymy**

*Liuixalus catbaensis* Nguyen, Matsui, and Yoshikawa, 2014.

**Holotype**

ZISP 10522, an adult male, collected near Viet Hai village, Cat Ba island, Hai Phong province, Vietnam, 29 – 30 April 2011 by Nikolai L. Orlov, Konstantin D. Milto, and Roman A. Nazarov.

**Paratypes**

ZISP 10523 – 10527, five adult males; ZISP 10528, one adult female, collected near Viet Hai village, Cat Ba island, Hai Phong province, Vietnam, 29 – 30 April 2011 by Nikolai L. Orlov, Konstantin D. Milto, and Roman A. Nazarov; ZISP 10667, one adult female, collected at Cat Ba island, Hai Phong province, Vietnam, April 2008 by Nikolai L. Orlov; ZMMU A-4940-1; ZMMU A-4940-2; ZMMU A-4940-3, three males and ZMMU A-4940-4, one adult female, collected at Cat Ba island, Hai Phong province, Vietnam, 10 – 22 October 2011 by Nikolay A. Poyarkov and Eduard A. Galoyan.

**Etymology**

The Latin adjective *calcarius* means “limestone,” referring to the limestone karstic habitat of the species.

**Diagnosis**

This species is assigned to the genus *Liuixalus* based on molecular evidence, and has morphological characteristics common to the congeners: intercalary cartilage present

between terminal and penultimate phalanges of digits; tips of digits expanded into large discs bearing circum-marginal grooves; fingers free of web; toes very poorly webbed; no web between outer metatarsals; vomerine teeth absent. *Liuixalus calcareus* is distinguishable from all its congeners by the brick-red dorsum lacking dark markings.

#### **Description of unsexed juvenile specimen (VNMN 3684)**

Unsexed juvenile; SVL 12.8 mm; head longer (HL 5.3 mm, 41.5%SVL) than wide (HW 5.1 mm, 39.8%SVL), wider than body; snout rounded in dorsal and lateral view, length (SL 2.3 mm, 18.1%SVL) longer than eye length (EL 2.2 mm, 17.2%SVL, ED 1.9 mm, 14.6%SVL), projecting beyond mouth; canthus rostralis clear; loreal region vertical and concave; nostrils slightly nearer to tip of snout (S-NL 1.1 mm; 8.4%SVL) than to eye (N-EL 1.0 mm, 7.5%SVL); internarial distance (IND 1.8 mm, 13.9%SVL) more than interorbital distance (IOD 1.6 mm, 12.7%SVL) and upper eyelid (UEW 1.2 mm, 9.2%SVL); eye large, protuberant; pupil horizontal; tympanum distinct, circular, diameter (TD 0.8 mm, 6.4%SVL) about one-third of eye length and separated from eye by one-third of tympanum diameter (T-EL 0.2 mm, 1.8%SVL); vomerine teeth absent; choana oval; tongue notched posteriorly; vocal sac absent.

Forelimb slender and long (FLL 7.8 mm, 61.2%SVL); hand and forearm long (LAL 6.2 mm, 48.6%SVL); finger length formula: I < II < IV < III (Fig. 2-5A); expanded disks each with a circummarginal groove and a transverse ventral groove; disks on third finger (3FDW 0.38 mm, 3.0%SVL,) slightly wider than those on second (2FDW 0.35 mm, 2.7%SVL) and fourth (4FDW 0.33 mm, 2.6%SVL), and much wider than first (1FDW 0.24 mm, 1.9%SVL), all much narrower than tympanum; webbing between fingers and lateral fringes on fingers absent; subarticular tubercles moderately developed; inner palmar tubercle flat (IPTL 0.39 mm, 3.0%SVL), outer one much smaller (OPTL 0.23 mm, 1.8%SVL); nuptial pad absent.

Hindlimb moderately long (HLL 19.5 mm, 152.3%SVL); tibiotarsal articulation reaching to point between eye and nostril when fully stretched leg adpressed to body; heels touching each other when thigh (THIGH 6.8 mm, 53.2%SVL) and tibia (TL 6.9 mm, 53.6%SVL) placed at right angle to body; foot (FL 5.3 mm, 41.7%SVL) much shorter than tibia; toe length formula I < II=V < III < IV; toe tips expanded into enlarged disks each with a circummarginal groove and a transverse ventral groove, width of fourth and third toe disks (4TDW 0.41 mm, 3.2%SVL; 3TDW 0.39 mm, 3.1%SVL) wider than those of

finger disks and remaining toe disks (1TDW 0.25 mm, 2.0%SVL; 2TDW 0.30 mm, 2.4%SVL; 5TDW 0.32 mm, 2.5%SVL); webbing formula I 2+–2++ II 2+–3++ III 2<sup>1</sup>/<sub>2</sub>–4 IV 3<sup>1</sup>/<sub>3</sub>–1<sup>3</sup>/<sub>4</sub> V (Fig. 2-5B); subarticular tubercles rounded; inner metatarsal tubercle oval, small (IMTL 0.39 mm, 3.0%SVL), less than half length of first toe (1TOEL 0.99 mm, 7.7%SVL); no outer metatarsal tubercle.

Dorsal surface nearly smooth, sparsely scattered with minute, blunt tubercles; strongly developed supratympanic fold between eye and arm insertion; flank scattered with flat tubercles; ventral surface smooth on lower jaw and breast, composed of flat granules on abdomen; no protruded tubercle at heel.

### **Color**

In life, dorsal ground color on body brick-red with few faint brown spots (Figs. 2-3, 4A); narrow dark stripe between eye and nostril below canthus; lips barred with white and black; side of head from snout through below eye to temporal region dusted with brown; darker brown stripe on supratympanic fold between eye and arm insertion, including posterodorsal one-third of tympanum and continuing to dark mark at base of upper arm; dorsal red fading to ventral gray at side of trunk, where several irregular dark markings scattered (Fig. 2-4C); forearm and hindlimb dorsally brick-red with faint dark brown bars on forearm and hindlimb; black mark at groin extending to base of thigh; thigh posteriorly dark brown, scattered with white posteroventrally and forming black marking below cloaca; distal half of toe tips darker; ventral side grayish white sparsely dotted with melanophores, more densely on throat than abdomen (Fig. 2-4B); iris golden with black reticulations. In preservative, pattern has not obviously changed, although all dorsal surfaces darkened.

### **Range**

Known from the type locality, Ao Ech (Frog Pond) within the Cat Ba National Park, Cat Ba Island, the largest island of the Cat Ba Archipelago in Ha Long Bay, northern Vietnam (Fig. 2-1).

### **Natural history**

All specimens of the type series were found at the locality on limestone placers in the tropical forest. A juvenile specimen used for molecular analysis was also from the type locality, and was found during the daytime, in a disturbed primary forest surrounded by large limestone rocks. There was a small pool formed in a small stream (ca. 1 m wide)

in the forest, around which the frog was found. The air temperature at the time of finding was not recorded, but was 27°C at 19:40 h. At night *Hylarana guentheri* (Boulenger, 1882), *Microhyla fissipes* Boulenger, 1884, *Microhyla heymonsi* Vogt, 1911, *Polypedates mutus* (Smith, 1940), and *Chiromantis hansenae* (Cochran, 1927) were found there, but no additional specimen of *L. calcareus* was encountered.

### Comparisons

*Liuixalus calcareus* is easily distinguished from *L. romeri* from Hong Kong and Guangxi, China, *L. ocellatus* from Hainan Island, and *L. hainanus* from Hainan Island by: having dorsally and laterally rounded snout, whose length slightly longer than eye (vs. snout slightly constricted in *L. hainanus* and tip bluntly pointed in *L. romeri*, snout shorter than eye in *L. ocellatus* and *L. hainanus*), tympanum diameter about one-third of eye length and separated from eye by one-third of tympanum diameter (vs. tympanum diameter about half eye diameter in *L. romeri* and *L. hainanus*, distance between tympanum and eye about half tympanum diameter in *L. romeri*), hand and forearm 49% of body length (vs. about 40% in *L. romeri*), no webbing between fingers (vs. slight webbing between outer two fingers in *L. romeri*), tibiotarsal articulation reaching between eye and nostril (vs. articulation reaching to anterior corner of eye in *L. ocellatus*, to nostril or snout tip in *L. romeri*, and exceeding snout tip in *L. hainanus*), heels touching each other when thigh and tibia placed at right angle to body (vs. heels remarkably overlapping in *L. hainanus*), webbing between fourth and fifth toes not reaching to third articulation of fourth (vs. reaching to second articulation in *L. ocellatus*), outer metatarsal tubercle absent (vs. tubercle small or indistinct in *L. romeri*, and small in *L. ocellatus*), dorsal surface nearly smooth (vs. skin with a few tubercles in *L. romeri* and relatively rough, scattered with large and small tubercles in *L. hainanus*) with only sparsely scattered minute, blunt tubercles between shoulder and sacral regions (vs. upper eyelid with tubercles relatively clear in *L. ocellatus* and numerous in *L. hainanus*, and a pair of black, small rounded warts posterior to eye in *L. ocellatus*), dorsal ground color brick-red with few, vaguely defined small brown spots (vs. ground color mostly olive brown or light reddish brown in *L. romeri*, yellowish, reddish, or dark brown in *L. ocellatus*, and brown in *L. hainanus*, with dark horizontal or triangle mark between eyes and a pair of X-shaped dark marks on shoulder, followed with a reverse-V mark in *L. romeri*, a pair of reverse triangular or V-shaped dark markings between eyes, posteriorly with a pair of small black marking in *L. ocellatus*, and with irregular or X-shaped blackish brown markings, medially with a clear,

light brown elliptical mark, followed by a triangular back brown mark in *L. hainanus*), and grayish white ventrum sparsely dotted with melanophores (vs. ventrally cream white with gold-yellow tinge, scattered with several dark spots in *L. romeri*).

## 2.4 Discussion

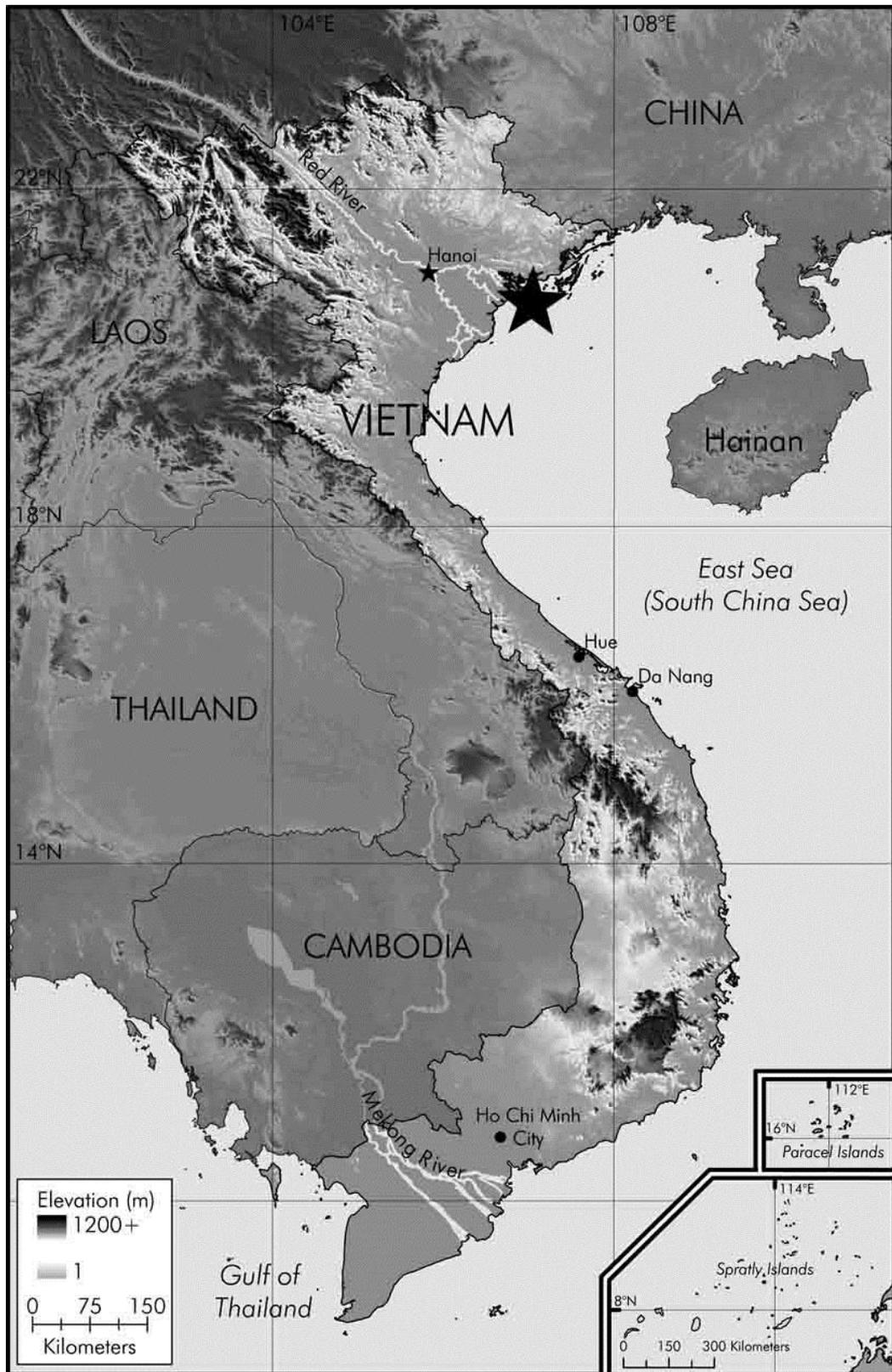
The family Rhacophoridae is the most diverse group of amphibians in Vietnam with a total of 10 genera and 61 recognized species recognized so far (Orlov, 2012; Rowley et al., 2010, 2011b; Nguyen et al., 2013; Nguyen et al., 2014a). Indeed the increase in the number of Vietnamese amphibian species greatly indebted to the discovery of new rhacophorids. Description of recent new species is usually made based on molecular and acoustic data (e.g., Rowley, 2011a, b). In case of the species newly reported here, the molecular, as well as morphological, data obtained clearly demonstrate its distinct species status.

Discovery of *Liuixalus* from northern Vietnam is not surprising, since the region is close to southern China, where the genus was restricted previously. In a megophryid frog, *Leptobrachium (Vibrissaphora) chapaense* (Bourret, 1937), some Vietnamese population proved to be conspecific with *L. (V.) hainanense* Ye and Fei in Ye, Fei, and Hu, 1993 from Hainan Island by Matsui et al. (2010), who estimated their separation at 2.6 MYBP, conforming to the date of separation of these regions in the early Pleistocene. Genetic distances on which this dating was made were 1.8–3.0% in 12S and 16S rRNA genes (Matsui et al., 2010). Compared with these values, distances obtained for 16S rRNA genes between *Liuixalus calcareus* and *L. romeri* from Jinxiu are small (1.8%), suggesting their separation later than in *L. (V.) hainanense*. However, 12S and 16S rRNA genes on which dating for *Leptobrachium (Vibrissaphora)* was made in Matsui et al. (2010) would generally give lower values than 16S rRNA gene alone. Apart from this, it is noted that interspecific genetic distances reported for *Liuixalus* are very small, even much smaller than in other rhacophorine genera (e.g., Li et al., 2008, 2013; this study).

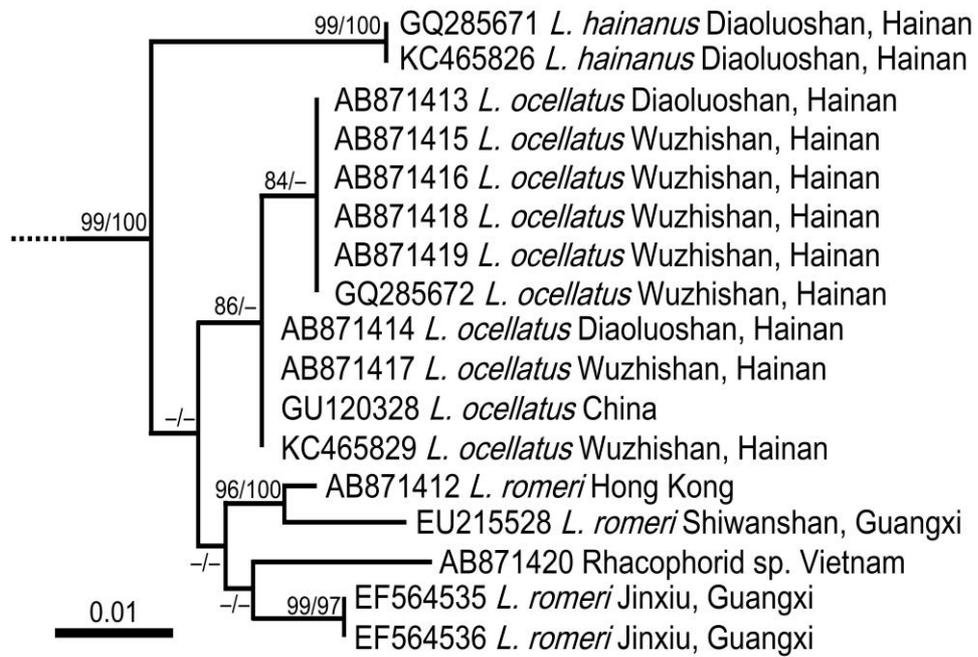
The family Rhacophoridae is split into two subfamilies, Buergeriinae Channing, 1989 and Rhacophorinae Hoffman, 1932 (Channing, 1989). Members of Buergeriinae are restricted to the eastern periphery of Asian continent. Within Rhacophorinae, *Liuixalus* is now generally considered as the sister taxon of all the remaining rhacophorines (Li et al.,

2008, 2009; Yu et al., 2008, 2009; Pyron and Wiens, 2011; Hertwig et al., 2012). Meanwhile the range of the genus is restricted to southeastern China from Hong Kong through Guangxi to Hainan, and now northern Vietnam, all in the eastern periphery of the Asian continent. Although the origin and subsequent evolutionary history of Rhacophoridae are in debate (e.g., Bossuyt and Milinkovitch, 2001; Li et al., 2013), the fact that phylogenetically very old groups, subfamily Buergeriinae and the genus *Liuixalus*, occur on the eastern periphery of Asian continent is biogeographically interesting.

In spite of remarkable phylogenetic antiquity, degree of genetic divergence within the genus *Liuixalus* is very small as shown above. This low genetic diversity is suspected to be a result of past population bottlenecks. Probably, after very old separation from the ancestral stock leading to the other rhacophorine genera in the Eocene (around 50.0-36.9 MYBP: Li et al., 2013), ancestors of *Liuixalus* might have experienced very limited range expansions, severe range fragmentations, and local extinctions before much more recent speciation events occurred at 8.3-3.5 MYBP in the late Miocene (Li et al., 2013). In order to test this hypothesis, further analyses of additional specimens of *L. calcareus* are necessary.



