

**THE SEARCH FOR GENETIC STRUCTURE AND
PATTERNS IN VIETNAMESE FROGS**

by

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A thesis submitted in conformity with the requirements

for the degree of Doctor of Philosophy

Graduate Department of Ecology & Evolutionary Biology

University of Toronto

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ABSTRACT

The Search for Genetic Structure and Patterns in Vietnamese Frogs

Doctor of Philosophy, 2008

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Vietnam has the greatest biodiversity of any country in Indochina. This diversity may be due to its topographically complex nature, with hills and mountains, drained by several independent river systems, covering three quarters of its area. Topographic complexity has undoubtedly had profound effects on the flora and fauna of the region. Recent surveys have uncovered several cryptic species in what were previously considered single widespread species. These discoveries have led some researchers to propose that widespread forest species do not, in fact, exist in Southeast Asia. To test these hypotheses, I examined patterns of mitochondrial phylogeny in several groups of frogs, both at and below the species level. Additionally, these analyses helped clarify the otherwise chaotic picture of anuran taxonomy and systematics. The stream-tied waterfall frogs of the genera *Amolops* and *Odorrana* were examined, the monophyly of the ranid subfamily Amolopinae was rejected, and taxonomic adjustments were made. The phylogeny of the Vietnamese narrow-mouthed frogs of the genus *Microhyla* was recovered and the current taxonomy examined. Patterns of maternal dispersal and genetic differentiation in mitochondrial DNA were further examined within *Microhyla heymonsi*, revealing geographic

structuring and the existence of two sympatric lineages. Lastly, frogs of the *Polypedates leucomystax* complex were examined and two major, largely sympatric lineages recovered. Within these groups, 11 separate mitochondrial lineages identified. These represented separate species on the basis of advertisement call and allozyme evidence. The relationship of genetic differentiation and river systems was also investigated and common patterns among the different groups were explored. Clear genetic breaks occurred across both the Red River and the Annamite Mountain range, though most common patterns were groupings of populations along river drainages. While several cryptic species were identified, widespread groups likely representing single species still exist, and a phylogenetic component to broad distribution were noted.

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TABLE OF CONTENTS

THE SEARCH FOR GENETIC STRUCTURE AND PATTERNS IN	
VIETNAMESE FROGS	
	i
Abstract	ii
Acknowledgements	iv
Table of Contents.....	ix
List of Tables	xv
List of Figures	xviii
List of Appendices	xx
Chapter 1 — Introduction	1
Vietnam	1
Anuran biodiversity in Vietnam	5
Vietnam as a peninsula: the search for common patterns.....	7
Specimens, species, and groups examined in this thesis.	9
Objectives of the dissertation	12
Thesis organization.....	14
References	16
Chapter 2 — The Phylogenetic Relationships of the Chinese and Vietnamese	
Waterfall frogs of the genus <i>Amolops</i>.....	20
Abstract.....	20
Introduction	20

Materials and Methods	23
<i>Specimens Examined</i>	23
<i>DNA Amplification and Sequencing</i>	24
<i>DNA Sequence Analysis</i>	24
Results	25
<i>Sequence Variation</i>	25
<i>Phylogenetic Analysis</i>	26
Discussion.....	27
Conclusion	28
References	29
Acknowledgments.	34
Chapter 3 — The phylogenetic relationships of the Vietnamese narrow-mouthed frogs, Genus <i>Microhyla</i> Tschudi 1838, and the validity of Genus <i>Micryletta Dubois 1987</i>	45
Abstract.....	45
Introduction	45
Materials and Methods	47
<i>Specimens Examined</i>	48
<i>DNA gene selection</i>	48
<i>DNA Amplification and Sequencing</i>	48
<i>DNA Sequence Analysis</i>	50
<i>Maximum Parsimony Evaluation</i>	50
<i>Bayesian Inference Analysis</i>	51
Results	52

<i>Parsimony evaluation</i>	52
<i>Bayesian Inference Evaluation</i>	54
<i>Nodal Stability</i>	55
Discussion.....	55
<i>Phylogenetic Hypotheses</i>	55
<i>Taxonomic Implications</i>	56
Genus <i>Micryletta</i> , subgenera of <i>Microhyla</i> , and species groups	56
<i>Microhyla okinavensis</i> and <i>Mh. fissipes</i>	58
<i>Biogeographical implications</i>	58
Acknowledgments.	60
References	61
 Chapter 4 — Biogeographical Patterns of Maternal Lineages of <i>Microhyla heymonsi</i>	
Vogt 1911.....	84
Abstract.....	84
Introduction	84
Materials and Methods	86
<i>Specimens Examined</i>	86
<i>DNA gene selection</i>	86
<i>DNA Amplification and Sequencing</i>	87
<i>DNA Sequence Analysis</i>	88
<i>Maximum Parsimony Evaluation</i>	88
<i>Bayesian Inference Analysis</i>	89
Results	90
<i>Parsimony evaluation</i>	90

<i>Bayesian Inference Evaluation</i>	91
<i>Nodal Stability</i>	91
Discussion.....	91
<i>Taxonomic implications</i>	92
<i>Biogeographic patterns</i>	93
Acknowledgments.	94
References	95
Chapter 5 — Are There Any Widespread Forest Amphibian Species in Asia:	
Cryptic Species and the <i>Polypedates leucomystax</i> Complex.112	
Abstract.....	112
Introduction	112
Materials and Methods	117
<i>Specimens examined</i>	117
<i>DNA amplification and sequencing</i>	117
<i>DNA sequence alignment</i>	118
<i>Maximum parsimony evaluation</i>	119
<i>Bayesian inference evaluation</i>	119
Results	120
<i>Parsimony evaluation</i>	121
<i>Branching patterns</i>	121
<i>Bayesian inference evaluation</i>	122
<i>Nodal stability</i>	123
Discussion.....	123
<i>Phylogeny and patterns</i>	124

<i>Sympatric maternal lineages</i>	126
<i>Taxonomic implications</i>	126
<i>Biogeography</i>	128
Acknowledgments	129
References	130
Chapter 6 — Summary	162
Introduction	162
Geographic Barriers and Conduits	162
<i>Rivers as Conduits</i>	164
<i>Rivers as Barriers</i>	167
Widespread Forest Species	170
The historical biogeography of Vietnam: a clouded picture	174
References	177
Appendix 1 — Taxonomic Chaos In Asian Ranid Frogs: An Initial Phylogenetic Resolution	183
Introduction	183
Materials and methods.....	184
<i>Specimens Examined</i>	184
<i>DNA Amplification and Sequencing</i>	185
<i>DNA Sequence Analysis</i>	186
Results	187
<i>Parsimony evaluation</i>	188
<i>Assessing Nodal Stability</i>	189

Discussion.....	189
<i>Previous studies</i>	190
<i>Patterns of relationships</i>	192
<i>Monophyly of the Ranidae and relationships among Subfamilies</i>	192
Acknowledgements	203
References	205

LIST OF TABLES

Table 2.1. Specimens used, voucher specimen catalog numbers, and collecting locality.

Sequences for specimens marked with a dagger (†) were obtained from
GenBank..... 35.

Table 2.2. Primers used for amplifying and sequencing fragments of RNA genes in the
specimens included in this study. Sequence position indicates the starting position
of the primer on the *Xenopus laevis* mitochondrial genome and is preceded by the
direction of amplification (H=heavy / L=light strand)..... 39.

Table 3.1. Species of *Microhyla* (*Mh.*), *Micryletta* (*Ml.*) and *Kaloula* that were
sequenced, voucher specimen catalog numbers, and collecting localities. The
taxonomy generally follows Frost (2007) 71.

Table 3.2. Primers used for amplifying and sequencing fragments of genes from species
of *Microhyla*, *Micryletta*, and *Kaloula*. Sequence position indicates the starting
position of the primer in the *Xenopus laevis* genome and is preceded by the
amplification direction as indicated by (H) heavy or (L) light
strand..... 81.

Table 3.3. Summary of genes sequenced from the ingroup taxa, *Microhyla* and
Micryletta, and the outgroup, *Kaloula*. TS = total number of homologous sites
resolved; AS = number of ambiguous sites removed; NSR = number of
homologous sites retained; NVS = number of variable sites; NPPIS = number of
potentially phylogenetically informative sites; NMPTs = number of most
parsimonious trees resolved; LMPTs = Length of most parsimonious solution; CI
= consistency index; RI = retention index..... 83.

Table 4.1. Species sequenced, voucher specimen catalog numbers, and collecting locality. Sequences for specimens marked with a dagger (†) were obtained from GenBank. The taxonomy follows Frost (2007)..... 103.

Table 4.2. Primers used for amplifying and sequencing fragments of genes in this study. Sequence position indicates the starting position of the primer in the *Xenopus laevis* genome and is preceded by the amplification direction as indicated by (H) heavy or (L) light strand..... 109.