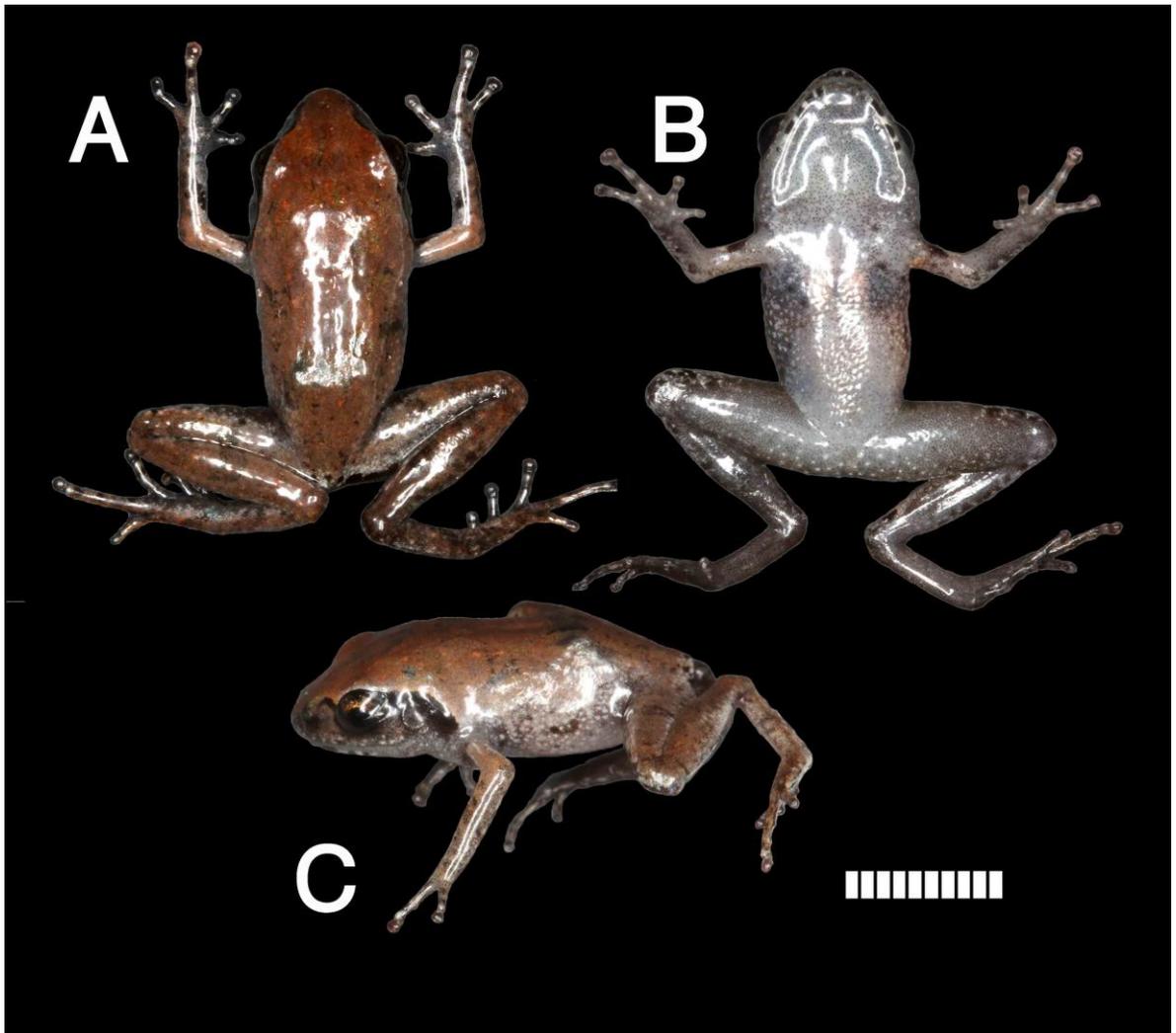
**Figure 2-1.** Map of Vietnam showing Cat Ba Island (filled star), where the rhacophorid sp. was found.



**Figure 2-2.** Maximum-likelihood (ML) tree from ca. 500 bp partial sequence of mitochondrial 16S rRNA gene for the rhacophorid sp. from Vietnam and *Liuixalus*. A rhacophorine *Chiromantis hansenae* and a buergeriine *Buergeria buergeri* were used as outgroup. Numbers above or below branches represent bootstrap supports for ML inferences and Bayesian posterior probabilities (ML-BS/BPP).



**Figure 2-3.** A juvenile specimen (VNMN 3684) of *Liuixalus calcareus* in life.



**Figure 2-4.** (A) Dorsal, (B) ventral, and (C) lateral views of a juvenile (VNMN 3684) of *Liuixalus calcareus* in an anesthetized condition. Scale bar=10 mm.



**Figure 2-5.** Ventral views of left (A) hand and (B) foot of juvenile (VNMN 3684) of *Liuxalus calcareus* after preservation. Scale bar=3 mm.

## CHAPTER 3

### A new tree frog of the genus *Kurixalus* from Vietnam

#### 3.1 Introduction

A rhacophorid frog genus *Kurixalus* Ye, Fei, and Dubois In Fei, 1999 occurs in Asia from the Ryukyus of Japan, Taiwan, the Philippines, Borneo, Sumatra, Malay Peninsula, Thailand, Laos, Cambodia, Vietnam, southern China, Myanmar, and eastern India. *Kurixalus* was originally established as a monotypic genus containing only Japanese and Taiwanese species *Chirixalus eiffingeri* (Boettger, 1895), but subsequent molecular studies have resulted in the placement in this genus of many small rhacophorids whose generic status was ambiguous.

At present, about 10 species are assigned to the genus *Kurixalus* (Yu et al., 2013), but there still remain several unnamed species from little explored regions. During our expedition to high mountains of southern Vietnam, we collected several specimens of small rhacophorids that proved to be a member of the genus *Kurixalus* by the later molecular phylogenetic analysis. Because the specimens are not only genetically distinct, but also morphologically easily distinguishable from all the other congeners by their unique immaculate green dorsum, we reported them as a new species (Nguyen et al., 2014b).

#### 3.2 Materials and methods

In order to assign the generic position of the rhacophorid specimens from southern Vietnam, we compared partial sequence of 16S rRNA (ca. 1300 bp) of representing rhacophorid genera, *Chiromantis*, *Feihyla*, *Gracixalus*, *Kurixalus*, *Liuixalus*, *Nyctixalus*, *Philautus*, *Polypedates*, *Rhacophorus*, *Raorchestes*, *Theلودerma*, and an outgroup *Buergeria* (Table 3-1).

Methods for DNA extraction, and amplification and sequencing of the mtDNA fragments followed Kuraishi et al. (2013). The resultant new sequences were deposited in GenBank (Accession numbers AB933284–933309: Table 3-1). The alignment matrices with 1316 sites for 16S rRNA were subjected to estimate phylogenetic relationships using maximum likelihood (ML) and Bayesian inference (BI). Pairwise comparisons of uncorrected sequence divergences (p-distance) were also calculated. Details for these procedures are given in Kuraishi et al. (2013).

For morphometric comparisons, we took the following 21 body measurements to the nearest 0.1 mm with dial calipers, following Matsui (1984) and Matsui (1994): (1) snout-vent length (SVL); (2) head length (HL); (3) head width (HW); (4) internarial distance (IND); (5) interorbital distance (IOD); (6) upper eyelid width (UEW); (7) nostril-eyelid length (N-EL); (8) snout length (SL); (9) eye length (EL); (10) eye diameter (ED), diameter of the exposed portion of the eyeball; (11) tympanum diameter (TD); (12) tympanum-eye length (T-EL); (13) forelimb length (FLL); (14) lower arm and hand length (LAL); (15) first finger length (1FL); (16) inner palmar tubercle length (IPTL); (17) hindlimb length (HLL); (18) tibia length (TL); (19) foot length (FL); (20) inner metatarsal tubercle length (IMTL); and (21) first toe length (1TOEL). For finger and toe disks, measurements were taken to the nearest 0.01 mm using a binocular dissecting microscope equipped with a micrometer: (22–25) first to fourth finger disk diameter (1–4FDW); and (26–30) first to fifth toe disk diameter (1–5TDW). We followed the system of description of toe-webbing states used by Savage (1975). Specimens examined are stored in the Vietnam National Museum of Nature, Hanoi (VNMN), the Natural History Museum, London (BM), the Muséum National d’Histoire Naturelle, Paris (MNHN), and the Graduate School of Human and Environmental Studies, Kyoto University (KUHE).

### **3.3 Systematics**

We obtained 1316 bp of 16S rRNA fragments of mtDNA gene for 40 samples, including outgroup (Fig. 3-2). Of 1316 nucleotide sites, 715 were variable, and 500 were parsimoniously informative within ingroup species. The best substitution model was J2+G with gamma shape parameter (G) of 0.280 for ML and GTR+G of 0.306 for BI. The

likelihood values (-lnLs) of the ML and BI trees were 20861.279 and 20915.613, respectively.

The specimens of rhacophorid species from southern Vietnam examined here showed some variations from each other (uncorrected p-distance of 0.4–5.4%), but formed a fully-supported clade to be regarded as a species. The clade of this species is a sister clade to *K. banaensis* (Bourret, 1939), and the species was confirmed to be a member of *Kurixalus*. Although relationships within the genus were not fully resolved (Fig. 3-2), the clade containing *Kurixalus* sp. from southern Vietnam and *K. banaensis* was sister to the clade including all the other congeners except for *K. eiffingeri*, *K. idiotocus* (Kuramoto and Wang, 1987), and *K. appendiculatus* (Günther, 1858). From the eight species of *Kurixalus* examined, *Kurixalus* sp. from southern Vietnam exhibited substantially large genetic distances (uncorrected p-distance of 6.9–18.1%), values larger than the distance between specimens of *K. bisacculus* (Taylor, 1962) and *K. baliogaster* (Inger, Orlov, and Darevsky, 1999) (3.0–5.2%). Furthermore, *Kurixalus* sp. from southern Vietnam is also clearly separated morphologically from all the other congeners, including its sister species, in congruence with genetic separation. Thus, we described *Kurixalus* sp. from southern Vietnam as new (Nguyen et al., 2014b).

### ***Kurixalus viridescens* Nguyen, Matsui, and Duc, 2014**

#### **Holotype**

Adult female VNMN 03802 (field number KH2011.04), collected by Duc Minh Hoang on 29 December 2011 from Hon Ba Nature Reserve (12°07'04" N, 108°56'46" E, 1540 m asl), Khanh Hoa Province, Southern Vietnam.

#### **Paratype**

Nine females: VNMN 03813–03816 (field number KHA 003–006) data same as the holotype; VNMN 03803 (field number TA0968), VNMN 03804 (field number TA0971) collected by Tao Thien Nguyen in November 2011 from the same locality as the holotype; VNMN 2013.25, 2013.72, 2013.74 (field number all TN-3-BQ) collected by Cuong The Pham in June 2013 from Bidoup of Bidoup-Nui Ba National Park (12°10'49" N, 108°40'24" E; 1590 m asl), Lam Dong Province, Southern Vietnam.

## Etymology

The specific name is a Latin adjective, referring to the uniformly greenish dorsal color of the species.

## Diagnosis

The species is assigned to the genus *Kurixalus* only by molecular phylogenetic evidence, but has the characteristics common to the genus: intercalary cartilage present between terminal and penultimate phalanges of digits; tips of digits expanded into large discs bearing circummarginal grooves; snout tip pointed; fingers poorly webbed; dermal fringes on forearm and tarsus present. *Kurixalus viridescens* can be distinguished from all other species of *Kurixalus* by the following combination of characteristics: female SVL 28.7–36.6 mm; snout tip pointed but not forming a corn; no dermal ridge around cloaca; vomerine teeth absent; solid green dorsum without any dark spots or markings; a lemon-yellowish venter without markings.

## Description of holotype

SVL 36.3 mm; body robust; head (HL 13.4, 36.9%SVL) slightly shorter than wide (HW 13.8, 38.0%SVL); snout (SL 5.2, 14.3%SVL) subequal to eye (EL 5.3, 14.6%SVL), dorsally pointed at tip (Fig. 3-4), sloping anteroventrally in profile, and projecting over lower jaw; canthus blunt; lore oblique, slightly concave; nostril slightly protuberant, nearer to tip of snout than to eye; internarial distance (IND 3.3, 9.1%SVL) narrower than interorbital (IOD 4.3, 11.8%SVL); latter wider than upper eyelid (UEW 3.3, 9.1%SVL); pineal spot absent; eye large, protuberant, diameter (ED 4.2, 11.6%SVL) much larger than eye-nostril (N-EL 2.2, 6.1%SVL); pupil horizontal; tympanum distinct, subcircular, length (1.7, 4.7%SVL) less than half eye diameter and separated from eye by one-third of tympanum diameter (T-EL 0.7, 1.9%SVL); vomerine teeth absent; tongue deeply notched posteriorly.

Forelimb long (FLL 24.1, 66.4%SVL); relative finger length I<II<IV<III; length of first finger (1FL 4.3, 11.8%SVL measured from distal edge of inner palmar tubercle) subequal to diameter of eye; tips of all fingers dilated into horizontally elongate large disks with circummarginal and transverse ventral grooves; disk of third (3FDW 2.5, 6.8%SVL) and fourth fingers (4FDW 2.3, 6.5%SVL) wider than tympanum; webs between fingers poorly developed, finger webbing formula I 2–2<sup>1</sup>/<sub>4</sub> II 2–3 III 2<sup>1</sup>/<sub>2</sub>–2<sup>1</sup>/<sub>2</sub> IV (Fig. 3-5A); fringe of skin on edge of fingers; subarticular tubercles distinct, rounded, formula 1, 1, 2,

2; no supernumerary tubercles on metacarpal; prepollex not prominent, oval; distinct inner (IPTL 1.7, 4.7%SVL) and two indistinct outer palmar tubercles.

Hindlimb long (HLL 56.8, 156.5%SVL), about 2.4 times length of forelimb; tibia not long (TL 18.1, 49.9%SVL), heels overlapping when limbs are held at right angles to body; tibiotarsal articulation of adpressed limb reaching middle of eye; foot (FL 16.2, 44.6%SVL) shorter than tibia; relative length of toes I<II<III<V<IV; tips of toes expanded into round disks with distinct circummarginal grooves, smaller than those of fingers (4TDW 2.1, 5.7%SVL; 5TDW 2.1, 5.7%SVL); webs between toes moderately developed, formula I 2–2<sup>3</sup>/<sub>4</sub> II 1<sup>1</sup>/<sub>2</sub>–2<sup>3</sup>/<sub>4</sub> III 1<sup>1</sup>/<sub>2</sub>–3 IV 2<sup>1</sup>/<sub>2</sub>–1<sup>3</sup>/<sub>4</sub> V (Fig. 3-5B); two outer metatarsals separated with webbing; subarticular tubercles partially distinct, rounded, formula 1, 1, 2, 3, 2; supernumerary tubercles absent; a small, oval inner metatarsal tubercle, length (IMTL 1.8, 5.0%SVL) about half length of first toe (1TOEL 3.7, 10.2%SVL), but no outer metatarsal tubercle.

Dorsum nearly smooth, with few sparsely distributed small tubercles; skin free of skull; a distinct, oblique supratympanic fold from eye above tympanum, ending at above arm insertion; skin of sides and abdomen areolate; weak ridge of skin on outer edge of forearm forming weak serration; hindlimb smooth, except for coarsely granular ventral side of thigh, weak serration along tarsus, and asperities on base of tarsus; no dermal appendage at vent.

### **Color**

In preservative, dorsal color of body slate blue, extending laterally on flanks and limbs, to lateral half of third and fourth fingers on forelimb, and to lateral half of fourth and fifth toes; serrations at lateral margins of forearm and tarsus light cream; groin to anterior surface of thigh cream; ventrally pinkish cream without marking; distal half of posterior thigh pinkish cream finely dotted with pale brown; ventral sides of hand and foot sparsely dotted with dark brown; webbing between third to fifth toes black, margined with cream, but cream between first to third toes. Color in life, based on a color transparency (Fig. 3-3), head and body dorsally light green without markings, fading to lemon yellow on sides to ventrum; limbs dorsally with very faint dark crossbars; iris gold with some black reticulations.

### **Variation**

Morphometric data are summarized in Table 3-2. Because the holotype and paratypes

are all female, sexual dimorphism could not be determined. The paratypes are similar to the holotype in the body proportion except for the following: SVL varies from 28.7–36.6 mm, with the mean of  $32.8 \pm 2.8$  mm. Relative sizes of characters on the head are larger in the paratypes. A few individuals have IOD smaller than UEW, and SL larger than EL. Some individuals have vaguely defined tympanum or indistinct supratympanic fold on its posterior half. The tibiotarsal articulation reaches the center of the eye, except for one specimen, in which the joint reaches the anterior end of the eye. Some individuals have conical dermal appendage at the heel (Fig. 3-3), which is lacking in the holotype. One specimen has trace of dark brown markings on interorbital, sacrum, and tibial regions. All the other specimens are nearly uniform in coloration and pattern.

### Comparisons

By its unique green and immaculate, and nearly smooth dorsum, *K. viridescens* can be easily distinguished from all the other congeners that have dorsum basically brown in color with darker spots, and covered by tubercles. In addition, *K. viridescens* is differentiated from the other congeners in the following way.

From *K. ananjevae* (Matsui and Orlov, 2004), *K. viridescens* differs by the size and color of body and size of eggs (female SVL 28.7–36.6 mm and ova large and unpigmented vs. female 43 mm, eggs bicolored and small, 1.3–1.5 mm in diameter in *K. ananjevae*). *Kurixalus viridescens* differs from *K. appendiculatus* in smaller body size, skin structure, and vomerine teeth (female SVL 28.7–36.6 mm, dermal appendage at cloaca or vomerine teeth absent vs. females 42–50 mm, conspicuous transverse, infra-cloacal dermal flap and vomerine teeth present in *K. appendiculatus*). From *K. baliogaster*, *K. viridescens* differs by the body size, ventral color, and shape of snout tip (female SVL 28.7–36.6 mm, ventral surface immaculate, and lacking a rostral cone vs. females 35–42 mm, ventral surfaces of head and trunk with black spots, females with a strong rostral cone in *K. baliogaster*). *Kurixalus viridescens* is similar to *K. banaensis* in body size (female SVL 28.7–36.6 mm vs. 34.2 mm in *K. banaensis*) and having large, unpigmented ova, but differs from it by the shape of roreal region and snout tip, and the dermal appendage at cloaca (loreal region normal, snout tip moderately pointed, and dermal appendage absent at cloaca vs. loreal region sloping and deeply concave, snout tip markedly pointed, and fringes present below cloaca in *K. banaensis*). *Kurixalus viridescens* overlaps in body size with *K. bisacculus* with the female SVL of 29 mm, but differs from it by the ventral color and vomerine teeth (ventrum immaculate and lacking vomerine teeth vs. ventral surface with black spots and

vomerine teeth present in *K. bisacculus*). *Kurixalus viridescens* also overlaps in body size with *K. eiffingeri* with female SVL of 32–44 mm, but differs from it in skin structure and color of eggs (a skin flap above cloaca present in females and eggs bicolored, 1.6–1.8 mm in diameter in *K. eiffingeri*). With *K. idiotocus*, *K. viridescens* again overlaps in body size but differs from it in skin structure (females 33–39 mm, granules around anus and on limbs, and dark markings on sides of body present in *K. idiotocus*). *Kurixalus viridescens* differs from *K. naso* (Annandale, 1912) by smaller body size, skin structure, poorly developed toe webbing, and ventral color (SVL 43 mm, snout with a cone, upper arm and tarsus fringed, toe webbing well-developed, and chin and breast marked with dark reticulation in *K. naso*). From *K. odontotarsus* (Ye and Fei, 1993), *K. viridescens* differs by smaller body size and different skin structure and ventral color (female SVL 43 mm, snout pointed and ventrum with dark markings in *K. odontotarsus*). *Kurixalus viridescens* is similar to *K. verrucosus* (Boulenger, 1893) in body size and absence of vomerine teeth, but differs from it in skin structure and ventral color (females 30–36 mm, dark ventral spots, especially on throat, and several whitish tubercles below cloaca present, and toe webbing much better developed in *K. verrucosus*).

### **Range**

Known only from the type locality, Hon Ba Nature Reserve, Khanh Hoa Province, and Bidoup – Nui Ba National Park, Lam Dong Province, southern Vietnam. At present, the species is known only from near the peak (>1500 m) of mountains and is likely to be restricted to high-elevation forests.

### **Natural history**

The type locality in the Hon Ba Nature Reserve is in the montane evergreen forest dominated by species of the Fagaceae, Theaceae, and Lauraceae. The holotype was collected on leaf of a shrub approximately 1 m above ground, away from ponds or streams. No tadpoles or eggs were found in the pond and calling males were absent from November to late December, but a paratype had large ovarian eggs about 3.8 mm in diameter and cream in color. Otherwise, no information for breeding is available. Associated species observed near the type locality (altitudes 1494–1557 m asl) were *Ingerophrynus galeatus* (Günther, 1864), *Theloderma truongsongense* (Orlov and Ho, 2005), *Rhacophorus calcaneus* Smith, 1924, *Rh. vampyrus* Rowley, Le, Thi, Stuart, and Hoang, 2010, *Polypedates megacephalus* Hallowell, 1861, and *Hylarana nigrovittata* (Blyth, 1856).

Besides, *Raorchestes gryllus* (Smith, 1924) and *Microhyla butleri* Boulenger, 1900 were found at lower elevations (1350–1363 m asl).

### 3.4 Discussion

Unfortunately, no reliable morphological synapomorphies have been established to define the genus *Kurixalus* until now and taxonomic assignment is made solely on molecular bases (Yu et al., 2013). *Kurixalus viridescens*, with monotonous green dorsum and without distinct dark patterns, is unusual in this genus and can be misidentified at a glance as some species of *Rhacophorus*. Because presence of many frogs, including rhacophorids, is noticed by the calls of males, it is strange that all specimens of *K. viridescens* are females and no male specimens were collected by now. However, since no remarkable sexual dimorphism has been reported in species of *Kurixalus*, the characteristics described on the basis of females would hold for males.