Table 4.3. Summary of genes sequenced from the ingroup and outgroup taxa. TS = total number of homologous sites resolved; AS = number of ambiguous sites removed; NSR = number of homologous sites retained; NVS = number of variable sites; NPPIS = number of potentially phylogenetically informative sites; NMPTs = number of most parsimonious trees resolved; LMPTs = Length of most parsimonious solution; Ts:Tv = transition to transversion ratio; CI = consistency index; RI = retention index..... 111.

Table 5.1. Specimens used, voucher specimen catalog numbers, and collecting locality. Sequences for specimens marked with a dagger (†) were obtained from GenBank..... 143.

Table 5.2. Primers used for amplifying and sequencing fragments of the Cytochrome b and ND1 genes in this study. Sequence position indicates the starting position of the primer on the *Xenopus laevis* mitochondrial genome and is preceded by the direction of amplification (H=heavy / L=light strand)..... 158.

Table 5.3. Summary of genes sequenced from the ingroup and outgroup taxa. TS = total number of homologous sites resolved; NVS = number of variable sites; NPPIS = number of potentially phylogenetically informative sites; NMPTs = number of

most parsimonious trees resolved; LMPTs = length of most parsimonious solution excluding uninformative characters; CI = consistency index; RI = retention index; RC = rescaled consistency index.....	159.
Table 5.4. Localities containing sympatric representatives of distinct lineages and the clades represented.....	160.
Table 5.5. Unnamed potential species of the <i>Polypedates leucomystax</i> species complex.....	161.

LIST OF FIGURES

- Figure 1.1.** Map of Southeast Asia showing sea levels at the hypothesized minimum during the Pleistocene (120 m below present level). Darker grey areas represent present day landmasses. At this level, Vietnam was connected by land to the islands of Hainan, Taiwan, Java, Sumatra, Borneo, as well as some of the Philippine islands. Map after Voris (2000)..... 8.
- Figure 2.1.** a. Manus of *Amolops mantzorum* illustrating the expanded digital discs. b. Drawing of a larval *Amolops mantzorum* illustrating the gastromyzophorous disc. After Liu (1951) Figs. 83 and 84..... 41.
- Figure 2.2.** A map of Vietnam and the southern provinces of China, showing collection localities of ingroup specimens from the genera *Amolops*, *Huia*, *Meristogenys*, and *Odorrana*. Numbers correspond to the specimen numbers in Table 1.1... 42.
- Figure 2.3.** The single most parsimonious tree resolved by analysis of the combined data for all genes sequenced. Outgroups have been pruned for clarity. Branch lengths represent the amount of change along the tree. Bootstrap Proportions (BSP > 50) are displayed above nodes and decay indices (DI) below..... 43.
- Figure 2.4.** A comparison of the phylogeny presented by Pang and Liu (1992) and the strict consensus of 99 MPTs resolved by a reanalysis of their data. Brackets delineate their groups A, B, and C..... 44.
- Figure 3.1.** Map of specimen localities. Numbers refer to the specimens in Table 1.1..... 68.
- Figure 3.2.** Maximum parsimony tree. Strict consensus of 576 most parsimonious trees. Bootstrap proportions are above the branches and Bayesian posterior probabilities

are below.....	69.
Figure 3.3. Bayesian 50% majority rule consensus tree showing relative branch lengths and species relationships.....	70.
Figure 4.1. Map of <i>Microhyla heymonsi</i> specimen localities. Numbers refer to the specimens in Table 4.1.....	100.
Figure 4.2. Maximum Parsimony tree. Strict consensus of 576 most parsimonious trees. Bootstrap proportions are above the branches and Bayesian posterior probabilities are below.....	101.
Figure 4.3. Bayesian 50% Majority Rule Consensus Tree showing relative branch lengths and species relationships. Bootstrap proportions are above the branches and Bayesian posterior probabilities are below.....	102.
Figure 5.1. Map of Specimen Localities. Numbers refer to the specimens in Table 5.1.....	138.
Figure 5.2. a) Phylogram of the relationships of ingroup specimens labeled with the clade names used in the text and figures. b) Phylogram of the relationships of the specimens in Clade A. Bootstrap proportions above 50% are placed above the branches and Bayesian posterior probabilities above 50% are below. c) Phylogram of the relationships of the specimens in Clade B. Bootstrap proportions above 50% are placed above the branches and Bayesian posterior probabilities above 50% are below.....	139.
Figure 6.1. Relief map of northern Vietnam, showing river systems and associated mountains, massifs, and deltas.....	169.
Figure 6.2. Maximum parsimony tree of Clade B of the <i>Polypedates leucomystax</i> complex showing the geographic breadth of the subclades.....	173.

LIST OF APPENDICES

Appendix 1. Taxonomic Chaos in Asian Ranid Frogs: An Initial Phylogenetic Resolution.....	183.
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Chapter 1

Introduction

Vietnam

Vietnam is situated entirely in the tropics of the northern hemisphere and spans an area of 330,363 km², 15 degrees latitude from 23°22'N to 8°30'N, and seven degrees longitude from 102°10'E to 109°21'E (Lap, 1999; Nguyen, 1995; Thao, 1998). It shares 1,150 km of land border with the People's Democratic Republic of China, 930 km with the Kingdom of Cambodia, and 1,650 km with the Lao People's Democratic Republic. Vietnam also has 3,260 km of coastline with the South China Sea to the east and Gulf of Thailand in the southwest (Nguyen, 1995). At its narrowest point in the middle, the country is only 50 km across, while at its widest in the North, it is approximately 600 km wide.

Hills and mountains cover approximately three-quarters of the country (Nguyen, 1995). Though southern Vietnam is characterized by lowlands, mountains and highlands are the dominant feature in northern and central Vietnam (Nguyen, 1995). Fan Si Pan Mountain, part of the Hoang Lien Range in northern Vietnam, is the highest peak in the country, reaching a height of 3,143 m. The highest peak in central Vietnam is Ngoc Linh Mountain, straddling Kon Tum and Quang Nam provinces and reaching a height of 2,598

m (Vu, 1994). More than ten other peaks in Vietnam exceed 2000 m, and more than 20 exceed 1,500 m (Orlov et al., in press). These peaks are separated by numerous valleys and basins in southern Vietnam and either deep ravines or wide valleys in the north potentially isolating the faunas of each (Nguyen, 1994).

The mountainous regions of Vietnam are divided into four zones: the Northwestern Zone, the Northeastern Zone, the Northern Truong Son Zone, and the Southern Truong Son Zone. In the Northwestern Zone, west of the Red River (Song Hong), the main mountain systems form massifs. The most notable is the Hoang Lien Son Range, which includes Fan Si Pan Mountain and the well known village of Sa Pa. This mountain range lies between the Black and Red Rivers until the point where they join. The mountains of the Northeastern Zone are generally lower in elevation, mostly 600–700 m high. Higher mountain ranges in this zone are concentrated along the Sino-Vietnamese border. The highest of these peaks, at 2,419 m, is Mount Tay Con Linh, part of the Chay River Massif, and the highest point in Vietnam east of the Red River.

The primary mountain range of the Northern and Southern Truong Son zones is the Annamite Range (Nui Truong Son). This range runs through central Vietnam parallel to the coast of the South China Sea. Together with the massifs of the Northwestern Zone, this mountain system forms a nearly monolithic ridge that stretches from the border with China southward to the Mekong River delta, essentially isolating Vietnam from the rest of Indochina (Laos, Cambodia, and Vietnam) and mainland Southeast Asia.

The Northern and Southern Truong Son Zones are separated by the Hai Van Pass, situated between the Thua Thien and Quang Nam provinces. The Southern

Truong Son Zone is also of note due to the presence of a large plateau, known as the Central Highlands. Ngoc Linh is the second highest mountain in Vietnam at 2598 m and the highest peak in the Central Highlands. It home to distinctive endemic lineages and recently described species (Genus *Acanthosaura*, Kalyabina-Hauf et al., 2004; *Microhyla nanopollexa* Bain and Truong, 2004; *Vibrissaphora ngoclinhensis* Orlov, 2005). It is connected to the southern Annamite Range via the southeast to northwest oriented Nam–Ngai–Dinh ridge. The peaks in this ridge are linked by a series of sharp ridges. These high mountains and the adjacent highland areas are referred to as the Kon Tum Plateau. The Kon Tum Plateau, and Ngoc Linh in particular, is a mountain isolate, separated by relatively long distances from other high altitude areas, and an effective island for the organisms living on it. It is a center of endemism (Tordoff et al., 2000).

South of the Central Highlands and the Southern Truong Son Zone, Vietnam is flatter, dominated by lowlands, floodplains and river deltas, draining both the Mekong and the Dong Nai–Vam Co river systems. While the Yok Don Nature Reserve is primarily lowland dipterocarp forest, the majority of this region is heavily cultivated or otherwise modified for human use (Nguyen, 1995). It is subject to regular flooding during the monsoon season.

There are nine major river systems in Vietnam. These include the Red River system, the Ky Cung / Bang Giang River system, and the Thai Binh River system in northern Vietnam, the Ma River, the Ca River, and the Thu Bon River (Ngoc Linh) in central Vietnam, and the Ba River, the Dong Nai / Vam Co River system, and the Mekong River in southern Vietnam.

The Northeastern and Northwestern zones are separated by the Red River (Song Hong), which coincides with the Ailao Shan shear zone. This river drains most of the montane portion of northern Vietnam. The 1,149 km-long Red River originates from Ngay Son Mountain, Dai Ly Lake in Tibet, and flows into Vietnam at Ha Khau, Lao Cai province. In Vietnam, this river is 550 km long. It is fed by two additional large tributaries the Black River (Song Da) and the Chay River (Song Chay). The sheer size of each of these three rivers forms potential barriers to frog dispersal and the rivers have been implicated as forming vicariant events responsible for speciation or genetic differentiation in the kukri snakes (*Oligodon*, Marc D. Green, pers. comm.) and the mountain horned dragons (*Acanthosaura lepidogaster*, Kalyabina-Hauf et al., 2004). Collecting sites along this drainage system include Sa Pa, Yen Bai, Pac Ban, and Hanoi. South and east of this, the northern lowlands are drained by the Tai Binh River system that joins the Red River in the Red River delta. The Red River Delta covers an area of 16,654 km². Collecting localities drained by this system include Ba Be National Park, Tam Dao National Park, the western portions of Hanoi, and Chi Linh.

The Ky Cung / Bang Giang River system, while also in northern Vietnam differs from the Red River drainage system and the Thai Binh river system in that it does not drain directly into the Gulf of Tonkin. Rather, these two rivers are confluent in Guangxi, China, forming the Ta Giang, a tributary of the Pearl River (Zhu Jiang), which, in turn, discharges into the South China Sea in Guangzhou, Guangdong province, China. Cao Bang, in Vietnam, is drained by the Bang Giang River and its tributaries, which empty via the Pearl River delta near both Hainan and Hong Kong in China.

In central Vietnam, most of the rivers are relatively short, flowing from the Annamite Mountains eastward into the South China Sea. The collection sites Con Cuong and Vinh are connected and drained by the 612 km Ca River, which originates in the Loi Mountains in Laos, passes thorough Nghe An province, Vietnam, and empties into the South China Sea. Further south, Ngoc Linh Mountain is drained by the Thu Bon River system and Kon Tum, Krong Pa, and Tram Lap are drained by the Da Rang into the South China Sea.

In southern Vietnam, the Cat Tien National Park is drained by the Dong Nai – Vam Co River system, which is the third longest river system after the Mekong and Red River systems in Vietnam. This system has over 200 tributaries, including the Da Dung, Dak Nong, La Nga, Sai Gon, and Vam Co rivers. Originating in the Central Highlands, this system discharges into the South China Sea via the Soai Rap River. Both Kon Tum and Yok Don National Park drain into the Mekong River by way smaller, eastwardly flowing tributaries.

Anuran biodiversity in Vietnam

Worldwide, amphibian diversity has become a major focus in the past ten years. Biodiversity surveys, conservation plans, and even barcoding have been the targets of significant funding, and with good reason. Amphibians, due to their semi-permeable skin and sensitivity to pollution, UV radiation, and other forms of habitat degradation have been identified as indicators of general environmental health. Further, there has been an unexplained increase in the decline of amphibian populations and number of species that

have disappeared (Houlahan et al., 2000). Extirpation and extinction have become serious risks for many amphibian species.

A general increase in species descriptions, constantly changing the number of recognized species, as well as the likelihood that many more species remain to be identified, makes estimates of the proportion of amphibian species at risk problematic (Duellman, 1999; Hanken 1999). Some of this increase is no doubt due to mounting efforts to find and document amphibian biodiversity before it disappears.

Indochina, and Vietnam in particular, has been the focus of recent surveys and species descriptions (Bain et al., 2003, 2004, 2006; Lathrop et al., 1998; Orlov, 2005; Orlov et al., 2002; Stuart et al., 2005). We need to know what is present to have informed conservation plans, as well as a stable taxonomy and phylogeny to implement conservation actions. Between 1997 and 2005, due to this focus on amphibian biodiversity, an increase biodiversity surveys, closer examination of museum collections, and the proliferation of molecular techniques, 53 new amphibian species were described from Indochina as well as 36 range extensions into the region (Bain et al., 2007).

In addition to instability in simple counts of species, there have been significant, recent changes made to higher level amphibian taxonomy (Appendix 1; Frost et al. 2006). These and future phylogenetic and population level evaluations and resulting taxonomic adjustments are of paramount importance. A stable taxonomy is required not only for biodiversity assessment but also for conservation. In practice, conservation is the domain of law informed by science. Currently, most legal systems are taxonomically based or they require the designation of specific lineages or populations (e.g. US Endangered Species Act of 1973 or the Canadian Species at Risk Act of 2002).

Proper assessment of biodiversity is more complicated than simple counts of species, genera, or families (Balmford et al. 2003). By focusing solely on the numbers of species, we may miss important genetically distinct lineages or populations that are either ecologically important or even at risk. Therefore, it is desirable to incorporate population level diversity and measures of phylogenetic distance into our biodiversity concepts and metrics (Dybas, 2001; Faith, 1992; Luck et al. 2003; Velland and Geber, 2005). To achieve this, additional characterization of the fauna from a phylogenetic and population level is required.

Vietnam as a peninsula: the search for common patterns

Vietnam is effectively a peninsula, with the Annamite Mountain Range to the West and the South China Sea to the East. However, this was not always the case. At times of maximum glaciation during the Quaternary, sea level was 80–120 m lower than present and as much as 5.4 m higher during interglacial periods (Boyd and Lam, 2004; Voris et al 2000). Fluctuating water levels since the Pleistocene have alternately rendered this area essentially a long peninsula and a single land mass, connected to the islands of Java, Sumatra, Taiwan, Hainan, Borneo, and the Philippines (Figure 1.1; Voris et al., 2000; Inger and Voris, 2001). This accordion-like expansion and contraction of potential habitat had the potential to alternately isolate lineages, leading to localized differentiation, and merge them, allowing the lineages to mix. Given that approximately 94% of the Pleistocene was glaciated (Van Devender and Burgess, 1985), the current interglacial isolation of Vietnam is atypical in geological time.

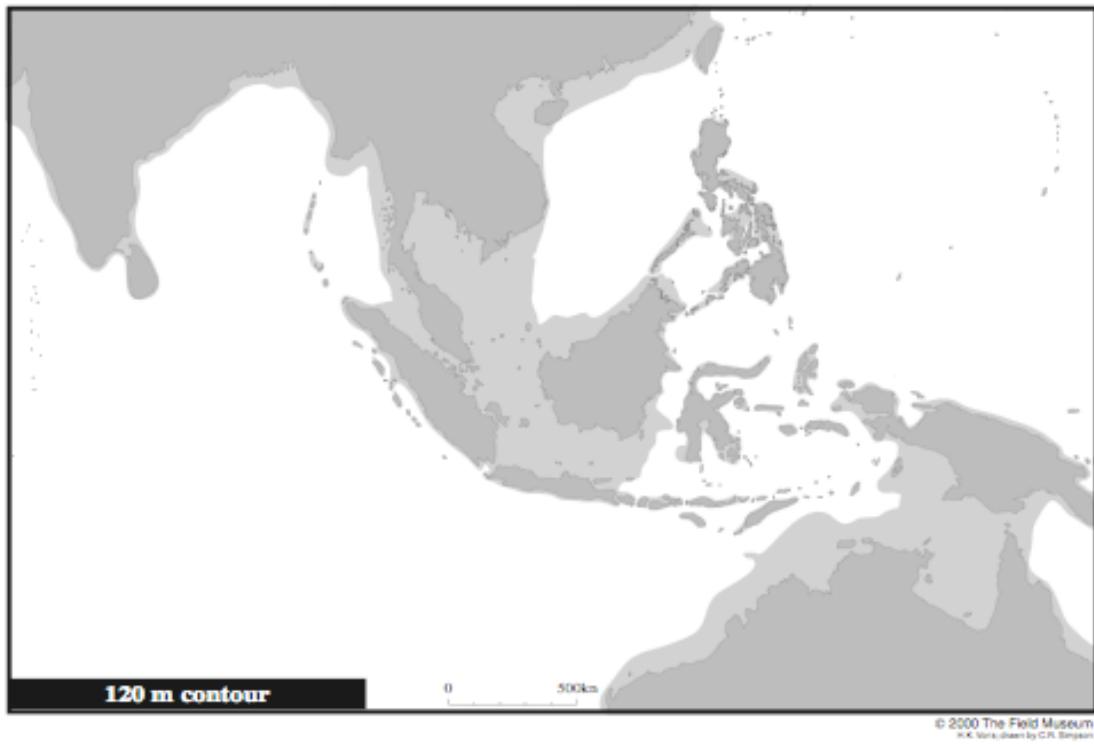


Figure 1.1. Map of Southeast Asia showing sea levels at the hypothesized minimum during the Pleistocene (120 m below present level). Darker grey areas represent present day landmasses. At this level, Vietnam was connected by land to the islands of Hainan, Taiwan, Java, Sumatra, Borneo, as well as some of the Philippine islands. Map after Voris (2000).