Due to limited data available at present, phylogenetic relationships with the genus *Kurixalus* and the other rhacophorid genera were poorly resolved in the tree constructed. However, this preliminary result suggested some interesting phylogenetic problems within the genus *Kurixalus*. Remote relationships of *K. appendiculatus*, *K. eiffingeri*, and *K. idiootocus* from the other species, suggested by Yu et al. (2013), were supported in our tree, but at the same time we confirmed a clade of the remaining species, which Yu et al. (2013) could not resolve. Two large clades were recognized in this major clade, and one of these composed of *K. banaensis* and *K. viridescens*. In this clade, the sequence of *K. banaensis* from Bi Doup Nui Ba National Park (KC465795: Li et al. [2013]) kept in the Kunming Institute of Zoology (KIZ 359) was embedded in a clade with *K. viridescens*. The genetic distances between this sequence and those of *K. viridescens* (1.8–5.2%) were within the range of *K. viridescens* (0.4–5.4%), and the fact that two specimens of *K. viridescens* were collected in Bidoup, one of two known localities of the species, strongly indicates their conspecific status. Future analyses including enigmatic *K. ananjevae* and *K. naso* are badly needed to understand more concrete relationships with the genus.

Vietnam encompasses the largest number of *Kurixalus* species, and six species (*K. ananjevae*, *K. appendiculatus*, *K. baliogaster*, *K. banaensis*, *K. odontotarsus*, and *K. verrucosus*) have been recorded from the country (Orlov et al., 2008: under various generic

names). Of these, *K. appendiculatus* has been recorded outside Vietnam from a very wide range including the Philippines, Borneo, Sumatra, Malaysia, Thailand, Myanmar, Northern India, and Cambodia. From the pattern of its distribution, it is probable that the records from Vietnam, as well as Cambodia, are based on misidentification. In this study we confirmed the presence in this country of *K. bisacculus*, sequence of which is very similar to Thai samples of the species (p-distances of 2.1–2.8%). We suspect the previous record of *K. appendiculatus* in Vietnam may be based on misidentification of *K. bisacculus*.

Some of other Vietnamese species also occur in neighboring Laos (*K. baliogaster*, *K. bisacculus*, and *K. odontotarsus*), and China (*K. bisacculus*, *K. odontotarsus*, and *K. verrucosus*), as well as in Thailand (*K. bisacculus* and *K. verrucosus*) and Myanmar (*K. verrucosus*), and only three species (*K. naso* from northern India, Myanmar, and China; *K. eiffingeri* from Ryukyu Is. of Japan and Taiwan; and *K. idiotocus* from Taiwan) are absent from the country. Thus, Vietnam can be regarded as the center of speciation of the genus *Kurixalus*, and the present addition of *K. viridescens* further strengthens such an idea.

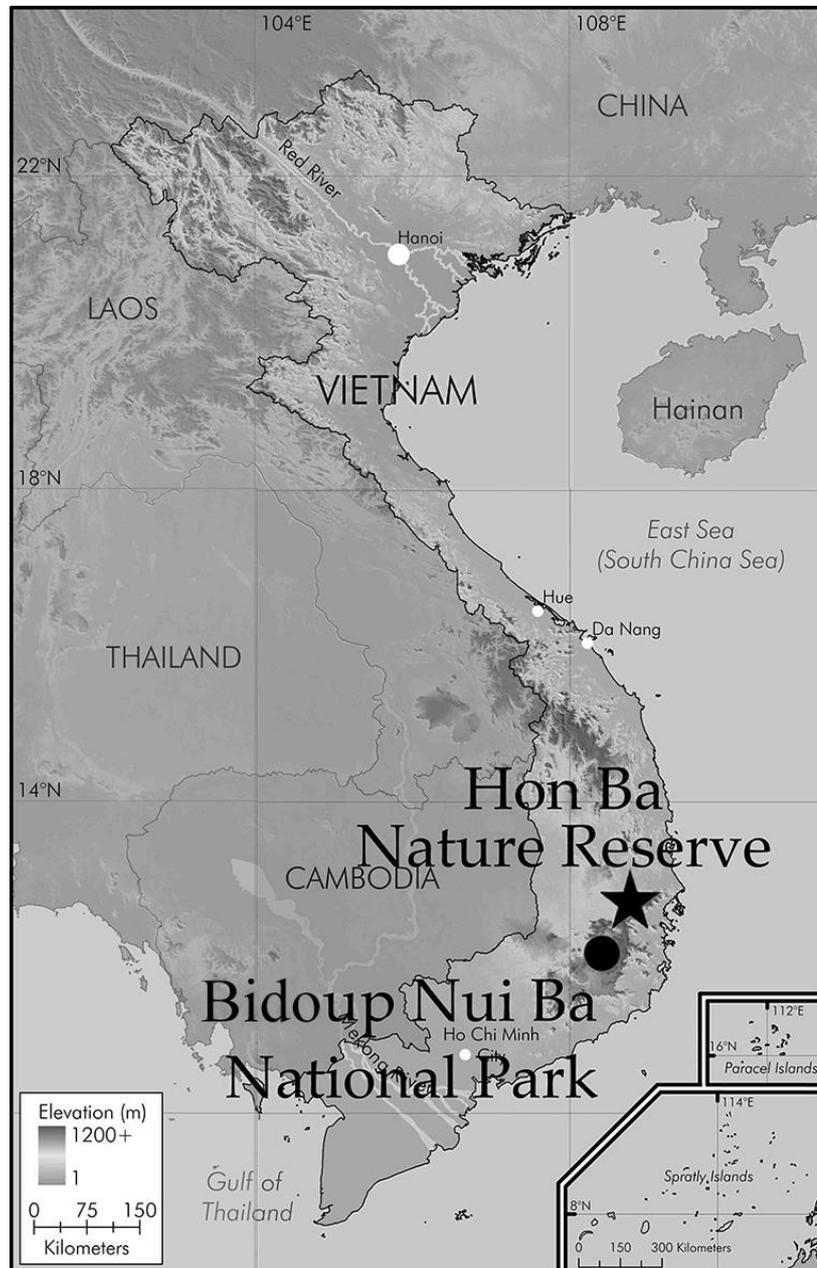
**TABLE 3-1.** Sample of rhacophorid sp. and other species used for DNA analysis in this study together with the information on voucher, collection locality, and GenBank accession numbers. Voucher abbreviations: AMNH=American Museum of Natural History, BORN=BORNEENSIS Collection, University Malaysia Sabah, CAS=California Academy of Science, CIB=Chengdu Institute of Biology, FMNH=Field Museum of Natural History, IABHU=Institute for Amphibian Biology, Hiroshima University, KIZ=Kunming Institute of Zoology, KUHE=Graduate School of Human and Environmental Studies, Kyoto University, RAO=field number of Ding-qi Rao, ROM=Royal Ontario Museum, VNMN=Vietnam National Museum of Nature.

Voucher	Species	GenBank	Locality	Reference
VNMN 03802	Rhacophorid sp.	AB933284	Vietnam, Khanh Hoa	This study
VNMN 03813	Rhacophorid sp.	AB933285	Vietnam, Khanh Hoa	This study
VNMN 03814	Rhacophorid sp.	AB933286	Vietnam, Khanh Hoa	This study
VNMN 01561	<i>Kurixalus bisacculus</i>	AB933287	Vietnam, Ha Giang	This study
VNMN 03805	<i>Kurixalus bisacculus</i>	AB933288	Vietnam, Cao bang	This study
CIB K 2805	<i>Kurixalus bisacculus</i>	AB933289	China, Guangxi	This study
KUHE 19428	<i>Kurixalus bisacculus</i>	AB933290	Thailand, Nakhon Sri Tamarat	This study
KUHE 35069	<i>Kurixalus bisacculus</i>	AB933291	Thailand, Kanchanaburi	This study
VNMN 03806	<i>Kurixalus bisacculus</i>	AB933292	Vietnam, Thanh Hoa	This study
VNMN 03807	<i>Kurixalus bisacculus</i>	AB933293	Vietnam, Thanh Hoa	This study
VNMN 03808	<i>Kurixalus bisacculus</i>	AB933294	Vietnam, Thanh Hoa	This study
VNMN 03809	<i>Kurixalus bisacculus</i>	AB933295	Vietnam, Thanh Hoa	This study
VNMN 03810	<i>Kurixalus bisacculus</i>	AB933296	Vietnam, Thanh Hoa	This study
VNMN 03811	<i>Kurixalus baliogaster</i>	AB933297	Vietnam, Gia Lai	This study
VNMN 03812	<i>Kurixalus baliogaster</i>	AB933298	Vietnam, Gia Lai	This study
VNMN 03652	<i>Kurixalus baliogaster</i>	AB933299	Vietnam, Kon Tum	This study
VNMN 03618	<i>Kurixalus baliogaster</i>	AB933300	Vietnam, Kon Tum	This study
VNMN 03636	<i>Kurixalus baliogaster</i>	AB933301	Vietnam, Kon Tum	This study
CIB 201307012	<i>Kurixalus odontotarsus</i>	AB933302	China, Yunnan	This study
CIB 201307071	<i>Kurixalus odontotarsus</i>	AB933303	China, Yunnan	This study
CAS 231489	<i>Kurixalus verrucosus</i>	KC465823	Myanmar, Kachin	Li et al. (2013)
RAO 6305	<i>Kurixalus verrucosus</i>	KC465825	China, Xizang	Li et al. (2013)
ROM 32986	<i>Kurixalus banaensis</i>	GQ285667	Vietnam, Gia Lai	Li et al. (2009)
VNMN JJ07(NK)	<i>Kurixalus banaensis</i>	AB933304	Vietnam, Lam Dong	This study
KIZ 359	<i>Kurixalus banaensis</i>	KC465795	Vietnam, Bidoup	Li et al. (2013)
KUHE 12910	<i>Kurixalus eiffingeri</i>	AB933305	Japan, Iriomote Is.	This study
KUHE 12979	<i>Kurixalus idiotocus</i>	AB933306	Taiwan, Jiayi	This study
KUHE 53614	<i>Kurixalus appendiculatus</i>	AB847125	Borneo, Sarawak	Matsui et al. (2014)
KUHE 52141	<i>Kurixalus appendiculatus</i>	AB933307	Malaysia, Johor	This study
KUHE 46345	<i>Chiromantis xerampelina</i>	AB813157	pet trade	Matsui et al. (2014)
KUHE 53591	<i>Feihyla kajau</i>	AB847122	Borneo, Sarawak	Matsui et al. (2014)
AMNH A163897	<i>Gracixalus gracilipes</i>	DQ283051	Vietnam, Ha Giang	Frost et al. (2006)

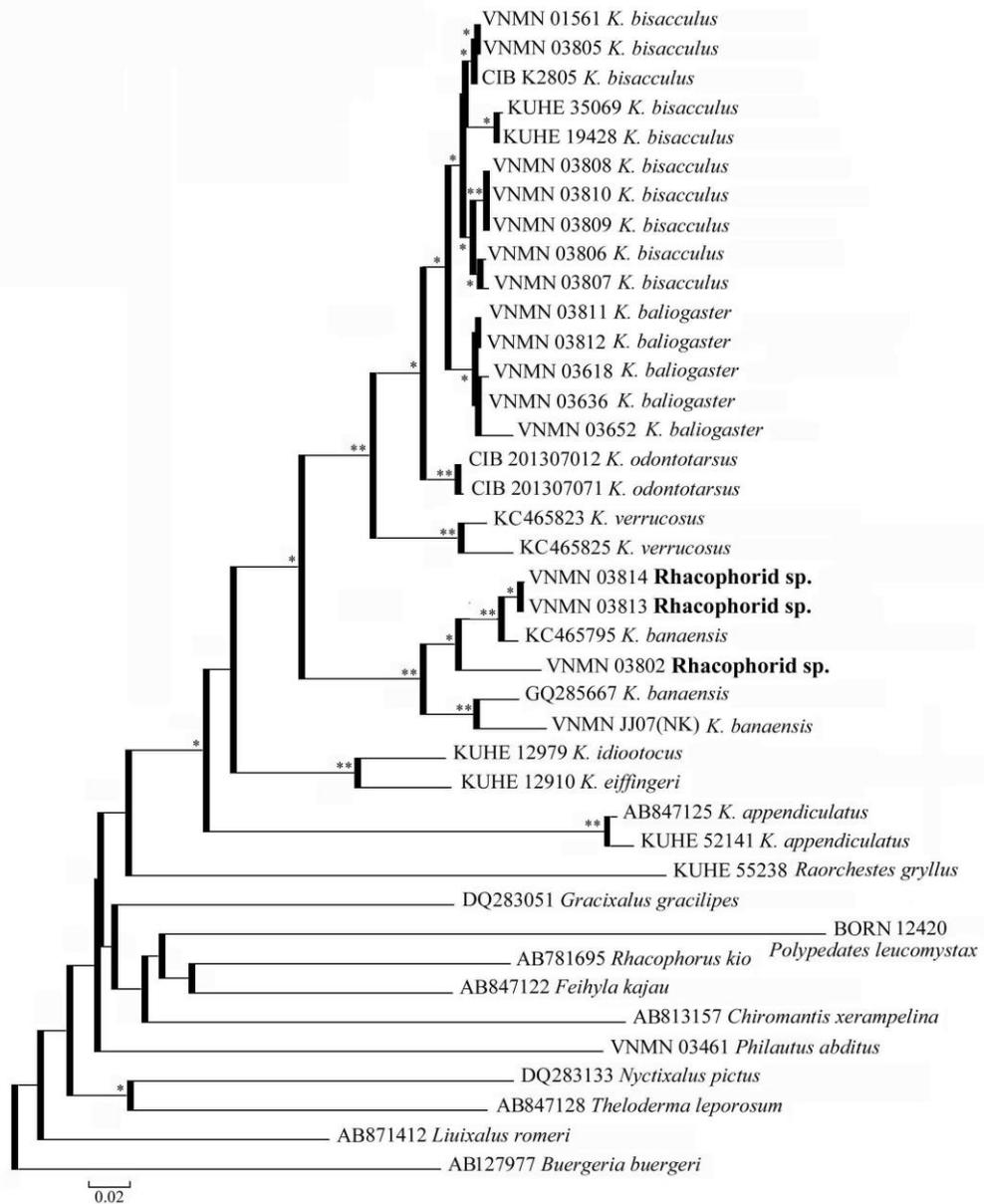
<b>Voucher</b>	<b>Species</b>	<b>GenBank</b>	<b>Locality</b>	<b>Reference</b>
CIB 20080048	<i>Liuixalus romeri</i>	AB871412	China, Hong Kong	Nguyen et al. (2014)
FMNH 231095	<i>Nyctixalus pictus</i>	DQ283133	Borneo, Sabah	Frost et al. (2006)
VNMN 03461	<i>Philautus abditus</i>	AB933308	Vietnam, Kon Tum	This study
BORN 12420	<i>Polypedates leucomystax</i>	AB728138	Borneo, Sabah	Kuraishi et al. (2013)
KUHE 55165	<i>Rhacophorus kio</i>	AB781695	Vietnam, Xuan Lien	Matsui et al. (2013)
KUHE 55238	<i>Raorchestes gryllus</i>	AB933309	Vietnam, TamDao	This study
KUHE 52581	<i>Theloderma leporosum</i>	AB847128	Malaysia, Kenaboi	Matsui et al. (2014)
IABHU 41011	<i>Buergeria buergeri</i>	AB127977	Japan, Hiroshima	Sano et al. (2004)

**TABLE 3-2.** Measurements of female *Kurixalus viridescens*. (in mm).\*Holotype.

	VNMN 03804	VNMN 03803	VNMN 2013.25	VNMN 03815	VNMN 03814	VNMN 2013.72	VNMN 2013.74	VNMN 03816	VNMN 03802*	VNMN 03813
SVL	28.7	28.8	30.9	31.0	32.8	34.0	34.1	34.5	36.3	36.6
HL	11.7	11.8	12.6	12.7	12.9	14.9	14.3	13.2	13.4	14.6
HW	11.2	12.2	12.6	13.4	13.0	13.6	14.1	13.9	13.8	14.2
IND	3.1	3.1	2.8	3.2	3.3	3.2	3.5	3.5	3.3	3.2
IOD	3.3	3.6	3.4	3.8	3.7	4.0	3.8	4.2	4.3	3.8
UEW	3.2	3.3	3.5	4.0	3.6	3.7	3.3	3.6	3.3	3.6
N-EL	2.7	2.5	2.8	2.5	2.5	3.0	2.7	3.3	2.2	2.8
SL	4.7	5.0	5.0	4.6	4.9	5.0	5.3	5.1	5.2	5.6
EL	4.5	4.6	5.9	5.2	5.3	6.0	5.6	5.3	5.3	6.5
ED	4.0	3.9	4.8	4.5	4.6	4.9	4.2	4.4	4.2	5.0
TD	1.4	1.7	2.1	1.7	1.9	1.7	2.1	2.0	1.7	2.0
T-EL	0.5	0.5	0.6	0.6	0.5	0.5	1.0	0.6	0.7	0.4
FLL	17.9	18.7	20.5	20.6	22.5	23.4	22.8	22.4	24.1	23.5
LAL	14.8	15.5	16.6	16.4	17.8	18.6	18.0	17.8	18.9	18.4
1FL	3.0	3.0	3.5	3.1	3.0	3.7	3.5	3.1	4.3	3.3
IPTL	1.5	1.7	1.9	1.6	1.7	2.3	1.9	1.9	1.7	1.9
HLL	45.2	47.6	48.5	50.3	52.4	51.7	54.2	55.4	56.8	53.2
TL	15.5	15.7	15.1	16.3	17.0	16.6	16.5	17.2	18.1	17.0
FL	12.4	12.7	13.7	13.8	13.7	15.5	15.7	14.0	16.2	15.0
IMTL	1.5	1.2	1.7	1.4	1.1	1.8	1.8	1.5	1.8	1.9
1TOEL	3.1	3.0	3.2	3.7	3.3	3.8	3.5	3.7	3.7	3.0
1FDW	1.02	0.83	1.40	1.27	0.93	.	.	1.43	1.26	1.00
2FDW	1.02	0.92	1.96	1.53	1.24	.	1.70	1.76	1.87	1.91
3FDW	1.09	1.38	1.91	1.89	1.32	.	1.77	2.01	2.46	1.88
4FDW	0.97	1.06	1.89	1.95	1.43	.	1.86	1.82	2.34	2.01
1TDW	0.71	0.83	0.90	1.16	0.92	.	0.65	1.29	1.27	1.16
2TDW	0.77	.	1.12	1.25	1.06	.	1.20	1.41	1.56	1.44
3TDW	0.88	1.11	1.52	1.31	1.29	.	1.16	1.43	1.80	1.51
4TDW	1.03	1.28	1.58	1.41	1.44	.	1.37	1.60	2.08	1.52
5TDW	0.69	1.00	1.49	1.56	1.56	.	1.64	1.49	2.06	1.61



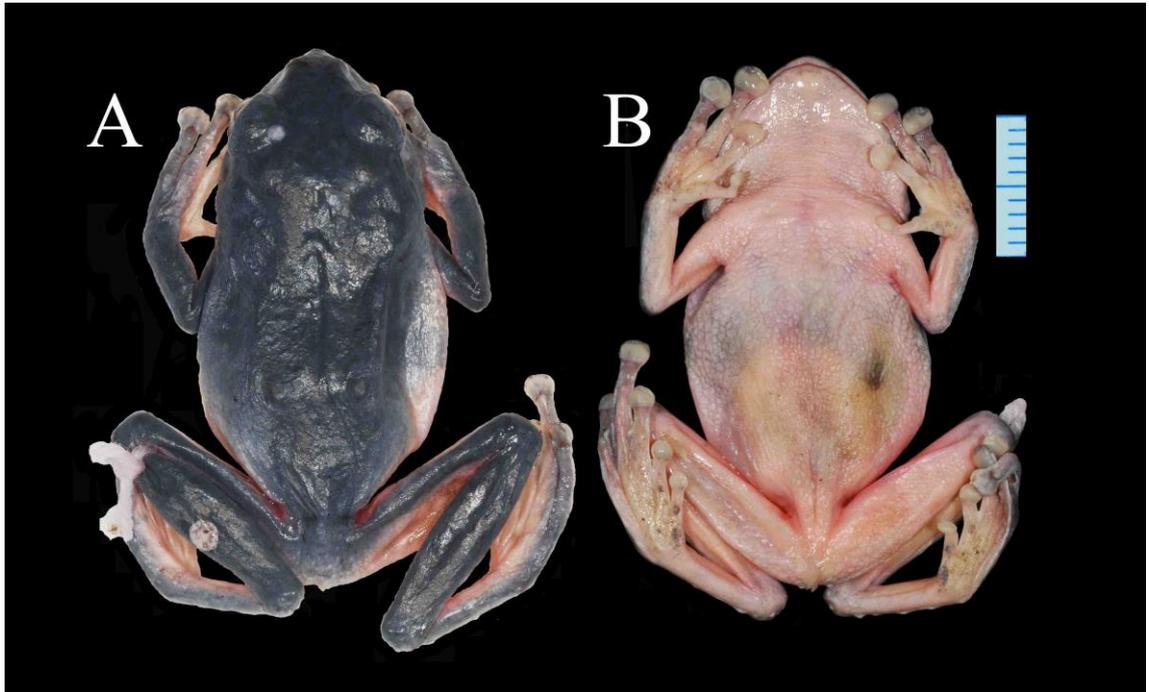
**Figure 3-1.** Map of Vietnam showing Hon Ba Nature Reserve (filled star=type locality) and Bidoup Nui Ba National Park (filled circle), where specimens of the rhacophorid sp. was found.