This repeated pattern of expansion and contraction predicts lack of structure and common pattern due to repeated mixing of lineages. Finding structure in the face of this implies additional factors that maintain lineage integrity and prompts questions regarding the processes involved in the generation and maintenance of species. This would be particularly expected in the case of pond breeding animals such as the frogs of the *Polypedates leucomystax* complex, *Microhyla*, and *Bufo*, which are not explicitly tied to river drainage systems. However, most of the studies of widespread species in Southeast Asia have focused on stream breeders, such as the *Odorrana livida* complex (Bain et al., 2003; Stuart et al., 2006), *Limnonectes* (Emerson et al., 2000; Evans et al., 2003; Gillespie et al., 2004; Toda et al., 1998; Tsuji, 2004), and *Rana chalconota* (Gillespie et al., 2004; Stuart et al., 2006). These animals would be expected to be tied to drainages and, therefore, show greater genetic structuring and likely represent more than one cryptic species. On the basis of these studies, broad statements have been made regarding the existence of widespread forest dwelling species in the region (Stuart et al., 2006).

Specimens, species, and groups examined in this thesis.

Selection of species and groups for inclusion in this thesis was based on a combination of factors. The search for broadly applicable biogeographic patterns requires the selection of groups with broadly overlapping distributions, different ecological tendencies and varied taxonomic levels. Species that are ecologically tied to streams and river systems are expected to show greater genetic structuring than pond breeding animals, which might move between drainages more readily. This combination further

allows for the testing of the hypothesis that there are no widespread forest species. Three groups of frogs were included in this thesis: the waterfall frogs of the genus *Amolops*; the microhylid frogs of the genera *Microhyla* and *Micryletta*; and the Javan whipping frogs of the *Polypedates leucomystax* species complex.

The waterfall frogs of the genus *Amolops* are a speciose group of ranid frogs that inhabit swift torrents and the splash zone of mountain cascades throughout Southeast Asia. These frogs possess a suite of characters associated with life in streams, further distinguishing them from other ranid frogs. Rather than having the “typical ranid” shape reminiscent of the edible frog, *Rana temporaria*, these frogs are flattened and possess drastically enlarged digital pads to help them adhere to rocks in fast moving torrents. The larvae are also unique in having a large gastromyzophorous adhesive disk (abdominal sucker). The tadpoles use this disk to affix themselves to the slippery surface of vertical rocks and boulders in the swiftly moving streams they inhabit (Liu, 1950; Yang, 1991a).

Narrow-mouthed frogs of the genera *Microhyla* and *Micryletta* are broadly distributed from South Asia across Southeast Asia to the Ryukyu Archipelago in Japan (Frost, 2007). They are small (Manthey and Grossman, 1997; Ziegler, 2002; Liu, 1950), locally abundant (Watanabe et al., 2005) and broadly distributed (Lee et al., 2006; Lever, 2003; Liu, 1950; Maeda and Matsui, 1999). Several species are either habitat generalists or associated with disturbed habitats. They breed in roadside ditches, grasslands, buffalo troughs, and rice paddies and may be prone to human mediated dispersal via the transport of crops, potted plants and lumber (Lee et al., 2006; Ota, 1999; Ziegler, 2002). *Microhyla heymonsi* was selected for further evaluation below the species level to look at patterns of

female dispersal due to its broad distribution, the large number of available samples, and preliminary results that showed some genetic structure between populations.

The Asian treefrogs allied to the Javan whipping frog, *Polypedates leucomystax* are a complex of species. They are geographically wide-ranging, locally abundant, and found in a variety of habitats, including ponds in primary forests, on the edges of secondary growth, and even in human settlements and metropolitan centers (Frost, 2007; Liu 1950; Manthey and Grossman, 1997; Pope 1930; Pope and Boring, 1940; Ziegler 2004). These frogs are also morphologically diverse. Populations of these frogs have either four clear longitudinal lines on the dorsum, six lines, an “X-shaped” marking on the dorsum, random flecking, a triangle pattern behind the head, a patternless dorsum, or some combination of these (Boulenger, 1890, 1912; Bourret, 1942; Church, 1963; Flower, 1896; Kirtisinghe, 1957). Their broad geographic range, combined with their apparently weedy nature and morphological polymorphism, has lead to significant controversy regarding their identity and taxonomic status (Frost, 2007; Matsui et al., 1986; Narins et al., 1998; Trépanier et al., 1999). Further confusing the situation, often multiple forms can be found at a single site, thereby clouding the picture, and individual *P. leucomystax* can change their color patterns in life, at least between striped and patternless forms (Church, 1963; Flower, 1896; Liu, 1950).

Specimens of these animals were selected from all Vietnamese river drainages and collection sites where tissues were available. In addition to Vietnamese animals, additional samples were obtained from Yunnan to further test the hypothesis that river drainages are both barriers to and conduits for gene flow since most of the major river drainages in northern Vietnam have upstream catchments in Yunnan. Additional

specimens from Laos, Cambodia, Thailand, Myanmar, China, and the Philippines were also included. These specimens serve to test the hypothesis that the Annamite mountain range acts as an eastern barrier, which, together with the South China Sea, isolates Vietnam from the remainder of Indochina and mainland Southeast Asia during interglacial periods. Due to their connection to the Mekong River, the Laotian and Cambodian specimens also serve as an additional test of the hypothesis that rivers can serve as a conduit for gene flow, connecting them to Vietnamese populations along the same drainage.

Objectives of the dissertation

In this thesis, I evaluate species and population level relationships of Vietnamese frogs to recover new hypotheses of evolutionary relationships, to test current implied phylogenetic hypotheses, and to provide necessary baseline biodiversity data. I also explore the geographical patterns of genetic structure in Vietnamese frogs.

The search for common patterns has long been a central focus of research programs. The presence of common patterns implies the existence of, and helps to identify common processes. Recent work in Baja California has shown common patterns across a broad variety of taxa, from lizards to mice and birds. Various hypotheses have been discussed for the common genetic break seen in mid-peninsular Baja California (Murphy and Aguirre León, 2002; Lindell et al., 2006). However, nature is not always that simple and many different processes act on different species and populations often having different effects. To evaluate this, I generated a comparative dataset and

framework from which these questions might begin to be addressed. Frogs that cover a range of habitat specialization, from torrent restricted frogs that are tied to specific rivers and drainages to widespread species that breed in ephemeral ponds, were selected for evaluation. I examined the patterns of relationships among the waterfall frogs of the genus *Amolops*. These frogs are specialized stream breeding frogs that would be expected to be tied to watersheds and river systems that might also serve as barriers to other species. I then examine Vietnamese representatives of the genus *Microhyla* to see if the same patterns are recovered in a widespread group of pond breeding frogs that, due to their dispersal abilities and generalist habit, might be less tied to drainage systems, though due to their small size and relatively poor swimming ability, may still respond to them as potential barriers. I focused more closely on *Microhyla heymonsi*, one of the species examined in the genus level analysis of *Microhyla*, looking at the effects of river systems on patterns of genetic structure and female dispersal within a species.

To further explore the response of widespread habitat generalists I examined the Vietnamese species complex of frogs related to *Polypedates leucomystax*. These animals are believed to be a complex of species based on call, allozyme, and DNA content data, though it has yet to be split (Matsui, et al., 1986; Nairns et al., 1998). Given the highly vagile nature of these frogs, their tolerance of human activity, and pond breeding habit, the Asian treefrogs serve as a further test the effects of river systems as genetic barriers and conduits both above and below the species level. This analysis also provides additional data in the search for common patterns and for biodiversity assessments.

Thesis organization

Following this general introduction, five chapters in publication format, are written in collaboration with my supervisor, Robert W. Murphy, our Vietnamese collaborator Ho Thu Cuc our Russian collaborator Nikolai Orlov and our Chinese colleague, Liu Wanzhao (Chapter 2).

Chapter 2 examines the phylogenetic relationships of the waterfall frogs, Genus *Amolops*, based on DNA sequence data from three mitochondrial genes (12S and 16S ribosomal RNA genes and the tRNA_{Val} gene) for samples collected from southern China through Vietnam. The recovered relationships are compared to the current taxonomy and provide a pattern of biogeographical relationships against which other studies may be compared. This study appears in *Amphibia-Reptilia* (Ngo et al. 2005).

In Chapter 3, the phylogenetic history of the widespread pond breeding narrow-mouthed frogs (genera *Microhyla* and *Micryletta*) is investigated. The relationships of 75 specimens from 14 species samples from across Indochina and the remainder of mainland Southeast Asia, with particular focus on Vietnamese specimens are evaluated based on 1392 base pairs from two mitochondrial genes (cytochrome *b* and the 16S ribosomal RNA gene). The generic and subgeneric taxonomy proposed by Dubois (1987) is evaluated in light of an explicit phylogeny and its validity is discussed. This study has been submitted for publication in *Zoologica Scripta*.