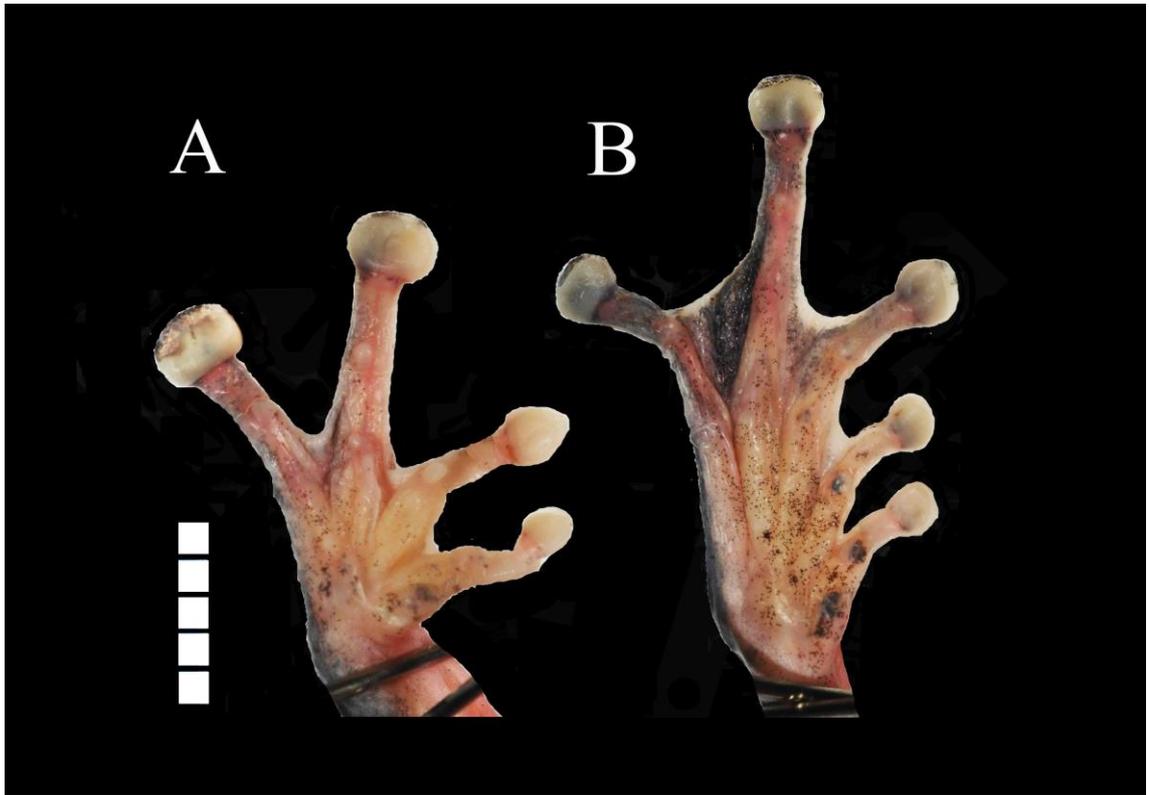
**Figure 3-2.** ML tree from a 1316 bp sequence of mitochondrial 16S rRNA gene for a rhacophorid sp. from southern Vietnam and representative rhacophorid members. \*: ML inferences (ML-BS) between 70% and 95%, and Bayesian posterior probabilities (BPP) between 95% and 100%. \*\*: ML-BS  $\geq$ 95% and BPP=100%.



**Figure 3-3.** Female paratype (VNMN 03814) of *Kurixalus viridescens* in life.



**Figure 3-4.** Dorsal (A) and ventral (B) views of female holotype (VNMN 03802) of *Kurixalus viridescens* after preservation. Scale bar=10 mm.



**Figure 3-5.** Dorsal views of right hand (A) and foot (B) of female holotype (VNMN 03802) of *Kurixalus viridescens* after preservation. Scale bar=5 mm.

## CHAPTER 4

### A new cryptic tree frog species allied to *Kurixalus banaensis* from Vietnam

#### 4.1 Introduction

The genus *Kurixalus* Ye, Fei, and Dubois, 1999 has a restricted range in Asia, from eastern India through Myanmar and mountainous southern China, southward to southern Cambodia and central Vietnam, through western and northern peninsular Thailand to Malaya, Sumatra, Borneo, and the Philippines (Frost, 2014). Recent phylogenetic analyses showed that *Kurixalus* species are taxonomically confused (Yu et al., 2010; Li et al., 2013; Nguyen et al., 2014b). From Vietnam, Nguyen et al. (2009) recorded five species of *Kurixalus*, viz. *K. ananjevae* (Matsui and Orlov), *K. baliogaster* (Inger, Orlov, and Darevsky), *K. carinensis* (Boulenger), *K. odontotarsus* (Ye and Fei), and *K. verrucosus* (Boulenger). Of these, *K. carinensis* has been placed in *Gracixalus* and *Philautus banaensis* Bourret was transferred to the genus *Kurixalus* (Li et al., 2009). Additionally, Yu et al. (2010) have suggested that previous records of *K. odontotarsus* and *K. verrucosus* from Vietnam should be assigned to *K. bisacculus*. Recently, Nguyen et al. (2014b) described the green *Kurixalus* form from southern Vietnam as *K. viridescens* Nguyen, Matsui, and Hoang. Thus, five *Kurixalus* species are left being valid in Vietnam at present, although eight species are listed in Frost (2014).

During our recent fieldwork in the Central Highlands of Vietnam, a small tree frog species was collected and provisionally identified as *Kurixalus banaensis*. However, the newly collected specimens have a smaller size and only weakly pronounced skin ornamentation compared with the latter. Molecular phylogenetic comparison revealed that they are genetically distinct from each other. Therefore, we described the tree frog from Kon Tum and Gia Lai provinces, Vietnam as a new species (Nguyen et al., 2014d).

## 4.2 Materials and methods

DNA sequence data from our new samples were combined with a previously collected mitochondrial sequence dataset for species of *Kurixalus* and relevant out-groups, including *Buergeria japonica* and *Raorchestes gryllus* (Li et al. 2013; Nguyen et al., 2014d; Table 4-1). We used the same methods for DNA extraction, and amplification and sequencing of the mtDNA fragments as those reported by Kuraishi et al. (2013). The resultant sequences (435 base pairs [bp] of partial sequences of mitochondrial 16S rRNA gene) were deposited in GenBank (Accession numbers LC002885–002915).

Measurements were taken with digital callipers to the nearest 0.1 mm. Morphological terminology follows Matsui (1984, 1994) and Nguyen et al., (2014b). The following abbreviations were used: (1) snout-vent length (SVL); (2) head length (HL); (3) head width (HW); (4) internarial distance (IND); (5) interorbital distance (IOD); (6) upper eyelid width (UEW); (7) nostril-eye distance (N-EL); (8) snout length (SL); (9) eye length (EL); (10) eye diameter (ED); (11) tympanum diameter (TD); (12) tympanum-eye length (T-EL); (13) forelimb length (FLL); (14) lower arm and hand length (LAL); (15) first finger length (1FL, measured from distal edge of inner palmar tubercle); (16) inner palmar tubercle length (IPTL); (17) hindlimb length (HLL); (18) tibia length (TL); (19) foot length (FL); (20) inner metatarsal tubercle length (IMTL); and (21) first toe length (1TOEL). Measurements of finger and toe disks in the holotype were taken to the nearest 0.01 mm using a binocular dissecting microscope equipped with a micrometer: first to fourth finger disk diameter (1–4FDW); and first to fifth toe disk diameter (1–5TDW).

Statistical comparisons were made between two groups separated by molecular analysis. In the univariate comparisons, SVL was compared by Tukey-Kramer test, but the remaining characters, converted to ratios to SVL (R), were compared by Dunn's multiple comparisons test. For description of toe-webbing states we followed the system of Savage (1975). Specimens examined are deposited in the collection of the Vietnam National Museum of Nature (VNMN), Hanoi, Vietnam.

### 4.3 Systematics

In our phylogenetic analysis, the undescribed species of *Kurixalux* from Vietnam was clustered in the same group with *K. banaensis* and *K. viridescens*, although their relationships were not resolved (Fig. 4-2). This group is a sister clade of the *K. verrucosus* group, containing *K. verrucosus* from southern China and Myanmar, *K. odontotarsus* from southern China, *K. baliogaster* from Central Highlands of Vietnam and *K. bisacculus* from China, Vietnam and Thailand. The specimens of *Kurixalux* sp. from Central Highlands exhibited distinctly large genetic distances from the nine examined species of *Kurixalux* with uncorrected p-distance of 8.4–17.2% (see Table 4-2). Whereas, genetic distance (uncorrected p-distance) between specimens of *Kurixalux* sp. from Gia Lai and Kon Tum provinces ranged from 0 to 1.5%. In addition, *Kurixalux* sp. is also distinguished from other congeners by some morphological characters, indicating its unnamed species status.

#### ***Kurixalux motokawai* Nguyen, Matsui, and Eto, 2014**

##### **Holotype**

Adult male VNMN 03458, collected by Tao Thien Nguyen, at 20:00 on 8 September 2012 in Mang Canh forest (14°40'33" N, 108°14'37" E, 1230 m asl.), Kon Plong District, Kon Tum Province, Central Highlands of Vietnam.

##### **Paratypes**

VNMN 03416, 03457, 03557, 03575, 03576, 03577, 03590, 03606, the same data as the holotype; VNMN 04028 (2012.113), collected by Tao Thien Nguyen and Chung Van Hoang between 19:00–22:00 h on 12 October 2012 near Dak Man Ranger Station (15°12'45" N, 107°44'15" E, 1150 m asl), Ngoc Linh Nature Reserve, Dak Glei District, Kon Tum Province; VNMN 03574, 04006 (KKK 2012.101), collected by Tao Thien Nguyen on 23 February 2012 from Kon Ka Kinh National Park (14°13'23" N, 108°19'15" E, 1050 m asl), K'Bang District, Gia Lai Province.

## **Etymology**

The species name is dedicated to Associate Professor Dr. Masaharu Motokawa from the Kyoto University, who is an eminent mammalogist and continuously supports the senior author in his study and his outstanding contribution to biodiversity research in Vietnam.

## **Diagnosis**

The species in question was assigned to the genus *Kurixalus* by a combination of the following morphological characters: small-sized rhacophorid (SVL < 50 mm); tips of digits enlarged to the discs, bearing circummarginal grooves; snout tip pointed; finger webbing poorly developed; dermal fringes present on forearm and tarsus (Bourret, 1939; Taylor, 1962, Matsui and Orlov, 2004, Yu et al, 2010). The species is unambiguously nested in the genus *Kurixalus* by molecular phylogenetic evidence, closely related to *K. banaensis* and *K. viridescens*. The specimens from Central Highlands differ from *K. viridescens* by the color pattern and body size and from *K. banaensis* by having a smaller size and weakly developed skin ornamentations.

## **Description of holotype**

SVL 27.8 mm; body moderately robust; head as long as wide (HL 10.8 mm, HW 10.8 mm, 38.8% of SVL); snout (SL 4.2 mm, 15.1% of SVL) shorter than eye (EL 4.5 mm, 16.2% of SVL), dorsally pointed at tip, sloping anteroventrally in profile, projecting over lower jaw; canthus slightly blunt; loreal region oblique, concave; nostril protuberant, closer to the tip of snout than to eye; internarial distance (IND 3.0 mm, 10.8% of SVL) as wide as interorbital (IOD 3.0 mm, 10.8% of SVL); narrower than upper eyelid (UEW 3.2 mm, 11.5% of SVL); pineal spot present; eye large, protuberant, diameter (ED 3.8 mm, 13.7% of SVL) much larger than nostril-eye distance (N-EL 1.8 mm, 6.5% of SVL); pupil horizontal; tympanum distinct, subcircular, its diameter (TD 1.7 mm, 6.1% of SVL) less than half eye diameter and separated from eye by one-third of tympanum diameter (E-TL 0.6 mm, 2.2% of SVL); vomerine teeth absent; tongue deeply notched posteriorly.

Forelimb moderate (FLL 18.1 mm, 65.1% of SVL); relative finger lengths I<II<IV<III; length of first finger (1FL 2.4 mm, 8.6% of SVL) much shorter than diameter of eye; tips of all fingers dilated into horizontally elongate large disks with circummarginal and transverse ventral grooves; third and fourth finger disks (3FDW 1.1 mm, 3.8% of SVL; 4FDW 1.1mm, 4.0% of SVL) narrower than tympanum diameter; fingers with poorly

developed webbing, formula  $I2-2^{3/4}II2-2^{1/2}III2^{1/2}-2IV$ ; fringe of skin on edge of fingers; subarticular tubercles distinct, rounded, formula 1, 1, 2, 2; supernumerary tubercles on metacarpal absent; prepollex prominent, oval; inner palmar tubercle distinct (IPTL 2.1 mm, 7.6%SVL), outer palmar tubercles two, small.

Hindlimb long (HLL 42.6, 153.2%SVL), about 2.4 times length of forelimb; tibia not long (TL 13.8, 49.6%SVL), heels overlapping when limbs are held at right angles to body; tibiotarsal articulation of adpressed limb reaching middle of eye; foot (FL 10.6, 38.1%SVL) shorter than tibia; relative length of toes  $I < II < III < V < IV$ ; tips of toes expanded into round disks with distinct circummarginal grooves, subequal to those of fingers (3TDW 1.1 mm, 3.9% of SVL; 4TDW 1.1 mm, 4.0% of SVL; 5TDW 1.0 mm, 3.7% of SVL); toe webbing moderately developed, formula  $I2-2II^{1/3}-2III1-2^{1/2}IV2^{1/2}-1^{1/2}V$ ; subarticular tubercles distinct, rounded, formula 1, 1, 2, 3, 2; supernumerary tubercles absent; inner metatarsal tubercle distinct, oval (IMTL 1.3 mm, 4.7% of SVL), about half length of first toe (1TOEL 2.7 mm, 9.7% of SVL); outer metatarsal tubercle absent.

Dorsal skin with sparsely distributed small tubercles; supratympanic fold distinct, running from eye above tympanum, ending at above arm insertion; lateral sides and abdomen areolate; a weakly developed ridge of skin on outer edge of forearm forming a weak serration; hindlimb smooth, except for a weak serration along outer edge of tarsus and fifth toe; tubercles including two pairs of large conical ones posteroventral to cloaca; heel with small triangle dermal appendage.

### **Coloration**

In preservative, ground color of head and body grayish brown with large dark brown marking except for upper eyelid, shoulder, and post-sacral regions. Lateral head and tympanic region grayish brown with a dark bar below canthus. Limbs dorsally grayish brown with dark brown marking, forming crossbars on lower arm, tibia, and tarsus. Ventral surface white, scattered with dark brown spots on chin, lower part of flanks, and limbs except for thigh. Rear of thigh mottled with dark spots. Infra-cloacal region white. Hand and foot ventrally grayish brown scattered with dark spots.

### **Variation**

Morphometric data are summarized in Table 4-3. Because only one female was available, sexual dimorphism could not be determined statistically, but a single female has the size (SVL 25.1 mm) within the variation range of males (23.2–28.4 mm, mean

26.1±1.46 mm). In some paratypes, head is wider than long, IND is smaller than IOD, and IOD is smaller than UEW (2 specimens). The tibiotarsal articulation reaches the center of the eye (9 specimens), to anterior corner of the eye (6 specimens), or to between eye and nostril (1 specimen). Some individuals have conical dermal appendage at the snout and/or heel. Coloration in life is variable, some individuals have a dorsum tinged with green (see Fig. 4-5).

### Comparisons

The newly found *K. motokawai* is morphologically similar to *K. banaensis*, but they can be distinguished from each other in the body size. The males of *K. motokawai* are smaller than those of *K. banaensis* (SVL 23.2–28.4 mm, mean±SD 26.1±1.46 mm, versus 26.2–33.2 mm, mean±SD 29.7±2.43 mm, Tukey-Kramer test,  $P<0.01$ ) (see Tables 4-4, 5). In terms of proportions, *K. motokawai* has a higher value of RFL in both sexes (ratio of forelimb length/SVL) but smaller value of R1TOEL in males (ratio of first toe length/SVL) (Dunn's multiple comparison test,  $P<0.05$ ) (see Table 4-6). These species also differ from each other in the shape of the snout tip and the dermal appendage at cloaca (snout tip less markedly pointed and lateral fringes on limbs and infra-cloacal tubercles less developed in *K. motokawai* than in *K. banaensis*).

*Kurixalus motokawai* differs from *K. viridescens* by having a smaller size in females (SVL 25 mm vs. 29–37 mm in *K. viridescens*) and a brown dorsum with dark markings (vs. green dorsum without markings in *K. viridescens*); from *K. ananjevae* (Matsui and Orlov, 2004) by having a smaller size (male SVL 23–28 mm, female SVL 25 mm vs. male 32 mm, female 43 mm in *K. ananjevae*) and the presence of dermal ornamentations (absent in *K. ananjevae*); from *K. appendiculatus* in having a smaller size in males (SVL 23–28 mm vs. 30–37 mm), infra-cloacal appendage with several conical tubercles (vs. dermal flap), and vomerine teeth absent (vs. present in *K. appendiculatus*); from *K. baliogaster* by having a smaller size in males (SVL 23–28 mm vs. 33 mm), ventral surface scattered with small dark spots and lacking a rostral cone in females (vs. ventral surface with conspicuous, large black spots, females with a strong rostral cone in *K. baliogaster*); from *K. bisacculus* by having a smaller size in females (SVL 25 mm vs. 29 mm), belly immaculate white (vs. belly with black spots), and vomerine teeth absent (vs. present in *K. bisacculus*).

*Kurixalus motokawai* differs from *K. eiffingeri* by having a smaller size (male SVL

23–28 mm vs. 31–35 mm and female SVL 25 mm vs. 32–44 mm) and serrated lateral skin on lower arm, tibia, and tarsus (vs. lateral skin with a row of tubercles in *K. eiffingeri*); from *K. idiootocus* by the presence of serrated dermal fringe on lower arm, tibia, and tarsus; from *K. naso* (Annandale, 1912), *K. motokawai* differs by body size, snout shape, toe webbing, and ventral color (male SVL 23–28 mm and female SVL 25 mm, snout tip weakly pointed, toe webbing poorly developed, and ventral surface scattered with small dark spots vs. SVL 43 mm, snout with a cone, toe webbing well-developed, and chin and breast marked with dark reticulation in *K. naso*); from *K. odontotarsus* by having a smaller size (male SVL 23–28 mm and female 25 mm vs. 30–33 mm and 35–43 mm, respectively), snout weakly pointed (vs. sharply pointed), and ventral surface scattered with small dark spots (vs., venter with large dark markings in *K. odontotarsus*); from *K. verrucosus* by having smaller size in female (SVL 25 mm vs. 43–45 mm), belly without dark spots (vs. present), and toe webbing moderately developed (well developed in *K. verrucosus*).

### **Distribution**

*K. motokawai* is currently known from several localities in the Central Highlands of Vietnam: Kon Plong forest and Ngoc Linh Nature Reserve in Kon Tum Province, and Kon Ka Kinh National Park in K' Bang District, Gia Lai Province, at elevations from 1050 m to 1230 m asl.

### **Natural history**

Surrounding habitat at the type locality of the species in Kon Plong District, Kon Tum Province was the primary rain-forest, inclined slopes of the mountains at elevations of 1100–1400 m asl. All specimens were collected at night, between 19:00 and 23:00. The specimens were found on leaves of scrub vegetation and young trees, about 0.5 - 1 m above the ground. Associated species observed at the type locality were *Gracixalus supercornutus* (Orlov, Ho, and Nguyen), *Raorchestes gryllus* (Smith) and *Rhacophorus robertingeri* Orlov, Poyarkov, Vassilieva, Ananjeva, Nguyen, Nguyen, and Geissler.

### **4.4 Discussion**

Generally, small rhacophorids with brown dorsum are difficult to identify and identification should be ideally made with reference to type specimens. *Kurixalus motokawai* has been confused with *K. banaensis* for a long time (e.g., Nguyen et al., 2009). *Kurixalus banaensis* was originally described by Bourret (1939) in the genus *Philautus* from Bana (= Ba Na, Da Nang City, Vietnam). In the Muséum national d'Histoire naturelle

(MNHN) in Paris, currently three syntypes (MNHN 1948.0159 - 0161) are deposited, together with a topotype (MNHN 1948.0162). Bossuyt and Dubois (2001) designated the lectotype (MNHN 1948.0160) and provided a redescription of this species. Our examination of the lectotype revealed some additional characters for the original description of Bourret (1939): snout clearly pointed; canthus distinct; tympanum indistinct; webbing between second and third fingers, and third and fourth fingers not rudimentary; dermal fringe on side of upper arm and tarsus serrated; triangular skin projections present below cloaca; interorbital with dark markings. Furthermore, the topotype has a pineal spot and round snout, and toe webbing formula of  $I1^{1/2}-2II1^{1/3}-2III1^{1/2}-2^{1/2}IV2-1^{1/2}V$ .

In our molecular analyses, *K. motokawai*, *K. viridescens*, and *K. banaensis* tended to form a group, which we propose to call the *K. banaensis* species group. Because *K. motokawai* and *K. banaensis* are superficially similar, previous records of *K. banaensis* in Vietnam need to be verified by further studies. Finally, comparison of advertisement calls may help to elucidate bioacoustic differences between *K. motokawai* and *K. banaensis*.

**TABLE 4-1.** Samples used in molecular analyses. Voucher abbreviations: CAS = California Academy of Science; CIB = Chengdu Institute of Biology; KIZ = Kunming Institute of Zoology; KUHE = Graduate School of Human and Environmental Studies, Kyoto University; RAO = field number of Ding-qi Rao; URE = Faculty of Education, University of the Ryukyus; VNMN = Vietnam National Museum of Nature.

Voucher	Species	GenBank	Locality	Reference
VNMN 03416	<i>Kurixalus</i> sp.	LC002886	Vietnam, Kon Tum	This study
VNMN 03457	<i>Kurixalus</i> sp.	LC002887	Vietnam, Kon Tum	This study
VNMN 03458	<i>Kurixalus</i> sp.	LC002888	Vietnam, Kon Tum	This study
VNMN 03575	<i>Kurixalus</i> sp.	LC002889	Vietnam, Kon Tum	This study
VNMN 03576	<i>Kurixalus</i> sp.	LC002890	Vietnam, Kon Tum	This study
VNMN 03577	<i>Kurixalus</i> sp.	LC002891	Vietnam, Kon Tum	This study
VNMN 03606	<i>Kurixalus</i> sp.	LC002892	Vietnam, Kon Tum	This study
VNMN 03722	<i>Kurixalus</i> sp.	LC002893	Vietnam, Kon Tum	This study
VNMN 03726	<i>Kurixalus</i> sp.	LC002894	Vietnam, Kon Tum	This study
VNMN 03735	<i>Kurixalus</i> sp.	LC002895	Vietnam, Kon Tum	This study
VNMN 03739	<i>Kurixalus</i> sp.	LC002896	Vietnam, Kon Tum	This study
VNMN 03757	<i>Kurixalus</i> sp.	LC002897	Vietnam, Kon Tum	This study
VNMN 03758	<i>Kurixalus</i> sp.	LC002898	Vietnam, Kon Tum	This study
VNMN 04006	<i>Kurixalus</i> sp.	LC002899	Vietnam, Gia Lai	This study
VNMN 04015	<i>Kurixalus</i> sp.	LC002900	Vietnam, Kon Tum	This study
VNMN 04027	<i>Kurixalus</i> sp.	LC002901	Vietnam, Kon Tum	This study
VNMN 04028	<i>Kurixalus</i> sp.	LC002902	Vietnam, Kon Tum	This study
VNMN 03802	<i>Kurixalus viridescens</i>	AB933284	Vietnam, Khanh Hoa	Nguyen et al. (2014)
VNMN 03813	<i>Kurixalus viridescens</i>	AB933285	Vietnam, Khanh Hoa	Nguyen et al. (2014)
VNMN 03814	<i>Kurixalus viridescens</i>	AB933286	Vietnam, Khanh Hoa	Nguyen et al. (2014)
VNMN 03461	<i>Kurixalus banaensis</i>	LC002903	Vietnam, Kon Tum	This study
VNMN 03909	<i>Kurixalus banaensis</i>	LC002904	Vietnam, Kon Tum	This study
VNMN 04016	<i>Kurixalus banaensis</i>	LC002905	Vietnam, Lam Dong	This study
VNMN 04017	<i>Kurixalus banaensis</i>	LC002906	Vietnam, Gia Lai	This study
VNMN 04018	<i>Kurixalus banaensis</i>	LC002907	Vietnam, Binh Phuoc	This study
VNMN 04019	<i>Kurixalus banaensis</i>	LC002908	Vietnam, Kon Tum	This study
VNMN 04020	<i>Kurixalus banaensis</i>	LC002909	Vietnam, Kon Tum	This study
VNMN 04021	<i>Kurixalus banaensis</i>	LC002910	Vietnam, Kon Tum	This study
VNMN 04022	<i>Kurixalus banaensis</i>	LC002911	Vietnam, Kon Tum	This study
VNMN 04023	<i>Kurixalus banaensis</i>	LC002912	Vietnam, Kon Tum	This study
VNMN 04024	<i>Kurixalus banaensis</i>	LC002913	Vietnam, Kon Tum	This study
VNMN 04025	<i>Kurixalus banaensis</i>	LC002914	Vietnam, Kon Tum	This study
VNMN 04026	<i>Kurixalus banaensis</i>	LC002915	Vietnam, Kon Tum	This study
VNMN 03809	<i>Kurixalus bisacculus</i>	AB933295	Vietnam, Thanh Hoa	Nguyen et al. (2014)
KUHE 35069	<i>Kurixalus bisacculus</i>	AB933291	Thailand, Kanchanaburi	Nguyen et al. (2014)
CIB K 2805	<i>Kurixalus bisacculus</i>	AB933289	China, Guangxi	Nguyen et al. (2014)
VNMN 03618	<i>Kurixalus baliogaster</i>	AB933300	Vietnam, Kon Tum	Nguyen et al. (2014)
VNMN 03652	<i>Kurixalus baliogaster</i>	AB933299	Vietnam, Kon Tum	Nguyen et al. (2014)
VNMN 03811	<i>Kurixalus baliogaster</i>	AB933297	Vietnam, Gia Lai	Nguyen et al. (2014)
KIZ 201307012	<i>Kurixalus odontotarsus</i>	AB933302	China, Yunnan	Nguyen et al. (2014)
KIZ 201307071	<i>Kurixalus odontotarsus</i>	AB933303	China, Yunnan	Nguyen et al. (2014)
CAS 231489	<i>Kurixalus verrucosus</i>	KC465823	Myanmar, Kachin	Li et al. (2013)
RAO 6305	<i>Kurixalus verrucosus</i>	KC465825	Cina, Xizang	Li et al. (2013)
KUHE 12910	<i>Kurixalus eiffingeri</i>	AB933305	Japan, Iriomote Is.	Nguyen et al. (2014)
KUHE 12979	<i>Kurixalus idiootocus</i>	AB933306	Taiwan, Jiayi	Nguyen et al. (2014)
KUHE 53614	<i>Kurixalus appendiculatus</i>	AB847125	Borneo, Sarawak	Matsui et al. (2014)
KUHE 55238	<i>Raorchestes gryllus</i>	AB933309	Vietnam, TamDao	Nguyen et al. (2014)
URE 1185	<i>Buergeria japonica</i>	LC002885	Japan, Ammaioshima	This study

**TABLE 4-2.** Uncorrected p-distances (%) for fragment of 16S rRNA between *Kurixalus* and related taxa.

	1	2	3	4	5	6	7	8	9	10	11
1 <i>Kurixalus</i> sp.											
2 <i>K. banaensis</i>	8.6-9.9										
3 <i>K. viridescens</i>	9.9-11.4	7.6-9.4									
4 <i>K. bisacculus</i>	9.1-9.9	10.1-11.1	9.1-11.1								
5 <i>K. baliogaster</i>	8.9-9.4	8.6-10.1	8.4-10.9	2.8-3.5							
6 <i>K. odontotarsus</i>	9.4-9.9	8.1-9.6	8.6-11.1	3.8	3.8-4.1						
7 <i>K. verrucosus</i>	8.4-10.9	8.6-9.6	8.6-9.9	6.1-6.6	6.1-6.3	5.3-6.3					
8 <i>K. idiootocus</i>	10.4-10.6	10.1-12.2	10.6-12.9	10.1-11.1	10.1-10.4	9.4	8.1-9.4				
9 <i>K. eiffingeri</i>	12.2-12.4	10.4-11.1	11.1-13.2	10.6-11.6	9.6-9.9	9.6	8.9-9.6	5.3			
10 <i>K. appendiculatus</i>	16.5-17.2	16.7-17.7	18.7-19.5	18.0-19.0	19.0-19.2	18.2	16.5-17.0	15.9	16.7		
11 <i>Raorchestes gryllus</i>	18.0-18.5	17.0-18.7	17.2-17.7	17.5-18.5	17.7-18.0	18.0	18.5-18.5	17.2	14.9	18.5	
12 <i>Buergeria japonica</i>	17.5-18.2	15.7-17.7	19.0	15.4-16.2	15.9-16.2	16.7	14.7-16.7	16.7	15.2	17.0	16.7

**TABLE 4-3.** Measurements of adult specimens of *Kurixalus motokawai* and *K. banaensis*. SVL (mean±SD, in mm) and medians of percentage ratios (R) of other characters to SVL, followed by ranges in parenthesis. See text for character abbreviations.

	<i>K. motokawai</i>		<i>K. banaensis</i>	
	17 M	1F	6 M	4 F
SVL	26.1±1.46 (23.2–28.4)	25.1	29.7±2.43 (26.2–33.2)	33.9±2.71 (30.5–37.0)
RHL	39.8 (36.4–42.4)	41.4	40.1 (39.2–41.6)	38.8 (37.7–42.5)
RHW	40.5 (36.4–44.2)	40.2	41.0 (37.5–42.4)	39.1 (37.0–42.8)
RIND	10.2 (8.8–11.7)	11.6	10.1 (9.6–10.7)	10.2 (8.1–11.0)
RIOD	12 (10.8–14.1)	12.7	11.3 (10.3–13.4)	11.0 (9.8–12.1)
RUEW	11.3 (8.8–13.4)	11.6	11.8 (8.8–13.0)	11.2 (11.1–11.5)
RSL	15.6 (13.8–17.4)	16.3	15.8 (14.7–17.8)	15.0 (14.3–15.6)
RN-EL	8.4 (6.5–10.9)	8.8	9.2 (8.0–11.1)	8.6 (7.5–9.0)
REL	17.1 (15.3–20.3)	18.3	17.2 (16.8–18.4)	16.1 (14.6–17.4)
RED	14.7 (13.7–15.3)	14.7	14.5 (14.1–15.7)	13.8 (13.8–13.8)
RTD	6.1 (4.5–7.1)	6.4	5.8 (5.6–6.6)	6.1 (5.2–7.5)
RE-TL	1.5 (0.7–2.4)	2.0	1.4 (1.1–1.6)	1.7 (0.9–2.0)
RLAL	49.3 (46.8–54.7)	47.0	48.7 (44.2–49.8)	46.5 (45.1–49.2)
RFL	65.1 (61.6–70.3)	62.5	60.7 (59.1–63.6)	59.6 (58.9–60.5)
RIPL	5.8 (4.1–7.6)	6.4	5.3 (4.0–6.3)	4.8 (4.3–5.6)
R1FL	8.4 (7.6–9.9)	8.8	8.7 (8.0–12.8)	9.2 (7.8–10.2)
RTL	49.3 (45.5–52.8)	50.2	50.4 (44.9–51.6)	50.5 (47.0–55.4)
RFL	40.2 (37.7–51.6)	39.4	42.5 (38.5–44.9)	41.9 (35.4–43.3)
RHLL	152.0 (144.0–161.8)	155.4	157.2 (136.1–166.7)	156.2 (142.2–165.0)
RIMTL	4.6 (3.0–5.7)	6.0	4.8 (3.7–5.8)	5.4 (4.3–5.9)
R1TOEL	8.2 (7.3–10.3)	8.0	9.5 (9.0–11.6)	8.4 (8.1–10.8)

**TABLE 4-4.** Comparison of the size (average SVL) between *Kurixalus motokawai* and *K. banaensis* (M = male, F = female, SD = standard deviation).

Species	N	Mean	SD
1 <i>Kurixalus motokawai</i> (M)	17	26.017	1.465
2 <i>K. banaensis</i> (M)	6	29.683	2.428
3 <i>K. banaensis</i> (F)	4	33.900	2.712
Total	27	28.033	3.427

**TABLE 4-5.** One way ANOVA test and Tukey-Kramer test of the size (average SVL) between *Kurixalus motokawai* and *K. banaensis*.  
 One way ANOVA test:  $df = 2,24$ ,  $F = 30.6760$ ,  $p = 0.0000$   
 Tukey-Kramer test: if  $q > q(0.05,3,24) = 3.532$ , then significant (\*), else not significant (ns).

Comparison	Difference	SE	q	Conclusion
<i>Kurixalus. banaensis</i> (F) vs <i>K. motokawai</i> (M)	7.829	0.743	10.534 *	<i>K. banaensis</i> (F) > <i>K. motokawai</i> (M)
<i>K. banaensis</i> (F) vs <i>K. banaensis</i> (M)	4.217	0.863	4.884 *	<i>K. banaensis</i> (F) > <i>K. banaensis</i> (M)
<i>K. banaensis</i> (M) vs <i>K. motokawai</i> (M)	3.613	0.635	5.688 *	<i>K. banaensis</i> (M) > <i>K. motokawai</i> (M)

**TABLE 4-6.** Kruskal-Wallis test and Dunn's multiple comparison test of the ratio of forelimb length/SVL (RFL) and of first toe length/SVL (R1TOEL) between *Kurixalus motokawai*. and *K. banaensis*.

Kruskal-Wallis test:

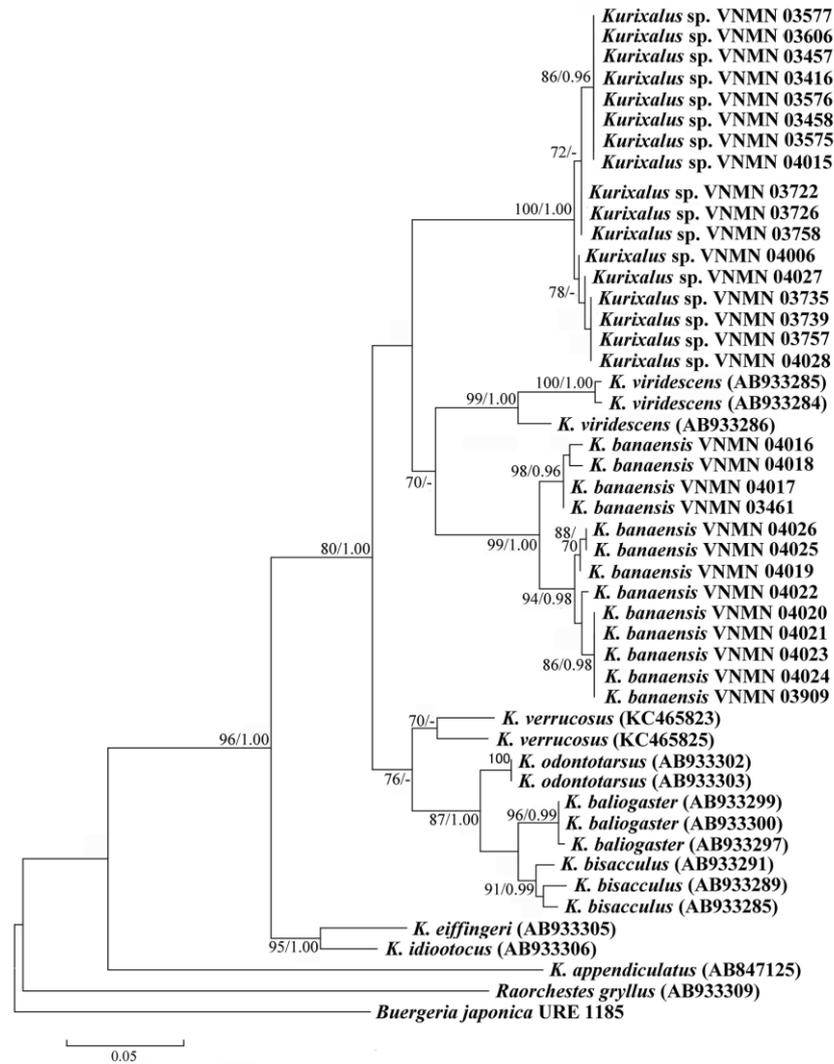
RFL:  $df = 2$ ,  $H = 16.4585$ ,  $p = 0.0003$  (significant)

R1TOEL:  $df = 2$ ,  $H = 6.18939$ ,  $p = 0.0453$  (significant)

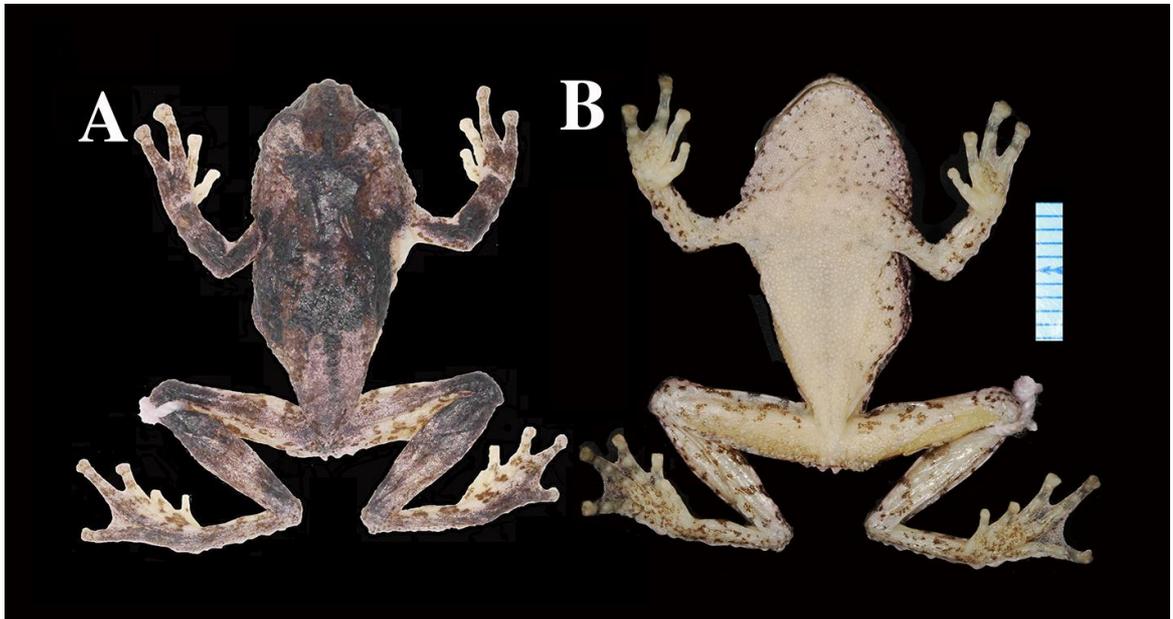
Comparison	Difference	SE	Q	Conclusion
RFL: if $Q > Q(0.05,3) = 2.394$ , significant (*), not significant (ns)				
1 vs 3	14.456	4.411	3.277 *	1 > 3
1 vs 2	11.539	3.769	3.062 *	1 > 2
2 vs 3	2.917	5.123	0.569 ns	1 > 2
R1TOEL: if $Q > Q(0.05,3) = 2.394$ , significant (*), not significant (ns)				
2 vs 1	9.363	3.769	2.484 *	2 > 1
2 vs 3	6.333	5.123	1.236 ns	2 = 3
3 vs 1	3.029	4.411	0.687 ns	3 = 1



**Figure 4-1.** Map showing the distribution of *Kurixalus motokawai* in Vietnam: 1) Kon Plong in Kon Tum Province (type locality), 2) Dak Glei in Kon Tum Province, and 3) K' Bang in Gia Lai Province.