Chapter 4 is an extension of Chapter 3 focusing on the within species mitochondrial genealogy of one species of *Microhyla*: *M. heymonsi*. This study has been submitted for publication in *Hamadryad*.

A widespread complex of Asian treefrogs is examined in Chapter 5. Nine distinct lineages are identified. These are proposed to be distinct species based on patterns of sympatric distribution, call data, allozymes and biogeographic patterns. This study has been submitted for publication in *Molecular Phylogenetics and Evolution*.

In Chapter 6, I consolidate the findings of my work on the genetics of Vietnamese anurans, compare biogeographic patterns within and between groups, comment on unresolved issues, and suggest opportunities for future research.

Appendix 1 contains a paper that I co-authored with several others (a Chinese collaborator, Liqiao Chen, my advisor Robert W. Murphy, Amy Lathrop, Russian collaborator Nikolai Orlov, Vietnamese collaborator Ho Thu Cuc, and Ildiko M. L. Somorjai). It frames the remaining chapters, examining generic and subfamilial relationships of Vietnamese and Chinese ranid and dicroglossid frogs. It serves to provide a philosophical framework for the dissertation as it demonstrates the chaotic state of anuran taxonomy in Asia as well as provides a phylogenetic background for the specimens analyzed in Chapters 1 and 5. I was the third author on the manuscript, but was significantly involved in the data gathering, analysis and rewriting of the manuscript. Given the applicability of the content, and the extent of my contribution, I believe it relevant enough to include here as an appendix. This study appears in the *Herpetological Journal* (Chen et al. 2005).

Organization of Chapters:

- 1) Introduction
- 2) *Amolops*, species relationships in specialized stream breeding frogs

- 3) *Microhyla*, species relationships in a genus of widespread pond breeding frogs
- 4) *Microhyla heymonsi*, the search for genetic structure within a single widespread species of pond breeding frog
- 5) *Polypedates leucomystax* species complex, evolutionary lineages and cryptic species in a putatively widespread pond–breeding frog.
- 6) Summary chapter.

Appendix 1) A phylogenetic assessment of Southeast Asian ranid frogs

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Chapter 2

The Phylogenetic Relationships of the Chinese and Vietnamese Waterfall frogs of the genus *Amolops*

Abstract

Ranid frogs of the genus *Amolops* occur in Southeast Asia and are typically found near waterfalls. Their phylogenetic relationships have not been resolved. We include 2213 aligned nucleotide sites of the 12S, 16S and tRNA^{val} gene regions of the mitochondrial DNA genome from 43 individuals of Chinese and Vietnamese *Amolops*, *Huia*, *Hylarana*, *Meristogenys*, *Odorrana* and *Rana*. The outgroup species were from the genera *Chaparana*, *Limnonectes*, *Nanorana*, and *Paa*. The data were analyzed within the framework of a refutationist philosophy using maximum parsimony. Four clades of waterfall frogs were resolved. *Meristogenys* was not resolved as the sister group to either *Huia* or *Amolops*. The hypothesis of evolutionary relationships placed *Amolops chapaensis* and *Huia nasica* in the genus *Odorrana*.

Introduction

The genus *Amolops* is a speciose group of ranid frogs that inhabit swift torrents and the splash zone of mountain cascades throughout Southeast Asia. In addition to their peculiar habitat, these frogs possess a suite of characters that further distinguish them from other ranid frogs. Rather than having the “typical ranid” shape reminiscent of the edible frog, *Rana temporaria*, these frogs are dorsoventrally depressed and equipped with drastically enlarged digital pads (Figure 2.1a.) The larvae are also unique in having poison glands and a large gastromyzophorous adhesive disk (abdominal sucker), which the tadpoles use to affix themselves to the slippery surface of vertical rocks and boulders in the swiftly moving streams they inhabit (Figure 2.1b; Liu, 1950; Yang, 1991a). When threatened or approached by predators, the tadpoles release their hold and escape by dropping into the roiling water below.

Amolops have been known to science since 1855 (Anderson, 1878 (1879); Blyth, 1855; Dubois, 1992) and a considerable body of natural history information has been amassed. However, the phylogenetic relationships of *Amolops* remain obscure (Berry, 1966; Chen, 1991; Dubois, 1992; Inger and Voris, 1993; Liu and Yang, 1994a; Pan et al., 1985; Yang, 1991a). This is partially due to the remote distribution of some of the species, as well as a taxonomic debacle that surrounds the description of the genus. In 1865, Cope erected the genus *Amolops* for *Polypedates afganus* Günther (1858), a frog, which, despite its name and original type locality, is not found in Afghanistan, but in China (Anderson, 1871; Annandale, 1912; Boulenger, 1890; Cope, 1865; Günther, 1858, 1858(1859)). In his description of the genus, Cope (1865) provided the following diagnosis:

“terminal phalanges short; transverse limb long; tongue without median inferior prominence; no dorso-lateral glandular folds; vomerine teeth.”

Unfortunately, Cope also described another genus, *Staurois*, on the same page with the similar sounding diagnosis:

“terminal phalanges slender; with short transverse limb; tongue with median inferior prominence; no dorso-lateral folds nor vomerine teeth; ethmoid widely separating prefrontals, and these from frontoparietals.”

These two diagnoses were similar enough that when Noble (1929) dealt with tadpoles of *Amolops*, he managed to confuse them, using the genus name *Staurois* instead. His use of the name *Staurois* for this group was subsequently followed by numerous authors, further muddying the taxonomic waters for years (Bourret, 1942; Liu, 1950; Liu and Hu, 1961; Pope, 1927; Pope and Boring, 1940). Inger (1966) presented a series of reasons to recognize *Amolops* as distinct from *Staurois*.

There have been several, recent attempts to elucidate the relationships of these frogs. Yang (1991a) undertook an analysis of the group using larval characters. He recognized three genera, *Meristogenys* Yang 1991, *Huia* Yang 1991 and *Amolops* Cope 1865 in a new ranid subfamily, Amolopinae (Yang, 1991a). Dubois (1992), in a revision of the ranid frogs, reduced Yang’s genera to subgeneric status, and erected a fourth subgenus, *Amo*, for *Amolops larutensis*. However, most authors continue to recognize Yang’s genera (Inger et al., 1999; Inger and Stuebing, 1997; Liu et al., 2000a; Liu and

Yang, 1994b; Matsui et al., 1993; Zhao, 1995). Pang and Liu (1992) pursued a phylogenetic analysis of Chinese *Amolops*. Unfortunately, their study was limited by a paucity of variable characters making their conclusions questionable.

Chen et al. (2005) examined the phylogenetic relationships of Asian ranids within a rigorous phylogenetic framework. Their study confirmed that Yang's separation of *Huia* from *Amolops* was justified. However, the monophyly of amolopine frogs was called into question because the single representative of the genus *Huia*, *H. nasica*, fell out within a clade of Vietnamese *Odorrana*.

Recently, a number of new species of *Amolops* have been described (Inger and Kottelat, 1998; Inger et al., 1999; Liu et al., 2000a). Because of the existing uncertainty about the relationships and groups of amolopine frogs, we investigated the phylogenetic relationships of several species using mtDNA sequence data.

Materials and Methods

Specimens Examined

In total, 22 individuals of amolopine frogs representing 16 of 34 species of *Amolops*, from China and Vietnam were sequenced for this study. Additionally, 14 specimens of *Amolops*, *Meristogenys*, and *Huia* from GenBank were included in the study. To further test the monophyly of the Amolopinae, three specimens of *Hylarana*, five specimens of *Odorrana*, and two specimens of *Rana* were included as ingroup taxa. The ranid frogs *Chaparana fansipani*, *Limnonectes blythi*, *L. cancrivorus*, *Nanorana parkeri*, *N. pleskei*, and *Paa yunnanensis* were selected as outgroup taxa based on their

relationship to the ingroup and the availability of sequences. Locality and voucher data for all specimens examined are presented in Table 2.1, and on the map in Figure 2.2. Tissue samples were either frozen or ethanol preserved heart, skeletal muscle, or liver. Voucher specimens are preserved in the Royal Ontario Museum (ROM) and the Kunming Institute of Zoology (KIZ).

DNA Amplification and Sequencing

Segments of two ribosomal RNA genes, 12S and 16S, and the tRNA^{Val} gene, from the mitochondrial genome were selected to reconstruct the phylogeny of species and the genealogies of the females from species where multiple specimens were available. Protocols for DNA extraction and PCR amplification of the gene segments follow Liu et al. (2000b). These mitochondrial gene regions were amplified using the primers in Table 2.2. Double stranded DNA was sequenced directly using ³³P labeled ddNTP cycle sequencing (Amersham).

Sequence data for all specimens belonging to *Hylarana*, *Meristogenys*, *Odorrana*, *Rana*, and the outgroup were obtained from GenBank, as well as two specimens of *A. spinapectoralis*, one specimen each of *A. cremnobatus*, *A. hongkongensis*, *A. loloensis*, *A. mantzorum*, *A. ricketti*, *A. wuyiensis*, and *Huia nasica* (Table 2.1.)