**Figure 4-2.** ML tree from a 435 bp sequence of mitochondrial 16S rRNA gene for *Kurixalus* species and out-groups. ML inferences (ML-BS)  $\geq 70\%$  /Bayesian posterior probabilities (BPP)  $\geq 95\%$  are shown near the node.



**Figure 4-3.** Dorsal (A) and ventral (B) views of the male holotype (VNMN 03458) of *Kurixalus motokawai* in preservative. Scale bar = 10 mm.



**Figure 4-4.** Dorsal views of right hand (A) and foot (B) of the male holotype (VNMN 03458) of *Kurixalus motokawai* in preservative. Scale bar = 5 mm.



**Figure 4-5.** Male paratype (VNMN 04006) of *Kurixalus motokawai* in life.

## CHAPTER 5

### A preliminary study of phylogenetic relationships and taxonomic problems of Vietnamese *Rhacophorus*

#### 5.1 Introduction

Through intensive field survey in many regions (e.g., Nguyen et al., 2009; Ohler et al., 2002; Orlov et al., 2001, 2008, 2012; Rowley et al., 2010, 2012b; Ziegler and Köhler, 2001), Vietnam now encompasses as many as one-fourth (22 of 86) of known species of *Rhacophorus* Kuhl and Van Hasselt, 1822 (Frost, 2014). In recent studies of molecular phylogeny of this genus, Vietnamese species have been almost always included (e.g., Li et al., 2008, 2009, 2012 a,b; Yu et al., 2008, 2009). However, phylogenetic relationships of three species have never been reported. First, no sequence data have been reported for *R. robertingeri* Orlov, Poyarkov, Vassilieva, Ananjeva, Nguyen, Sang, and Geissler, 2012. Second, there are unpublished sequence data of *R. exechopygus* Inger, Orlov, and Darevsky, 1999, but these data seem to have never been compared with other species. Third, sequence data of *R. vampyrus* Rowley, Le, Thi, Stuart, and Hoang, 2010 (HQ656815-19, as *Rhacophorus* sp.) were used only for adults-larvae matching in the original description of the species, but relationships with other species using these data were not given (Rowley et al., 2010). In addition, taxonomic relationships of Chinese species related to *R. dugritei* (David, 1872) have been closely examined (Li et al., 2012a), but related species from Vietnam have never been seriously studied. In addition, taxonomic relationships of *R. rhodopus* Liu and Hu, 1960 and *R. bipunctatus* Ahl, 1927 are not well understood. We therefore attempted to clarify these problems based on an analysis of partial sequences of mtDNA in this short paper.

#### 5.2 Materials and methods

In order to solve above problems of the Vietnamese *Rhacophorus*, we compared partial sequences of mitochondrial 16S rRNA (ca. 450 bp) of representing *Rhacophorus*

species and outgroup species, *Buergeria buergeri* (Temminck and Schlegel, 1838) and *Mantella madagascariensis* (Grandidier, 1872) (Table 5-1). Methods for DNA extraction, and amplification and sequencing of the mtDNA fragments are the same as those reported by Kuraishi et al. (2013). The resultant new sequences were deposited in GenBank (Accession numbers LC10566–010616; Table 5-1). The alignment matrices with 434 sites for 16S rRNA were subjected to estimate phylogenetic relationships using maximum likelihood (ML) and Bayesian inference (BI). Pairwise comparisons of uncorrected sequence divergences (p-distance) were also calculated. Details for these procedures are given in Kuraishi et al. (2013).

### 5.3 Results

We obtained 434 bp of 16S rRNA fragments of mtDNA gene for 66 samples from Vietnam and neighboring countries, including outgroup (Fig. 5-2). Of 434 nucleotide sites, 164 were variable, and 142 were parsimoniously informative within ingroup species. The best substitution model was J2+G with gamma shape parameter (G) of 0.251 for ML and GTR+G of 0.167 for BI. The likelihood values (-lnLs) of the ML and BI trees were 3061.534 and 3222.215, respectively.

The two phylogenetic analyses yielded slightly different topologies (only the ML tree is shown in Fig. 5-1). The following relationships were indicated by the two analyses:

(i) Monophyly of the *Rhacophorus* with respect to the outgroup species was supported (95% and 100% support in ML bootstrap value and Bayesian posterior probability, respectively).

(ii) Monophyly of Clade A, including *R. kio* Ohler and Delorme, 2006, *R. helenae* Rowley, Tran, Hoang, and Le, 2012a, *R. rhodopus*, and *R. bipunctatus* was moderately supported (72% and 96% support).

(iii) Monophyly of Clade B including the remaining species was also moderately supported (72% and 97% support).

(iv) *Rhacophorus robertingeri* tended to form a monophyletic group with *R. orlovi* Ziegler and Köhler, 2001, but the support was not significant (64% and 89% support).

(v) *Rhacophorus exechopygus* formed a well-supported monophyletic group with *R. annamensis* Smith, 1924 (99% and 100% support).

(vi) *Rhacophorus vampyrus* tended to be grouped as sister species to the clade including *R. dugritei*, *R. dennysi* Blanford, 1881, *R. feae* Boulenger, 1893, *R. maximus* Günther, 1858, and their relatives, but the support was weak (74% and 84% support).

(vii) *Rhacophorus dugritei*, *R. minimus* Rao, Wilkinson, and Liu, 2006, *R. hungfuensis* Liu and Hu, 1961, *R. puerensis* (He, 1999), and *R. duboisi* Ohler, Marquis, Swan, and Grosjean, 2000 formed a well-supported clade (85% and 99% support). Within this clade, clades of *R. dugritei* and *R. minimus* (93% and 99% support), *R. hungfuensis* (99% and 100% support), and *R. puerensis* and *R. duboisi* (82% and 96% support) showed trichotomous relationships.

(viii) *Rhacophorus pingbianensis* (Kou, Hu, and Gao, 2001) and *R. omeimontis* (Stejneger, 1924) formed a well-supported clade (89% and 100% support), but the relationship between the two species was unresolved.

(ix) *Rhacophorus rhodopus* from China and Vietnam were remotely related and Chinese samples formed a fully supported clade with *R. bipunctatus*. In contrast, Vietnamese samples included two lineages and resulted in unsupported monophyly of *R. rhodopus* and *R. bipunctatus* (58% and 62% support).

## 5.4 Discussion

### Phylogeny of Vietnamese *Rhacophorus*

In our results, two moderately supported clades were recognized, in which *R. annamensis* was in the Clade B, containing *R. dugritei* and *R. omeimontis*, and not in the Clade A, containing *R. kio*, *R. rhodopus*, and *R. bipunctatus*. This is not concordant with Li et al. (2008, 2012b), who found a clade containing *R. annamensis*, *R. kio*, *R. rhodopus*, and *R. bipunctatus*, although their relationships were unresolved.

### Phylogeny and taxonomy of *R. robertingeri*

As far as we are aware, there are no sequence data for *R. robertingeri*. This species is endemic to moderate to high elevations of northern to southern Vietnam, but has been

confused with *R. calcaneus* Smith, 1924 and many doubtful locality records exist (Ziegler et al., 2014). In our result, *R. robertingeri* was closer to *R. orlovi* than to *R. calcaneus*, although their sister species relationship was not confirmed. Now *R. robertingeri* is assigned to the *R. calcaneus* group (Orlov et al., 2012), but the clade was not supported, either (59% and 80% support).

#### **Phylogeny and taxonomy of *R. exechopygus***

This species occurs moderate to high mountains of Vietnamese Central Mountains and adjacent Annamite Mountains of Laos (Orlov et al., 2002, 2008; Frost, 2014). The DNA sequence data of this species in GenBank (GQ469976-80) seem to have never been compared with the other species. Analysis of our own data clarified sister species relationship of *R. exechopygus* and *R. annamensis*. Both the two species have broad webbing on hand, but are different in body size and dermal appendages (small and present in *R. exechopygus* vs. large and absent in *R. annamensis*: Inger et al., 1999), and their close relationship was not easily supposed morphologically.

#### **Phylogeny and taxonomy of *R. vampyrus***

*Rhacophorus vampyrus* is known from high mountains on the Langbian Plateau of southern Vietnam, and is reported to have unique larval life history (Rowley et al., 2012b). Rowley et al. (2010) studied DNA sequence of this species (HQ656815-19, as *Rhacophorus* sp.) for larval and adult barcoding. Our own sample had sequences very similar to those reported by Rowley et al. (2010). No molecular phylogenetic studies have been made and Orlov et al. (2012) placed the species in their *Rhacophorus calcaneus* group from morphology. However, our result suggested its very remote position not only from *R. calcaneus*, but also from any other species studied (p-distances=12.0–14.9%). As shown above, the *Rhacophorus calcaneus* group of Orlov et al. (2012) was not supported molecular phylogenetically, and requires reassessment.

#### **Phylogeny and taxonomy of the *R. dugritei* and *R. omeimontis* groups**

Taxonomic problem of tree frogs in the *R. dugritei* group of Dubois (1987) or the *R. dugritei* group and *R. omeimontis* group of Fei et al. (2010) is complicated because of their close morphological similarity. These include *R. dugritei*, *R. omeimontis*, *R. puerensis*, *R. duboisi*, and *R. pingbianensis*. Recently, Li et al. (2012a) performed detailed molecular phylogeny, including mtDNA and nuDNA, of the species related to *R. dugritei* from

China, of which there were many controversial taxonomic ideas. They found three well-supported lineages, and described a new species.

Our main purpose was to determine relationship of *R. duboisi* occurring mainly from Vietnam and partly in neighboring China. This is because Li et al. (2012a) did not include *R. duboisi* in their study, although this species was originally described as closely related to the *R. dugritei* group (Ohler et al., 2000). In contrast, Li et al. (2012b) studied *R. duboisi* and found its very close relationship with *R. omeimontis*. In our result, *R. duboisi* formed a substantially supported clade with *R. puerensis*, which showed trichotomous relationships with *R. hungfuensis* and the clade of *R. dugritei* and *R. minimus*. At the same time, our samples of *R. duboisi* were remotely related to the clade of *R. omeimontis*. Thus the species should be considered a member of the *R. dugritei* group sensu Li et al. (2012a).

Another problem is the taxonomic position of *R. pingbianensis*, which was originally described as *Polypedates* and a close relative of *R. omeimontis* (as *Polypedates*). However, it was later synonymized with *R. omeimontis* by Yu et al. (2009) and Fei et al. (2009), or *R. duboisi* by Orlov et al. (2002) and Li et al. (2012b). In our results, *R. pingbianensis* from Sapa formed a well-supported clade with *R. omeimontis* (89% and 100% support) with very small genetic divergence (p-distances=0.7–1.0%), more favoring for the idea of Yu et al. (2009) than that of Li et al. (2012b). The reason for discordance of our results from those given by Li et al. (2012b) is unknown, and future examination of voucher specimens is necessary. In relation to this, *R. pingbianensis* is differentiated from *R. omeimontis* by the lack of vocal sac, hence vocal opening (Kou et al., 2001). Our specimens include two females and a male, which lacks vocal slit, and was hence morphologically identified as *R. pingbianensis*. Future studies should include range of morphological variation in these two species.

### **Phylogeny and taxonomy of *R. rhodopus***

Bordoloi et al. (2007) morphologically studied moderate-sized, red-webbed *Rhacophorus* including *R. rhodopus* and *R. bipunctatus* and provided a key to this group. *Rhacophorus rhodopus* was described from Yunnan (Liu and Hu, 1960), without detailed comparisons with *R. bipunctatus*, which has been considered widely distributed from Malaysia to Assam. Inger et al. (1999) considered Vietnamese population and Thai populations of *R. bipunctatus* are identical, and relegated *R. rhodopus* as a synonym of *R. bipunctatus*. In contrast, Bordoloi et al. (2007), while recognizing distinct status of the two

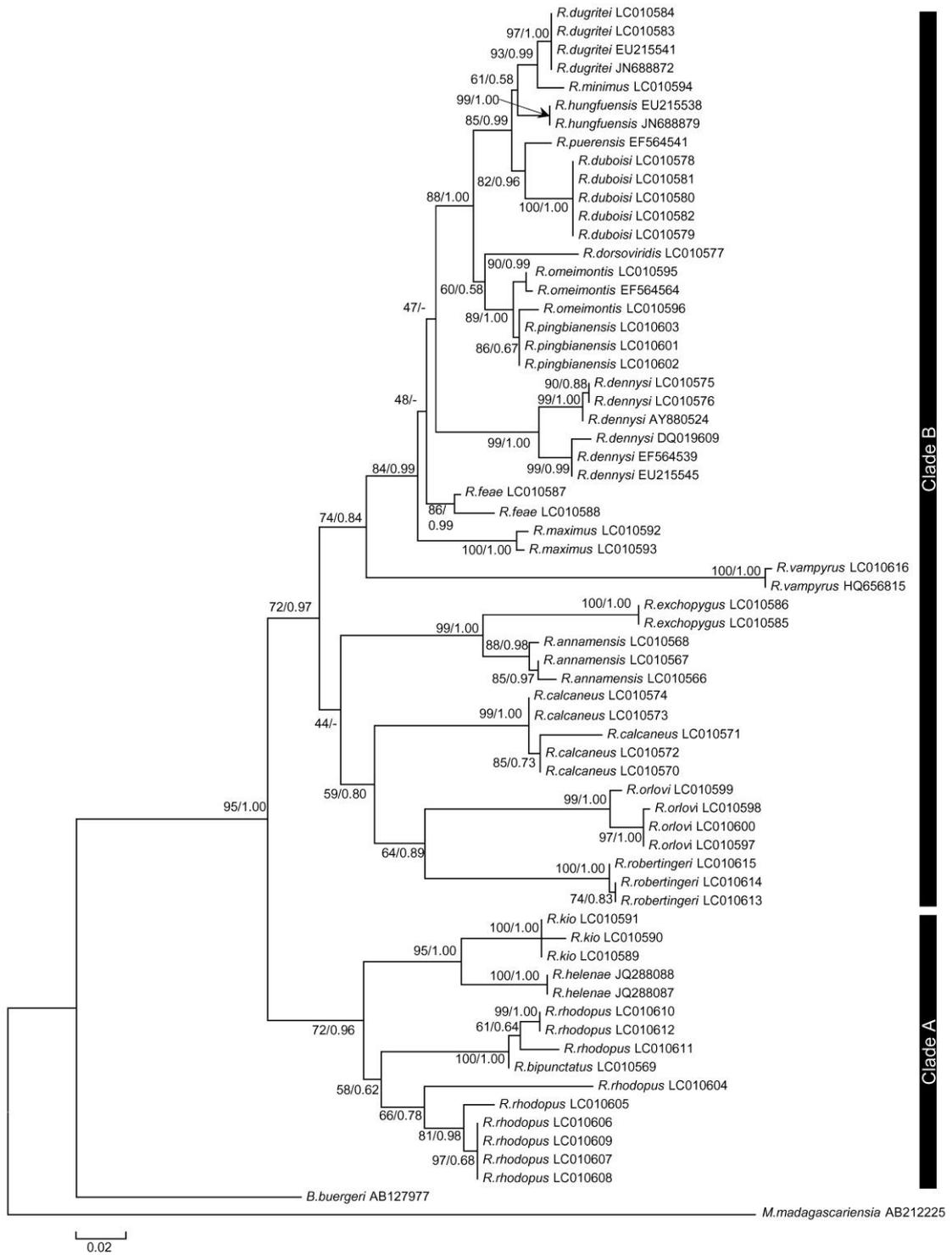
species, identified *R. bipunctatus* from Thailand (Taylor, 1962) as *R. rhodopus*. Bordoloi et al. (2007) concluded that Vietnamese specimens are to be assigned to the same biological species as *R. rhodopus* from Yunnan.

However, the taxonomic problems seem to be not so easily solved. In our results, *R. rhodopus* exhibited unresolved trichotomous relationships: (1) a Vietnamese clade from Nghe An, Thanh Hoa, and Bac Giang, (2) a sample from Gia Lai, and (3) a clade from Yunnan and *R. bipunctatus* from Malaysia. At least some Vietnamese populations are not conspecific with *R. rhodopus* from Yunnan, which may possibly be conspecific with Malaysian *R. bipunctatus*. This result is similar to that already reported by Yu et al. (2007). Li et al. (2012b) also found paraphyly of *R. rhodopus* where the Chinese clade of samples from Yunnan and Xizang were split from samples from Hainan and Vietnam with *R. reinwardtii* (Schlegel, 1840) in between. Thus further studies both from morphology and molecular phylogeny based on many samples from their whole distribution range are necessary.

**TABLE 5-1.** Sample of Vietnamese *Rhacophorus* and other species used for DNA analysis in this study together with the information on voucher, collection locality and GenBank accession numbers. Voucher abbreviations: AMS=Australian Museum; CIB=Chengdu Institute of Biology; IABHU=Institute for Amphibian Biology, Hiroshima University; KIZ=Kunming Institute of Zoology; KUHE=Graduate School of Human and Environmental Studies, Kyoto University; LJT=field number of Jia-tang Li; MNHN=Muséum National d’Histoire Naturelle, Paris; NCSM=North Carolina Museum of Natural Sciences; SCUM=Sichuan University Museum; UNS=University of Science, Ho Chi Minh City; VNMN=Vietnam National Museum of Natural History; ZFMK=Zoologisches Forschungsinstitut und Museum A. Koenig.