DNA Sequence Analysis

Sequences were entered into BioEdit (ver. 5.0.9, Hall, 2001) for assembly, aligned with the computer algorithm Clustal W (ver. 1.6, Thompson et al., 1994) and

subsequently adjusted by eye. Potentially phylogenetically informative sites were retained for analysis using PAUP* (ver. 4.0b10 Swofford, 2003).

All characters were evaluated as unordered because there is no *a priori* reason to assume order of evolutionary change between nucleotide bases (Swofford et al., 1996). Character covariation was evaluated using the global permutation tailed test (PTP, Faith, 1991). The data were analyzed within a refutationist framework using a maximum parsimony methodology. The maximum parsimony analysis using PAUP* employed an heuristic search, with random addition sequence, 50 replicates, retaining minimal trees only, using tree bisection–reconnection branch swapping with steepest descent and collapsing zero length branches. Ratios of transitions to transversions were calculated in MacClade (ver. 4.0.5; Maddison and Maddison, 2002).

Nodal support was assessed for the data sets. Bootstrap proportions (BSP, Felsenstein, 1985), using 1,000 replicates, and Decay Indices (DI, Bremer, 1988; Bremer, 1994) were calculated in PAUP*.

Results

Sequence Variation

Nucleotide composition of the individual and combined gene sequences is summarized in Table 2.3. A total of 2213 bp were resolved and aligned. Of these sites, 1172 (53.0%) were variable and 887 (40.1%) were potentially phylogenetically informative. The transition to transversion ratio was calculated across the MPT and found to be 1.63:1.

A total of 607 aligned base pairs from the 12S ribosomal RNA gene and 1548 base pairs from the 16S rRNA gene were sequenced. Of these, 294 (48.4%) and 835 (53.9%) were variable, and 215 (35.4%) and 639 (41.3%) were potentially phylogenetically informative. The transition to transversion ratios for the 12S and 16S rRNA gene regions were found to range from 1.79–1 and 1.45–1, respectively. Of the intervening 58 base pairs of the tRNA^{Val}, 43 (74.1%) were variable and 33 (56.9%) were potentially phylogenetically informative.

Phylogenetic Analysis

Analysis of the combined data, excluding uninformative characters, yielded a single most parsimonious tree (Figure 2.3) of 4,991 steps in length (CI=0.33, RI=0.62). The ingroup was comprised of six clades. The two specimens of *Meristogenys* fell out together as the sister clade to *Hylarana* plus the remaining ingroup taxa. *Rana pipiens* and *R. johnsi* formed the sister group to a clade consisting of a paraphyletic *Odorrana*, *Amolops chapaensis*, and the five specimens of *Huia nasica*. This group was, in turn, the sister group to a monophyletic *Amolops*. Within *Amolops*, there were two distinct clades. Within the first clade, the type species of the genus *A. marmoratus* was found to be the sister to the specimens of *A. cremnobatus*. These two species then fell out as the sister group of a clade of Chinese *Amolops* from Sichuan and Yunnan. The second clade consisted of the Vietnamese *A. spinapectoralis* plus a clade of Chinese species from Fujian, Guangdong, and Hainan, and the widespread species *A. ricketti* from Vietnam.

Discussion

The results of this analysis provide support for Yang's (1991a) separation of *Huia* from *Amolops*, and for the arrangement of *Huia* and *Odorrana* found by Chen et al. (2005). Unfortunately, our study, like Chen et al., lacks the other three species of *Huia*. If the genus *Huia* is a monophyletic grouping, then taxonomic adjustment will be necessary to avoid making *Odorrana* paraphyletic. If *H. nasica* is not a sister species of other *Huia*, then it is possible that the remaining species of *Huia* will retain the name. To further maintain the monophyly of the genus *Odorrana* and not render the genus *Amolops* paraphyletic, *Amolops chapaensis* must be placed within the genus *Odorrana*.

Additionally, if all species of *Huia* branch off within *Odorrana*, the evolutionary significance of the larval gastromyzophorous disc used to diagnose amolopine frogs (Yang, 1991a) will form an interesting puzzle. Because the structure is not known from tadpoles of *Odorrana* (Fei, 1999) its presence in larval *Huia* requires a separate evolutionary origin or several independent losses since they shared a common ancestor with *Amolops*. The hypothesis of an independent origin of the gastromyzophorus disc is further supported by the placement of *Meristogenys*. Since the tadpoles of *Hylarana erythraea*, *H. lateralis*, *H. maosonensis*, *Rana johnsi*, and *R. pipiens* are known and all lack this structure,

A set of relationships within *Amolops* was proposed by Pang and Liu (1992). However, their phylogenetic analysis was not particularly successful because only nine characters were used to elucidate relationships among 14 taxa, and four of these were identical with respect to their character states. One node not resolved by the data was

arbitrarily split, and paraphyletic species groupings, A and B, were identified (Figure 2.4). When we reevaluated their data using maximum parsimony, 99 most parsimonious trees were found. A strict consensus tree of these resembles the tree they presented (Figure 2.4). The terminal relationships in their tree are consistent with those found in this study. Both analyses resolved *A. hainanensis* and *A. torrentis* as sister taxa, and *A. daiyunnensis* as sister to them. However, the lack of resolution and the limited number of taxa employed in their study do not allow for further comparison.

Potential conflict exists with the classification of Yang (1991a). He synonymized *A. hongkongensis* with *A. daiyunnensis* because they were similar in all characters examined except size, which showed some overlap. In contrast, Fei (1999) removed *A. hongkongensis* from synonymy with *A. daiyunnensis* without discussion. Our study supports Fei's recognition of the two species as evidenced by a 14.9% pairwise sequence divergence, a genetic difference that is more than triple that of some putative species (e.g. *A. lifanensis* and *A. granulosus*, 1.5%). While this percent divergence is not justification in its own right for the recognition of *A. hongkongensis*, it does suggest that the two taxa may well be evolutionarily diverging or separate, and that further investigation is warranted.

Conclusion

This study provides a historical perspective for future character evolution analyses. Further resolution and clarification of amolopine relationships may be obtained by broadening the geographic and taxonomic scope, especially by additional species of

Amolops, *Meristogenys*, and *Odorrana*. An expanded data set will serve to test Dubois' (1992) claim that Yang's three amolopine genera form a monophyletic grouping, a hypothesis challenged here and by Chen et al. (2005).

Further investigation is also required with regards to cryptic species and phenotypic plasticity in this genus. Specifically, the extent of genetic divergence between *A. daiyunnensis* and *A. hongkongensis*, and the morphological diversity of specimens of the *ricketti* complex raise interesting questions about species boundaries.

There are two important future directions of research to pursue. The first would be to use nuclear markers, such as allozymes and microsatellites, to investigate fixed differences. Nuclear gene data would allow an evaluation of the extent of gene flow between the nominate form of *A. daiyunnensis* and the Hong Kong form (*A. hongkongensis*). Another project that needs to be undertaken is the sampling of additional morphological characters to better determine the range of phenotypic plasticity within taxa. Only then will it be possible to act on the results of this study with confidence, identify cryptic species and develop a stable taxonomy.

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Table 2.1. Specimens used, voucher specimen catalog numbers, and collecting locality.

Sequences for specimens marked with a dagger ([†]) were obtained from GenBank.

Taxon #	GenBank Number 12S/tRNA/1	Species	Voucher No.	Locality
	6S			
1	DQ204429 204451 204473	<i>Amolops bellulus</i>	KIZ 9810021	Lushui, Yunnan, China
2	DQ204430 204452 204474	<i>Amolops chapaensis</i>	ROM 28321	Sa Pa, Lao Cai Prov., Vietnam
3	DQ204431 204453 204475	<i>Amolops chapaensis</i>	ROM 30943	Sa Pa, Lao Cai Prov., Vietnam
4	DQ204432 204454 204476	<i>Amolops chunganensis</i>	KIZ C93116	Nanjiang, Sichuan, China
5	DQ204433 204455 204477	<i>Amolops cremnobatus</i>	ROM 14528	Khe Moi, Nghe An Prov., Vietnam
6	DQ204434 204456 204478	<i>Amolops cremnobatus</i>	ROM 14542	Khe Moi, Nghe An Prov., Vietnam
7	AF206077 206122 206458	<i>Amolops cremnobatus</i> [†]	ROM 14534	Khe Moi, Nghe An Prov., Vietnam
8	DQ204435 204457 204479	<i>Amolops daiyunnensis</i>	KIZ F93069	Dehua, Fujian, China
9	DQ204436 204458 204480	<i>Amolops granulosus</i>	KIZ C92161	Dayi, Sichuan, China
10	DQ204437 204459 204481	<i>Amolops hainanensis</i>	KIZ 970512	Lingshui, Hainan, China
11	AF206072 206117 206453	<i>Amolops hongkongensis</i> [†]	ROM 16300	Hong Kong (Xianggang), China
12	DQ204438 204460 204482	<i>Amolops lifanensis</i>	KIZ C93156	Lixian, Sichuan, China