No.	Species	Voucher	Locality	Accession No.	Reference
1	<i>R. annamensis</i>	VNMN 4090	Vietnam, Dak Nong, Nam Nung	LC010566	This study
2	<i>R. annamensis</i>	VNMN 4091	Vietnam, Dak Lak, Chu Yang Sin	LC010567	This study
3	<i>R. annamensis</i>	VNMN 4092	Vietnam, Gia Lai, Kon Ka Kinh	LC010568	This study
4	<i>R. bipunctatus</i>	KUHE 53375	Malaysia, Pahang, Genting	LC010569	This study
5	<i>R. calcaneus</i>	VNMN 4093	Vietnam, Khanh Hoa, Hon Ba	LC010570	This study
6	<i>R. calcaneus</i>	VNMN 4094	Vietnam, Khanh Hoa, Hon Ba	LC010571	This study
7	<i>R. calcaneus</i>	VNMN 4095	Vietnam, Khanh Hoa	LC010572	This study
8	<i>R. calcaneus</i>	VNMN 4096	Vietnam, Dak Lak, Chu Yang Sin	LC010573	This study
9	<i>R. calcaneus</i>	VNMN 4097	Vietnam, Dak Lak, Chu Yang Sin	LC010574	This study
10	<i>R. dennysi</i>	KUHE 55211	Vietnam, Vinh Phuc, Tam Dao	LC010575	This study
11	<i>R. dennysi</i>	VNMN 4098	Vietnam, Vinh Phuc, Tam Dao	LC010576	This study
12	<i>R. dennysi</i>	MNHN 1440K	Vietnam	AY880524	Ohler et al., 2006
13	<i>R. dennysi</i>	KIZ 060821050	China, Guangxi, Jinxiu	EF564539	Yu et al., 2009
14	<i>R. dennysi</i>	ZFMK 65461	Pet trade	DQ019609	Van der Meijden et al., 2005
15	<i>R. dennysi</i>	SCUM060401L	China, Guangdong, Shaoguan	EU215545	Li et al., 2008
16	<i>R. dorsovireidis</i>	VNMN 4099	Vietnam, Son La, Ta Xua	LC010577	This study
17	<i>R. duboisi</i>	VNMN 4100	Vietnam, Lao Cai, Sapa	LC010578	This study
18	<i>R. duboisi</i>	VNMN 4101	Vietnam, Lao Cai, Sapa	LC010579	This study
19	<i>R. duboisi</i>	VNMN 4102	Vietnam, Lao Cai, Sapa	LC010580	This study
20	<i>R. duboisi</i>	VNMN 4103	Vietnam, Lao Cai, Sapa	LC010581	This study
21	<i>R. duboisi</i>	VNMN 4104	Vietnam, Lao Cai, Sapa	LC010582	This study
22	<i>R. dugritei</i>	SCUM 051001L	China, Sichuan, Baoxing	EU215541	Li et al., 2008
23	<i>R. dugritei</i>	CIB 20050194	China, Sichuan	LC010583	This study
24	<i>R. dugritei</i>	KUHE 27701	China, Sichuan	LC010584	This study
25	<i>R. dugritei</i>	LJT 051002	China, Sichuan, Baoxing	JN688872	Li et al., 2012a
26	<i>R. exechopygus</i>	VNMN 4107	Vietnam, Gia Lai, Kon Ka Kinh	LC010585	This study
27	<i>R. exechopygus</i>	VNMN 4108	Vietnam, Gia Lai, Kon Ka Kinh	LC010586	This study
28	<i>R. feae</i>	VNMN 4109	Vietnam, Lao Cai, Sapa	LC010587	This study
29	<i>R. feae</i>	VNMN 3462	Vietnam, Kon Tum, Kon Plong	LC010588	This study
30	<i>R. helenae</i>	AMS R 173230	Vietnam, Binh Thuan	JQ288087	Rowley et al., 2012
31	<i>R. helenae</i>	UNS 00450	Vietnam, Dong Nai	JQ288088	Rowley et al., 2012

No.	Species	Voucher	Locality	Accession No.	Reference
32	<i>R. hungfuensis</i>	SCUM 060425L	China, Sichuan, Wenchuan	EU215538	Li et al., 2008
33	<i>R. hungfuensis</i>	SCUM 060424L	China, Sichuan, Wenchuan	JN688879	Li et al., 2012a
34	<i>R. kio</i>	VNMN 4110	Vietnam, Gia Lai, Kon Ka Kinh	LC010589	This study
35	<i>R. kio</i>	VNMN 4111	Vietnam, Ha Giang, Bac Quang	LC010590	This study
36	<i>R. kio</i>	VNMN 4112	Vietnam, Cao Bang, Ha Lang	LC010591	This study
37	<i>R. maximus</i>	VNMN 1534	Vietnam, Bac Giang, Yen Tu	LC010592	This study
38	<i>R. maximus</i>	VNMN 4113	Vietnam, Nghe An, Pu Huong	LC010593	This study
39	<i>R. minimus</i>	CIB GX2009.05.11	China, Yuan Nam	LC010594	This study
40	<i>R. omeimontis</i>	KIZ 060821282	China, Sichuan, Hongya	EF564564	Yu et al., 2009
41	<i>R. omeimontis</i>	CIB 20060104	China, Sichuan	LC010595	This study
42	<i>R. omeimontis</i>	CIB 070039	China, Sichuan	LC010596	This study
43	<i>R. orlovi</i>	VNMN 4114	Vietnam, Thanh Hoa, Xuan Lien	LC010597	This study
44	<i>R. orlovi</i>	VNMN 3067	Vietnam, Ha Tinh, Huong Son	LC010598	This study
45	<i>R. orlovi</i>	VNMN 4116	Vietnam, Kon Tum, Ngoc Linh	LC010599	This study
46	<i>R. orlovi</i>	VNMN 4115	Vietnam, Nghe An, Pu Huong	LC010600	This study
47	<i>R. pingbianensis</i>	VNMN 4105	Vietnam, Lao Cai, Sapa	LC010601	This study
48	<i>R. pingbianensis</i>	VNMN 4106	Vietnam, Lao Cai, Sapa	LC010602	This study
49	<i>R. pingbianensis</i>	VNMN 3217	Vietnam, Lao Cai, Sapa	LC010603	This study
50	<i>R. puerensis</i>	KIZ 060821003	China, Yunnan, Yongde	EF564541	Yu et al., 2009
51	<i>R. rhodopus</i>	VNMN 4117	Vietnam, Gia Lai, K' Bang	LC010604	This study
52	<i>R. rhodopus</i>	VNMN 4118	Vietnam, Bac Giang, Yen Tu	LC010605	This study
53	<i>R. rhodopus</i>	VNMN 4119	Vietnam, Nghe An, Pu Huong	LC010606	This study
54	<i>R. rhodopus</i>	VNMN 4122	Vietnam, Thanh Hoa, Xuan Lien	LC010607	This study
55	<i>R. rhodopus</i>	VNMN 4121	Vietnam, Thanh Hoa	LC010608	This study
56	<i>R. rhodopus</i>	VNMN 4120	Vietnam, Nghe An, Pu Huong	LC010609	This study
57	<i>R. rhodopus</i>	CIB 1306005	China, Yunnan	LC010610	This study
58	<i>R. rhodopus</i>	CIB 1306006	China, Yunnan	LC010611	This study
59	<i>R. rhodopus</i>	CIB 1306010	China, Yunnan	LC010612	This study
60	<i>R. robertingeri</i>	VNMN 4123	Vietnam, Gia Lai, Kon Ka Kinh	LC010613	This study
61	<i>R. robertingeri</i>	VNMN 4124	Vietnam, Gia Lai, Kon Ka Kinh	LC010614	This study
62	<i>R. robertingeri</i>	VNMN 3446	Vietnam, Kon Tum, Kon Plong	LC010615	This study
63	<i>R. vampyrus</i>	NCSM 77318	Vietnam, Lam Dong, Bidoup-Nui Ba	HQ656815	Rowley et al., 2010
64	<i>R. vampyrus</i>	VNMN 4125	Vietnam, Khanh Hoa, Hon Ba	LC010616	This study
65	<i>B. buergeri</i>	IABHU 41011	Japan, Hiroshima	AB127977	Sano et al., 2004
66	<i>M. madagascariensis</i>	IABHU 6933	Madagascar	AB212225	Kurabayashi et al., 2006



**Figure 5-1.** ML tree from a 434 bp sequence of mitochondrial 16S rRNA gene for *Rhacophorus* and outgroup species. ML inferences (ML-BS)/Bayesian posterior probabilities (BPP) are shown near the node.

## CHAPTER 6

### First record of *Philautus petilus* from Vietnam and its phylogenetic position

#### 6.1 Introduction

In the checklist of amphibians and reptiles of Vietnam, Nguyen et al. (2009) reported eight species of the genus *Philautus*. However, recent phylogenetic studies suggested great changes in the generic assignment of many rhacophorid species including the species formerly nested in *Philautus*: *P. banaensis* Bourret was moved to *Kurixalus*, and *P. jinxiuensis* Hu and *P. quyeti* Nguyen, Hendrix, Böhme, Vu, and Ziegler were transferred to *Gracixalus* by Li et al. (2008, 2009); *P. gryllus* Smith, *P. longchuanensis* Yang and Li, and *P. parvulus* (Boulenger) were placed in the genus *Raorchestes* after Biju et al. (2010); and *P. truongsoneensis* Orlov and Ho was allocated to *Theلودerma* by Rowley et al. (2011a). Therefore, only two species of *Philautus* are currently known from Vietnam, viz. *P. abditus* Inger, Orlov and Darevsky and *P. maosonensis* Bourret (Orlov et al., 2012).

During our recent field surveys in Dien Bien Province, northwestern Vietnam in 2012 and in 2014, three specimens of small-sized rhacophorid were collected from Muong Nhe Nature Reserve. Close morphological examination of the specimens revealed them to be *Philautus petilus* Stuart and Heatwole, 2004, a species originally described from Laos and recently reassigned to the genus *Theلودerma* by Stuart et al. (2013) based on morphological characteristics. Our molecular analysis also showed that this species is separated distinctly from the species of *Philautus* but clustered within the genus *Theلودerma*. We reported this species for the first time from Vietnam and confirm the transfer this taxon from *Philautus* to *Theلودerma* on the basis of molecular phylogeny (Nguyen et al., 2014c).

## 6.2 Materials and Methods

### Sampling

Field surveys were conducted within Muong Nhe NR, Dien Bien Province, Vietnam in April 2012 and January 2014. Specimens were preserved in 80% ethanol after removing and fixing pieces of muscle in 95% ethanol for DNA sample. The specimen was transferred to 70% ethanol and was subsequently deposited in the collection of the Hanoi National University of Education (HNUE), Vietnam.

### Morphological analysis

Measurements were taken with a dial caliper to the nearest 0.1 mm. Terminology of morphological characters followed Stuart and Heatwole (2004). Abbreviations used are: SVL=snout-vent length; HL=head length (from tip of snout to the commissure of the jaws); HW=head width at the commissure of the jaws; SE=snout length (from tip of snout to the anterior corner of the eye); EL=eye diameter; TYD=horizontal diameter of tympanum; NS=distance from nostril to tip of snout; EN=distance from front of eye to nostril; UEW=maximum width of upper eyelid; IUE=minimum distance between upper eyelids; FLL=forelimb length (from the elbow to the base of outer tubercle); HAL=hand length (from the base of outer palmar tubercle to the tip of fourth toe); FL=femur length (from vent to knee); TL=tibia length; TW=tibia width; FOL=distance from the base of tarsus to the tip of fourth toe; fd1–4: width of discs of fingers I–IV, td1–5: width of discs of toes I–V. Webbing formula followed Glaw and Vences (2007).

### Molecular analysis

For molecular phylogenetic analyses, we obtained tissue samples of *Philautus* and *Theلودerma* from the newly collected specimens and sequences available from GenBank (trimmed to match the length of the fragment obtained here), including specimens of *Philautus aurifasciatus* (Schlegel) (type species of the genus *Philautus*) and *Theلودerma leporosum* Tschudi (type species of the genus *Theلودerma*). Specimens of other rhacophorid genera, *Buergeria buergeri* (Temminck and Schlegel), *Kurixalus eiffingeri* (Boettger), *Nyctixalus pictus* (Peters), *Polypedates leucomystax* (Gravenhorst), and *Rhacophorus feae* (Boulenger), were used as outgroups (Table 6-1).

We used the protocols of Matsui et al. (2010) for DNA extraction, amplification, and sequencing. Fragments containing 12S rRNA, tRNA<sup>Val</sup>, and 16S rRNA, approximately 2250bp long, were amplified and sequenced using six pairs of primers (Kuraishi et al., 2011). Sequences were aligned by ClustalX (Thompson et al., 1997) and manually checked using the original chromatograph data in the program BioEdit (Hall, 1999). Phylogenetic trees were constructed using Maximum likelihood (ML) and Bayesian Inference (BI). We used Treefinder ver.1.5 Oct. 2011 (Jobb, 2011) for ML and MrBayes ver 3.1.2 (Ronquist and Huelsenbeck, 2003) for BI. Tree nodes with bootstrap values of 70% or greater were considered as sufficiently resolved (Hillis and Bull, 1993). In the BI analysis, nodes with a BPP of 95% or greater were considered significant (Leache and Reeder, 2002). Uncorrected p-distances for 16S rRNA were calculated by using MEGA ver. 5.2.

### **6.3 Results**

Details of three Vietnamese specimens examined are as follows: HNUE MNA.2012.0001 (adult male) collected on 14 April 2012, and HNUE MNA.2014.0374 (adult female) and HNUE MNA.2014.0375 (adult male) collected on 21 January 2014, all by Dzung Trung Le, from near the Phy Thy stream (22°19'54"N 102°20'55"E, 620 m a.s.l.) within Muong Nhe Nature Reserve, Leng Su Sin Commune, Muong Nhe District, Dien Bien Province, Vietnam.

#### **Description of the specimen from Vietnam**

Habitus moderately slender, body dorsoventrally compressed (SVL 31.5–32.4 mm in two males; 30.3 mm in a female); head longer than wide (HL 9.8–10.1 mm, HW 8.2 mm, HL/SVL 0.31, HW/SVL 0.25–0.26 in males and HL 10.1 mm, HW 8.1 mm, HL/SVL 0.33, HW/SVL 0.27 in the female); snout slightly pointed anteriorly (SE 3.8–4.0 mm, SE/SVL 0.12–0.13 in males and SE 3.6 mm, SE/SVL 0.12 in female); eye large, shorter than snout length (EL 3.5 mm, EL/HL 0.35–0.36, EL/SE 0.88–0.92 in males and EL 3.1 mm, EL/HL 0.31, EL/SE 0.86 in the female), pupil round; tympanum round, small, clearly visible (TYD 2.2–2.3 mm, TYD/EL 0.63–0.66 in males and TYD 2.2 mm, TYD/EL 0.71 in the female); supratympanic fold distinct; nostril in lateral direction, much closer to tip of snout than to eye (NS 1.2–1.3 mm, EN 3.9–4.1 mm, NS/EN 0.31–0.32 in males and NS 1.3 mm, EN 4.2 mm, NS/EN 0.31 in the female); loreal region slightly concave, oblique;

interorbital distance narrower than upper eyelid (IUE 2.4 mm for all specimens, IUE/UEW 1.5–1.6 in males and 1.5 in the female); vomerine teeth very small, in oblique rows closer to choanae than to each other; tongue deeply notched posteriorly.

Forelimb slender and short (FLL/SVL 0.17–0.19 in males and 0.17 in the female); relative lengths of fingers:  $I < II < IV < III$ ; tips of fingers with round discs, with circummarginal grooves; disc of finger III smaller than tympanum (fd3/TYD 0.7–0.89 in males and 0.65 in the female); fingers free of webbing; fingers III and IV with large middle subarticular tubercle, inner palmar tubercle small, outer palmar tubercle distinct; fingers I and II with large palmar tubercle at base.

Thigh long; tibia approximately five times longer than wide; relative toe length  $I < II < III < V < IV$ ; tips of toes expanded into round discs, with circummarginal grooves, slightly smaller than finger discs; subarticular tubercles on toes I–V: 1, 2, 2, 4, 3; webbing formula  $Ie(1/2)(1)iIIe(1/2)(1)iIIIe(1/2)(1)iIVe(1)(0)iV$ ; inner metatarsal tubercle elongated, outer metatarsal tubercle very small, almost undiscernible.

Skin on dorsal and ventral surfaces smooth, except for distinct, white asperities on head, posterior part of back, dorsal surfaces of forelimb, thigh, tibia, fingers and toes, and anterior half of sides; dermal fringes, row of enlarged tubercles, or accessory flaps of skin absent on outer margins of limbs.

Coloration in life, head and body dorsally light brown with dark brown reticulations and black spots in irregular shape, larger and more distinct in posterior part of dorsum; dorsolateral zone of head and body light brown; lateral head and tympanum dark brown, darker from behind tympanum to groin; a black stripe present below edge of canthus extending from tip of snout to anterior corner of eye and from posterior corner of eye along supratympanic fold to flank, edged in white near level of mid-body; axillar region white; dorsal surface of limbs dark brown with white asperities; thigh and tibia with some black marbling; venter cream, chin with some dark spots, underside of limbs pigmented; pupil black; iris bicolored, upper part reddish brown, lower part grey (Fig. 6-1).

### **Natural history**

The first male (HNUE MNA.2012.0001) was found at 20:30 h on the tree, ca. 0.7 m above the ground. The others (HNUE MNA.2014.0374, 0375) were found in the water in a bamboo hole. Surrounding habitat is evergreen mixed forest of hardwoods and bamboos at an elevation of 623 m a.s.l. The advertisement call and larvae of this species are unknown.

## **Morphological identification**

The specimens from Vietnam agree well with diagnosis of the species although they differ from the female holotype from Laos by having a longer head (ratio of HL/SVL 0.31 in males and 0.33 in the female vs. 0.27 in the holotype). In contrast, Vietnamese specimens have smaller ratios of tympanum to eye diameter (TYD/EL 0.63–0.66 in males and 0.71 in the female vs. 0.80 in the holotype) (see Stuart and Heatwole, 2004). However, we suppose that these differences might be within the range of individual variations. Morphological characteristics of the Vietnamese specimens (e.g., the presence of dorsal dermal asperities and distinct tympanum, and the absence of finger webbing) also supported the removal of this species from *Philautus* to *Theلودerma* as suggested by Stuart et al. (2013).

## **Phylogenetic position of *Philautus petilus***

In order to identify the phylogenetic position of the species, we use molecular evidence to provide independent support of its generic assignment from morphology. The combined matrix contained 642 aligned characters, of which 340 were variable and 273 were parsimony-informative for ingroup. The best model selected by Kakusan for both analyses was GTR with gamma (0.341 for ML and 0.353 for BI). The ML and BI analyses produced essentially identical topology, therefore, only ML tree is shown in Fig. 6-2. The likelihood values (-lnL) for ML and BI tree were 4893 and 4920, respectively. In our analyses, the specimen from Dien Bien Province was embedded within the clade II of the genus *Theلودerma*, together with *T. asperum* (Boulenger) and *T. licin* McLeod and Norhayati. This clade is clearly separated from the clade I (*T. bicolor* [Bourret], *T. corticale* [Boulenger], *T. gordonii* Taylor, *T. rhododiscus* [Liu and Hu], and the type species of the genus, *T. leporosum* Tschudi, from Malaysia) and the clade III (*T. stellatum* Taylor) (see Fig. 6-2). The Vietnamese specimen of *Philautus petilus* is significantly divergent from others within the Clade II with the minimum p-distance of approximately 11.2% in the mitochondrial fragment of 16S rRNA (see Table 6-2). Based on these phylogenetic results, we confirm the placement of *Philautus petilus* in the genus *Theلودerma*.

## 6.4 Discussion

As mentioned above, recent systematic studies have made great changes in the generic allocation of many rhacophorid species, including the change in the contents of the genus *Theلودerma*. These changes were made mostly based on results of molecular phylogenetic analyses (e.g., Frost et al., 2006; Li et al., 2009; Yu et al., 2009), and generic diagnoses based on morphology are becoming more and more obscure within Rhacophoridae. This is also the case with *Theلودerma* as shown above and morphological synapomorphies to delimit the genus are still to be investigated. Although most authors agree that the genera *Theلودerma* and *Nyctixalus* are monophyletic (Pyron and Wiens, 2011; Rowley et al., 2011), some recent phylogenetic work threw doubt on the monophyly of *Theلودerma*. Our result, with three groups in this genus concurred this, although *Philautus petilus* was clearly nested within one group of *Theلودerma*.

Since the original description of Stuart and Heatwole (2004), only a single female specimen of *Theلودerma petilum* has been known from the type locality, Phou Den Din National Biodiversity Conservation Area in northern Laos. The specimens from Muong Nhe Nature Reserve are the first record in Vietnam and also provided knowledge on males of this species. It is noted that Muong Nhe Nature Reserve of Vietnam is contiguous with the Phou Den Din National Biodiversity Conservation Area of Laos and the newly recorded locality is approximately 30 km eastward from the type locality of the species (Fig. 6-3).

Vietnam is the type locality of 10 species (or 46% of the species number) of the genus *Theلودerma*. Since 2009, five species, *T. bambusicolum* Orlov, Poyarkov, Vassilieva, Ananjeva, Nguyen, Nguyen, and Geissler; *T. chuyangsinensis* Orlov, Poyarkov, Vassilieva, Ananjeva, Nguyen, Nguyen, and Geissler; *T. lateriticum* Bain, Nguyen, and Doan; *T. nebulosum* Rowley, Le, Hoang, Dau, and Cao; and *T. palliatum* Rowley, Le, Hoang, Dau, and Cao, have been described from the country (Bain et al., 2009; Rowley et al., 2011; Orlov et al., 2012), and our finding of *T. petilum* brings the species number of *Theلودerma* to 15 in Vietnam (Nguyen et al., 2009; Orlov et al., 2012). Vietnam has been considered as a hotspot of new species discovery in Asia (Nguyen, 2006; Ziegler and Nguyen, 2010), but new recordings of species known from adjacent regions like the present case also will further increase rich amphibian fauna of this country.

**TABLE 6-1.** Sample of *Theلودerma* and outgroup species used for DNA analysis in this study together with the information on voucher, collection locality and GenBank accession numbers. Voucher abbreviations=CIB: Chengdu Institute of Biology; FMNH: Field Museum of Natural History; HNUE: Hanoi National University of Education; IABHU: Institute for Amphibian Biology, Hiroshima University; KUHE: Graduate School of Human and Environmental Studies, Kyoto University; MZB: Museum Zoologicum Bogoriense; VNMN: Vietnam National Museum of Nature.

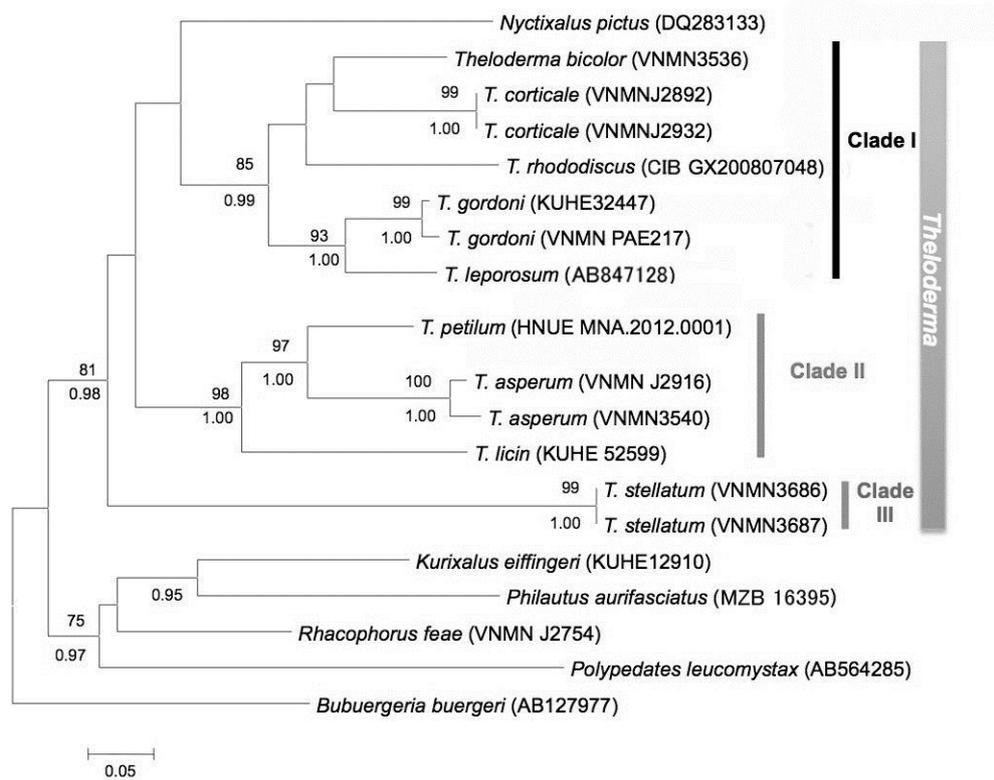
Species	Voucher No	Genbank No.	Locality
<i>Theلودerma asperum</i>	VNMN 3540	KJ802913	Vietnam, Lao Cai
<i>T. asperum</i>	VNMN J2916	KJ802914	Vietnam, Vinh Phuc
<i>T. bicolor</i>	VNMN 3536	KJ802915	Vietnam, Lao Cai
<i>T. corticale</i>	VNMN J2892	KJ802916	Vietnam, Tuyen Quang
<i>T. corticale</i>	VNMN J2932	KJ802917	Vietnam, Vinh Phuc
<i>T. gordonii</i>	VNMN PAE217	KJ802918	Vietnam, Son La
<i>T. gordonii</i>	KUHE 32447	KJ802919	Laos, Houapan
<i>T. leporosum</i>	KUHE 52581	AB847128	Malaysia, Negeri Sembilan
<i>T. licin</i>	KUHE 52599	KJ802920	Malaysia, Selangor
<i>T. rhododiscus</i>	CIB GX200807048	KJ802921	China, Guangxi
<i>T. stellatum</i>	VNMN 3686	KJ802922	Vietnam, Phu Yen
<i>T. stellatum</i>	VNMN 3687	KJ802923	Vietnam, Phu Yen
<i>Philautus aurifasciatus</i>	MZB 16395	KJ802924	Indonesia, Java, Central Java
<b><i>P. petilus</i></b>	HNUE MNA.2012.0001	KJ802925	Vietnam, Dien Bien
<i>Kurixalus eiffingeri</i>	KUHE 12910	KJ802926	Japan, Iriomote Is.
<i>Polypedates leucomystax</i>	MZB unnumbered	AB564285	Indonesia, Java, Depok
<i>Rhacophorus feae</i>	VNMN J2754	KJ802927	Vietnam, Lao Cai
<i>Nyctixalus pictus</i>	FMNH 231095	DQ283133	Malaysia, Sabah
<i>Buergeria buergeri</i>	IABHU 41011	AB127977	Japan, Hiroshima

**TABLE 6-2.** Uncorrected p-distances (in %) for short fragment of 16S rRNA among species in the genus *Theلودerma* and outgroup species.

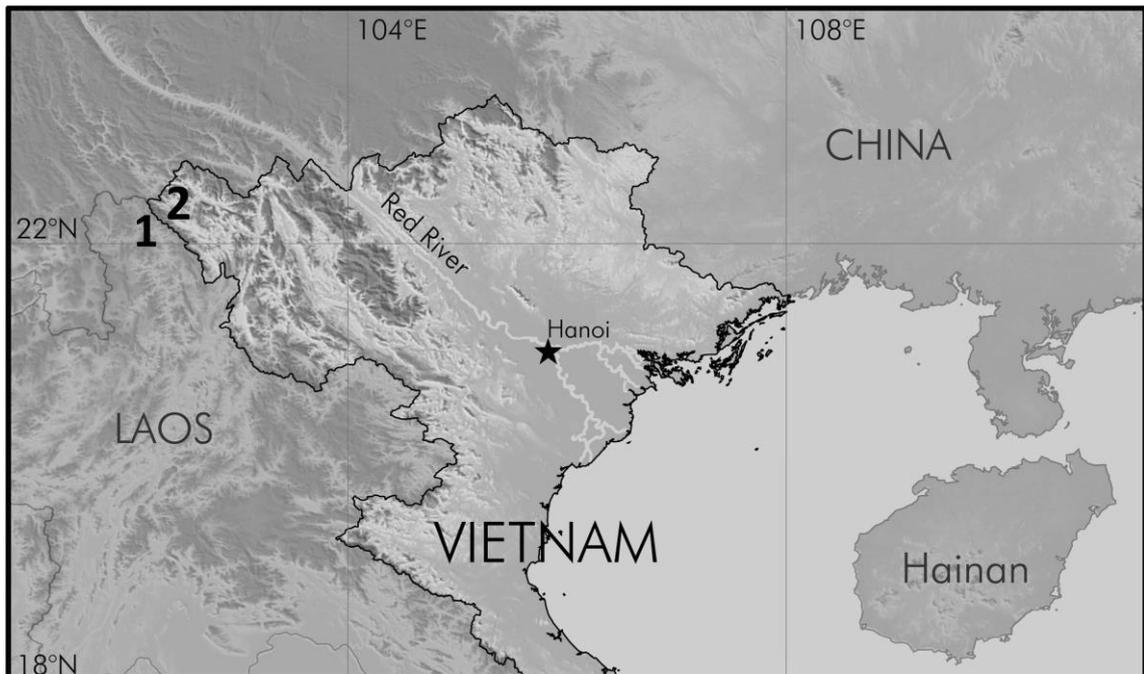
Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1 <i>Theلودerma asperum</i> (VNMN 3540)	-																	
2 <i>T. asperum</i> (VNMN J2916)	2.9	-																
3 <i>T. bicolor</i> (VNMN 3536)	18.5	19.2	-															
4 <i>T. corticale</i> (VNMN J2892)	18.5	18.5	10.1	-														
5 <i>T. corticale</i> (VNMN J2932)	18.5	18.5	10.1	0	-													
6 <i>T. gordonii</i> (VNMN PAE217)	17.4	17.6	12.1	13.5	13.5	-												
7 <i>T. gordonii</i> (KUHE 32447)	17.6	17.8	12.8	13.7	13.7	1.1	-											
8 <i>T. leporosum</i> (KUHE 52581)	19.2	19.8	12.4	13.5	13.5	8.3	7.6	-										
9 <i>T. licin</i> (KUHE 52599)	14.9	15.3	17.8	19.2	19.2	17.3	17.4	17.8	-									
10 <i>T. rhododiscus</i> (GX200807048)	18.2	18.3	13.5	13.1	13.1	14	14	13.7	18.5	-								
11 <i>T. stellatum</i> (VNMN 3686)	22.7	23	18.2	20	20	20.3	20.9	20.5	21.6	21.8	-							
12 <i>T. stellatum</i> (VNMN 3687)	22.7	23	18.2	20	20	20.3	20.9	20.5	21.6	21.8	0	-						
13 <i>Philautus aurifasciatus</i> (MZB 16395)	21	21.4	19.4	19.6	19.6	19.2	19.1	17.8	19.1	22.3	21.8	21.8	-					
14 <i>P. petilus</i> (HNUE MNA.2012.0001)	11.2	11.7	15.8	16.2	16.2	14.7	15.1	16.4	15.3	18.3	22.7	22.7	19.8	-				
15 <i>Kurixarus eiffingeri</i> (KUHE 12910)	21.4	20.3	17.8	18.7	18.7	17.6	18	17.8	20.1	20.7	22.1	22.1	16.9	18.9	-			
16 <i>Polypedates leucomystax</i> (AB564285)	21.8	21.4	20.3	20.7	20.7	20.5	21.4	21.4	22.5	20.9	25.5	25.5	21.9	21.9	18.7	-		
17 <i>Rhacophorus feae</i> (VNMN J2754)	18.7	18.5	18.5	18.2	18.2	15.5	15.6	16.5	18.2	17.8	20.1	20.1	16.4	18.2	13.7	20	-	
18 <i>Nyctixalus pictus</i> (DQ283133)	18.3	19.2	16	16.7	16.7	16	16	18.5	15.8	16.4	21.2	21.2	19.2	18.7	17.3	20.9	16.9	-
19 <i>Buergeria buergeri</i> (AB127977)	19.1	19.8	18.5	18.7	18.7	17.8	17.8	18	19.6	19.2	22.3	22.3	19.4	18.7	19.1	20.3	16	21.4



**Figure 6-1.** A male *Theloderma petilum* (HNUE MNA.2012.0001) from Dien Bien Province, Vietnam: A) Dorsal view and B) ventral view.



**Figure 6-2.** Maximum-likelihood (ML) tree of *Theloderma petilum* and related taxa based on the partial 16S rRNA mitochondrial gene. Numbers above and below branches are ML bootstrap values (values  $\geq 70$  shown) and Bayesian posterior probabilities (values  $\geq 0.95$  shown).



**Figure 6-3.** Distribution of *Theloderma petilum*: 1) Phou Dendin National Biodiversity Conservation Area, Phongsaly, Laos (type locality) and 2) Muong Nhe Nature Reserve, Dien Bien Province, Vietnam.

## CHAPTER 7

### General discussion

#### 7.1 Diversity of the rhacophorid fauna in Vietnam

As repeatedly stated in the above chapters, the family Rhacophoridae is one of the most diverse amphibian groups in the Vietnamese fauna. From the use of newly obtained large collections for the molecular and morphological analyses shown in chapters 2 to 6, I could elucidate the presence of the genus *Liuixalus* in northern Vietnam, which brought the total genus number of this family to 11. I also described additional two new species and documented one new country record, bringing the total number of 73 rhacophorid species (35% of the total species number of the Vietnamese amphibian fauna and approximately 20% of the total species number of the Rhacophoridae currently known in the world: Frost, 2014). Of these, markedly diverse genera, in terms of the species richness, are *Rhacophorus* (22 species), *Theلودerma* (15 species), and *Kurixalus* (9 species). With 16 new species having been described during recent five years (Milto et al., 2013; Nguyen et al., 2013a; Nguyen et al., 2014a, b, c; Orlov et al., 2012; Ostroshabov et al., 2013; Rowley et al., 2010, 2011a, b, 2012a, b, 2014), Vietnam is surely considered as a hotspot of new species discovery in Asia, which idea has been proposed by some authors (Nguyen, 2006; Ziegler and Nguyen, 2010). In addition to this current situation, future discovery of new species and new recordings of species occurring in adjacent regions will surely demonstrate further rich amphibian fauna of this country.

#### 7.2 Unresolved taxonomic problems in some species

##### 7.2.1 Genus *Kurixalus*

Because there are few usable external characteristics, the color pattern is important for identification in rhacophorids. However, my collection of a large number of *Kurixalus* species from Vietnam showed a wide spectrum of color pattern. In addition, the body color can change in frogs in life in response to the background color, while it

may disappear after preservation as time passes.

As my results in chapters 3 and 4 show, molecular phylogenetic analyses have proved the presence of two new species, *Kurixalus viridescens* and *K. motokawai*. Of these, *K. motokawai* is morphologically not only very similar to *K. banaensis*, but also closely resembles species of other genus such as *Raorchestes gryllus* (IUCN, 2012; Orlov et al., 2012). *Kurixalus banaensis* was originally described by Bourret (1939) in the genus *Philautus* from Bana (Ba Na, Da Nang City) Vietnam, while *Raorchestes gryllus* was originally described by Smith (1924) from Langbian Peaks, Langbian Plateau, S. Annam (Vietnam) also as a member of *Philautus*. In their redescription, Orlov et al., (2012) referred to a wide variation in the color patterns in life of *R. gryllus*, which includes patterns very similar to those frequently seen in *K. motokawai* and *K. banaensis*. Morphological diagnoses currently available are not so effective for these species, and morphological differentiation of *K. motokawai*, *K. banaensis*, and *R. gryllus* needs to be fixed based on new analytical techniques (Orlov et al., 2012). Another thing, *R. gryllus* has been recorded in a wide range of habitats, at elevations between 800 and 2200 m (Nguyen et al., 2009; Orlov et al., 2012) and the morphological characteristics of this species are also very similar to those of some other species like *Kurixalus bisacculus* (IUCN, 2012; Orlov et al., 2012) Most probably, acoustic characteristics would play a great role in differentiating them, and accumulation of recordings of their calls is of immediate importance.

### **7.2.2 Genus *Rhacophorus***

#### ***Rhacophorus calcaneus* and *R. robertingeri***

In the past, taxonomic studies were forced to rely heavily on morphological characteristics, especially on external ones. However, current taxonomy contrastingly relies on the results of phylogenetic relationships inferred from molecular analyses. Thus, when there are inconsistencies between the results drawn from these two approaches, current researchers easily treat morphological similarity as the result of convergence. In contrast, new taxonomic systems drawn from molecular phylogeny usually fail in showing synapomorphies among taxa grouped.

Based on morphological examination, Orlov et al., (2012), while synonymizing *Rhacophorus chuyangsinensis* with *R. calcaneus*, described *R. robertingeri* based on

“*Rhacophorus calcaneus*” in some of their previous reports (e.g., Orlov et al., 2008; Inger et al., 1999). They (Orlov et al., 2012) assigned *R. robertingeri* to their *R. calcaneus* group, and differentiated the two species by external characteristics such as texture and color patterns on dorsal surface and flank, although such features are changeable and difficult to recognize in long-preserved specimens because of fading of color along time.

Contrasting to Orlov et al.’s (2012) grouping from morphology, results of my phylogenetic analysis (Chapter 5) indicated closer relationship of *R. robertingeri* to *R. orlovi* than to *R. calcaneus*, although their sister species relationship was not confirmed. The discordance between morphology and mitochondrial phylogeny shown in these rhacophorid species, illustrates mere an example among large number of similar cases known in various animal taxa.

### ***Rhacophorus dugritei* complex**

In my result shown in chapter 5, based on molecular analyses, *R. duboisi* was considered a member of the *R. dugritei* group sensu Li et al. (2012a). Unlike the case in *R. robertingeri* and *R. calcaneus*, my result from molecular analyses agreed with one of two phylogenetic hypothesis from morphological evidence (Ohler et al., 2000), and disagreed with another hypothesis from molecular evidence (Li et al. 2012b). I could not find out reasons for this inconsistency between two molecular works, but the difference would be related to the differential genes compared and the size of gene fragments. In the worst case, incorrect identification of samples can be involved, and first correct morphological identification is very important in this kind of works.

Another member of the *R. dugritei* group, *R. pingbianensis* had similarly phylogenetic and taxonomic problems. My result strongly suggested its close relationship with *R. omeimontis*.

### ***Rhacophorus rhodopus***

Taxonomic problems in some Vietnamese rhacophorid frogs cannot be solved without examining same or closely related species occurring outside the country. As shown in the results in chapter 5, two groups were recognized within *R. rhodopus* from Vietnam, like the result already reported by Yu et al. (2007) among Chinese populations. As far as I have examined, specimens of *R. rhodopus* from Vietnam do not differ clearly in morphological characters. Therefore, in order to clarify the

taxonomy of this species in Vietnam, future studies both from morphology and molecular phylogeny should be done based not only on additional samples from their whole distribution range both in Vietnam and China, but also on adequate samples of its relative, *R. bipunctatus*, from surrounding countries.

### **7.2.3 Genus *Theلودerma***

As discussed above, recent molecular phylogenetic studies suggested great changes in the past taxonomic framework based virtually on morphology alone. This is particularly the case in the generic assignment of small rhacophorid species including those formerly nested in the genus *Philautus*. In case of Vietnamese species, *P. banaensis* was moved to *Kurixalus*, and *P. jinxiuensis* Hu and *P. quyeti* Nguyen, Hendrix, Böhme, Vu, and Ziegler were transferred to *Gracixalus* by Li et al. (2008, 2009). Whereas *P. gryllus*, *P. longchuanensis* Yang and Li, and *P. parvulus* (Boulenger) were placed in the genus *Raorchestes* after Biju et al. (2010). Furthermore, *P. truongsoneensis* Orlov and Ho was allocated to *Theلودerma* by Rowley et al. (2011a).

As a result of these drastic changes, the taxonomic relationships of species in the genus *Theلودerma* have become complicated. Due to the limited number of morphological synapomorphies within the genus, and unresolved past molecular phylogenies (Rowley et al., 2011a), generic allocation of some species within the genus is in want of confirmation. My molecular results shown in chapter 6 confirmed the placement of *Philautus petilus* in the genus *Theلودerma*, but there remain several other species that require similar examination. In addition, morphological reassessments of molecularly hypothesized taxonomy are inevitable to establish reliable taxonomy.

## Conclusions

This study was carried out in order to gain better understanding of the systematics of rhacophorids in Vietnam, and as a result, I could achieve the following contributions:

1. Discovery of new rhacophorid species and new locality records for Vietnam. Three new species, one new country record species and genus have been recently discovered from Vietnam, and have brought the total species number of Rhacophoridae known from Vietnam to 73 in 11 genera. With the newly described species, and newly recorded and generically combined species (*Theلودerma petilum* transferred from *Philautus*), Vietnam is now the country embracing nine species of the genus *Kurixalus* (75% of the species now recognized in the genus) and 15 species of the genus *Theلودerma* (65% of the known species, including an ambiguous Chinese species *T. moloch*, Li *et al.* 2009: Frost, 2014), and can be regarded as the center of speciation of these two genera.
2. Updates of molecular and morphological data for the Vietnamese rhacophorids. These baseline data include new species and the first molecular report of three *Rhacophorus* species endemic to Vietnam (*R. robertingeri*, *R. exechopygus*, and *R. vampyrus*). The data also provide taxonomic relationships of the *R. dugritei* group, including members from Vietnam. In addition, taxonomic relationship of *R. rhodopus* and *R. bipunctatus* is also discussed.

## Outlook

As pointed out in the discussion, the amphibian species richness of Vietnam is currently clearly underestimated. The surveys conducted in this country indicate that the species in the high elevation forests and adjacent regions are still not fully revealed. Hence, further intensive researches in this field are strongly recommended.

Many taxonomic problems seem to be not so easily solved and comprehensive revision of the rhacophoridae requires accumulation of much more data. Therefore, further studies both from morphology and molecular phylogeny based on additional samples from their whole distribution range are necessary to achieve these difficult, but important goals.

Acoustic data of the calls of rhacophorids would surely provide more usable traits for identifying species with similar morphological characters, such as *Raorchestes gryllus*, *Kurixalus banaensis*, and *K. motokawai*. Thus, future study should aim to link morphology and bioacoustics to support easier and reliable taxonomic identification. As suggested by previous studies (e.g., Matsui et al., 2001, Ohler et al., 2002, Gawor et al., 2009; Frost, 2014) studies on vocal properties are properly and effectively utilized to confirm taxonomic status of cryptic species forming a complex as seen in Vietnamese *Polypedates*.

The final goal of studies as treated in this thesis is to enumerate rhacophorid taxa occurring in Vietnam as far as possible, so that we can confirm actual biodiversity and estimate evolutionary history of this frog family in Vietnam. However, in order to achieve this, we must at the same time consider conservation of animals and environment surrounding them. Otherwise, it would be not possible even to collect basic data in future.

The principal threats to the amphibians, including Rhacophoridae, in Vietnam are habitat loss and degradation, unsustainable trade, followed by invasive species introduction, pollution, disease, and climate change. Currently, conservation studies for habitat protection are insufficient in this country. Also, activities for environmental impact mitigation of economic development projects, and linking isolated forest patches require more efforts. It is also recommended to enforce wildlife trade control laws, and implement biodiversity awareness campaigns. We, systematic herpetologists, must play a great role in these situations.

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