	DQ204439			
13	204461	<i>Amolops loloensis</i>	KIZ C92009	Baoxing, Sichuan, China
	204483			
	AF206112			
14	206157	<i>Amolops loloensis</i> [†]	JF101	Baoxing, Sichuan, China
	206493			
	DQ204440			
15	204462	<i>Amolops mantzorum</i>	KIZ 92064	Baoxing, Sichuan, China
	204484			
	DQ204441			
16	204463	<i>Amolops marmoratus</i>	KIZ	Longling, Yunnan, China
	204485			
	AF206072			
17	206117	<i>Amolops ricketti</i> [†]	ROM 16818	Tam Dao, Vinh Phu Prov., Vietnam
	206453			
	DQ204442			
18	204464	<i>Amolops ricketti</i>	ROM 26365	Cao Bang, Cao Bang Prov., Vietnam
	204486			
	DQ204443			
19	204465	<i>Amolops</i>	ROM 7513	Tram Lap, Gia Lai Prov., Vietnam
	204487	<i>spinapectoralis</i>		
	AF206076			
20	206121	<i>Amolops</i>	ROM	Ngoc Linh, Kon Tum Prov.,
	206457	<i>spinapectoralis</i> [†]	27456	Vietnam
	AF206075			
21	206120	<i>Amolops</i>	ROM 7555	Tram Lap, Gia Lai Prov., Vietnam
	206456	<i>spinapectoralis</i> [†]		
	DQ204444			
22	204466	<i>Amolops</i>	ROM	Ngoc Linh, Kon Tum Prov.,
	204488	<i>spinapectoralis</i>	27424	Vietnam
	DQ204445			
23	204467	<i>Amolops torrentis</i>	KIZ 970543	Lingshui, Hainan, China
	204489			
	DQ204446			
24	204468	<i>Amolops</i>	KIZ	Tenchong, Yunnan, China
	204490	<i>viridimaculatus</i>	930501	
	DQ204447			
25	204469	<i>Amolops wuyiensis</i>	KIZ F93009	Wuyishan, Fujian, China
	204491			
	AF205555			
26	315146	<i>Amolops wuyiensis</i> [†]		Huangshan, Anhui, China
	AF206093			
27	206138	<i>Chaparana fansipani</i> [†]	ROM 28286	Sa Pa, Lao Cai Prov., Vietnam
	206474			

	DQ204448			
28	204470	<i>Huia nasica</i>	ROM 18332	Tam Dao, Vinh Phu Prov., Vietnam
	204492			
	DQ204449			
29	204471	<i>Huia nasica</i>	ROM 18031	Tam Dao, Vinh Phu Prov., Vietnam
	204493			
	DQ204450			
30	204472	<i>Huia nasica</i>	ROM 20235	Tam Dao, Vinh Phu Prov., Vietnam
	204494			
	AF206080			
31	206125	<i>Huia nasica</i> [†]	ROM 16640	Tam Dao, Vinh Phu Prov., Vietnam
	206461			
	AF206082			
32	206127	<i>Limnonectes blythii</i> [†]	ROM 7144	Tram Lap, Gia Lai Prov., Vietnam
	206463			
	AF206092			
33	206137	<i>Limnonectes</i> <i>cancrivorus</i> [†]	ROM 1059	Dumaguete City, Negros Island, Philippines
	206473			
	AY322317	<i>Meristogenys</i> <i>kinabaluensis</i> [†]	VUB 0627	Borneo
	AY322319	<i>Meristogenys cf.</i> <i>orphrocnemis</i> [†]	VUB 0630	Borneo
	AF206110			
36	206155	<i>Nanorana parkeri</i> [†]	JF037	Lhasa, Tibet, China
	206491			
	AF206111			
37	206156	<i>Nanorana pleskei</i> [†]	JF118	Xinduqiao, Sichuan, China
	206492			
	AF206086			
38	206131	<i>Occidozyga</i> <i>martensii</i> [†]	ROM 22222	Yok Don, Dac Lac Prov., Vietnam
	206467			
	AF206106			
39	206151	<i>Odorrana banaorum</i> [†]	ROM 7472	Tram Lap, Gia Lai Prov., Vietnam
	206487			
	AF206101			
40	206146	<i>Odorrana daorum</i> [†]	ROM 19053	Sa Pa, Lao Cai Prov., Vietnam
	206482			
	AF206104			
41	206149	<i>Odorrana chloronota</i> [†]	ROM 14885	Hong Kong (Xianggang), China
	206485			
	AF206102			
42	206147	<i>Odorrana</i> <i>hmongorum</i> [†]	ROM 19112	Sa Pa, Lao Cai Prov., Vietnam
	206483			
	AF206100			
43	206145	<i>Odorrana</i> <i>megatypanum</i> [†]	ROM 13046	Khe Moi, Nghe An Prov., Vietnam

		206481		
		AF206103		
44	206148	<i>Odorrana morafkai</i> [†]	ROM 7446	Tram Lap, Gia Lai Prov., Vietnam
	206484			
		AF206089		
45	206134	<i>Paa yunnanensis</i> [†]	ROM 19128	Sa Pa, Lao Cai Prov., Vietnam
	206470			
		AF206094		
46	206139	<i>Rana erythraea</i> [†]	ROM 7296	Tram Lap, Gia Lai Prov., Vietnam
	206475			
		AF206096		
47	206141	<i>Rana johnsi</i> [†]	ROM 24230	Chi Linh, Hai Duong, Vietnam
	206477			
		AF206097		
48	206142	<i>Rana lateralis</i> [†]	ROM 22153	Yok Don, Dac Lac Prov., Vietnam
	206478			
		AF206107		
49	206152	<i>Rana cf. maosonensis</i> [†]	ROM 24274	Chi Linh, Hai Duong, Vietnam
	206488			
50	X86247	<i>Rana pipiens</i> [†]	UIMNH 9542	Locality unknown
	86318			
51	M10217	<i>Xenopus laevis</i> [†]	N/A	Locality unknown

Table 2.2. Primers used for amplifying and sequencing fragments of RNA genes in the specimens included in this study. Sequence position indicates the starting position of the primer on the *Xenopus laevis* mitochondrial genome and is preceded by the direction of amplification (H=heavy / L=light strand).

Name	Sequence 5'-3'	Sequence position	Reference
12S2H	AGGGTGACGGGCGGTGTGT	H2897	Kocher et al. (1989)
12S2L	ACACACCGCCCGTCACCCTC	L2917	Fu (1999)
16S3H	GTAGCTCACTGATTTCGGG	H3341	Fu (1999)
16S3L	CCCGAAATCAAGTGAGCTAC	L3362	Fu (1999)
16S1H	GGCTATGTTTGGTAAACAG	H3958	Modified From Palumbi (1996)
16S5H	CTACCTTGACGGTTAGGATACCGCGGC	H4040	Fu (1999)
16S1M	CCGACTGTTACCAAAAACAT	L3955	Fu (1999)
16S2H	CCGGATCCCCGGCCGGTCTGAACTCAGATCACG	H4552	Palumbi (1996)

Table 2.3. Summary of fragment length, base composition, variable sites, and substitution ratios for the combined data set and individual mitochondrial RNA genes. All numbers are those after omission of ambiguously aligned regions.

Gene	Unambiguously Aligned Base Pairs	Variable Sites	Potentially Parsimony Informative Sites	%GC	TS:TV
12S rRNA	607	294 (48.4%)	215 (35.4%)	39.0%	1.93:1
tRNA^{Val}	58	43 (74.1%)	33 (56.9%)	41.5%	N/A
16S rRNA	1548	835 (53.9%)	639 (41.3%)	31.4%	1.51 : 1
All RNA gene data	2213	1172 (53.0%)	887 (40.1%)	33.7%	1.63 : 1

Figure 1.

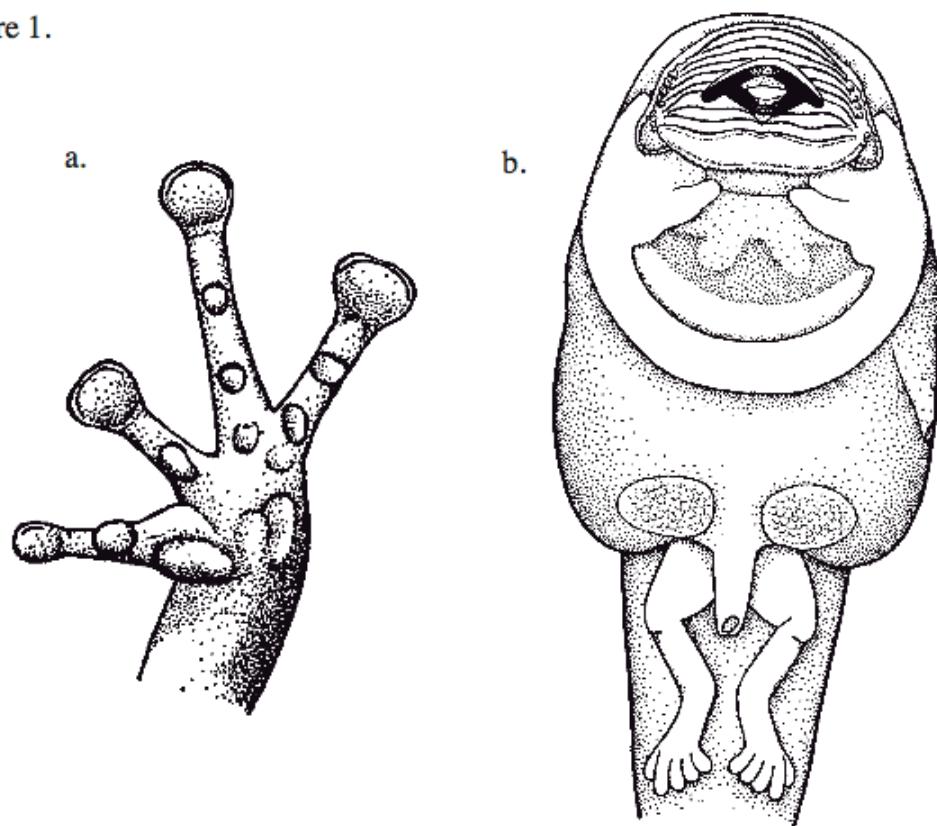


Figure 2.1. a. Manus of *Amolops mantzorum* illustrating the expanded digital discs.

b. Drawing of a larval *Amolops mantzorum* illustrating the gastromyzophorous disc.

After Liu (1951) Figs. 83 and 84.



Figure 2.2. A map of Vietnam and the southern provinces of China, showing collection localities of ingroup specimens from the genera *Amolops*, *Huia*, *Meristogenys*, and *Odorrana*. Numbers correspond to the specimen numbers in Table 1.1.

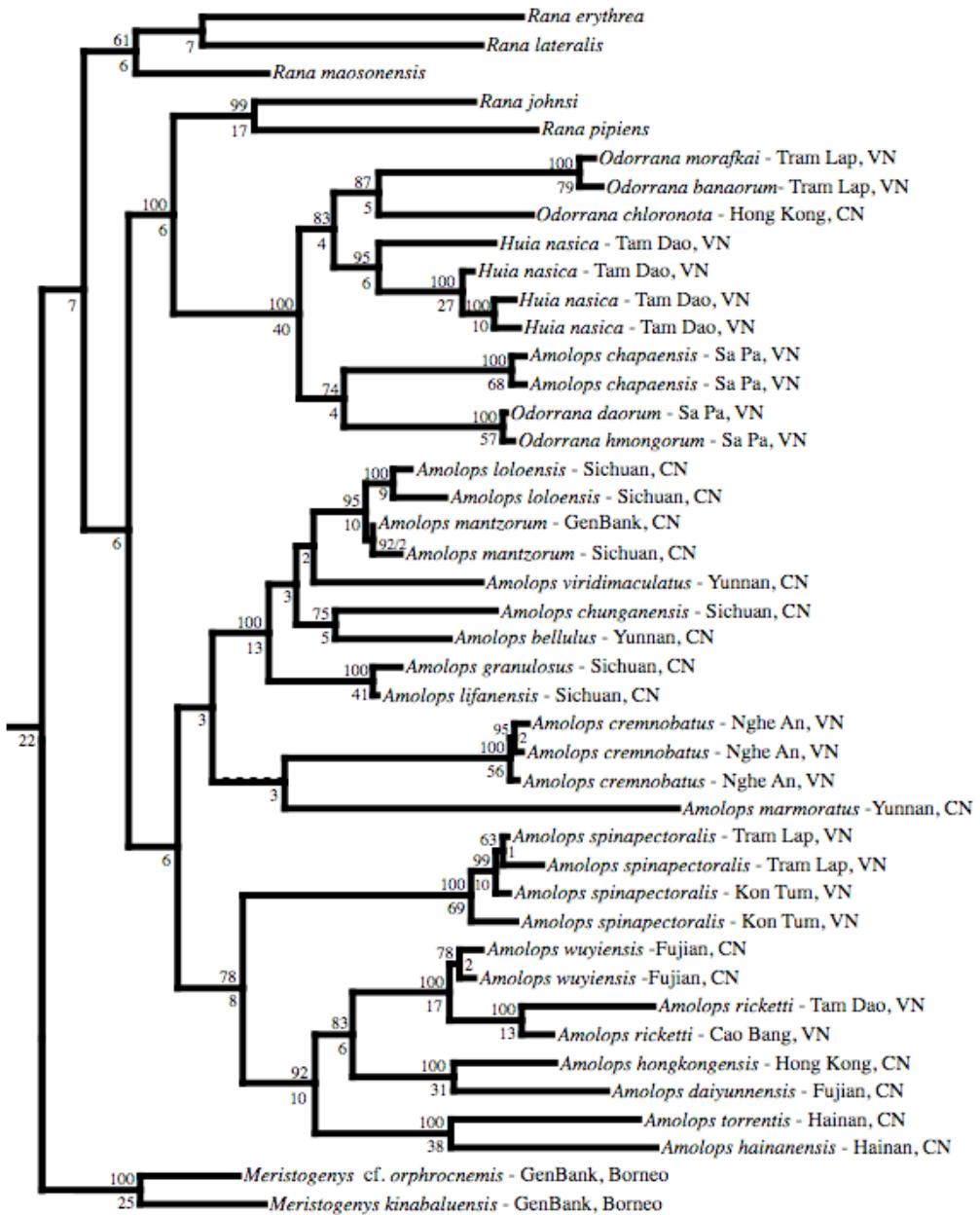


Figure 2.3. The single most parsimonious tree resolved by analysis of the combined data for all genes sequenced. Outgroups have been pruned for clarity. Branch lengths represent the amount of change along the tree. Bootstrap Proportions (BSP > 50) are displayed above nodes and decay indices (DI) below.

Figure 4.

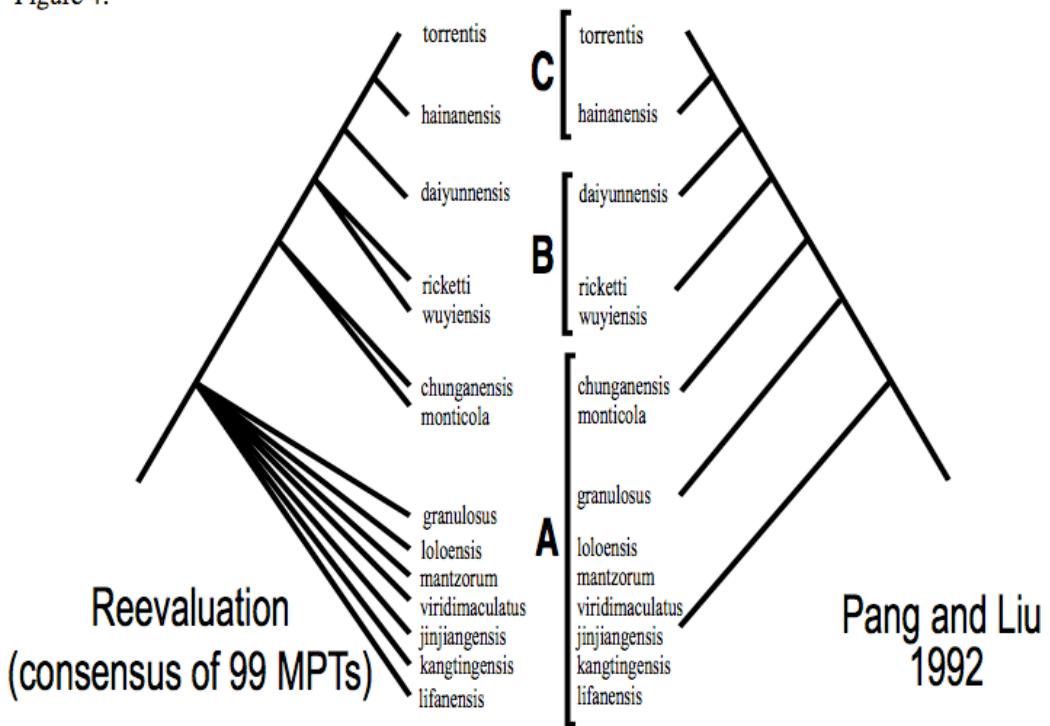


Figure 2.4. A comparison of the phylogeny presented by Pang and Liu (1992) and the strict consensus of 99 MPTs resolved by a reanalysis of their data. Brackets delineate their groups A, B, and C.

Chapter 3

The phylogenetic relationships of the Vietnamese narrow-mouthing frogs, Genus *Microhyla* Tschudi 1838, and the validity of Genus *Micryletta* Dubois 1987

Abstract

The narrow-mouthing frogs of the genera *Microhyla* and *Micryletta* are small, ant and termite feeding anurans that range widely in Southeast Asia. Their phylogenetic relationships have not been previously resolved, though recent taxonomic revisions were made. I include 1392 aligned nucleotide sites of the *16S* and *cytochrome b* gene regions of the mitochondrial DNA genome from 72 individuals of *Microhyla* and *Micryletta*. The outgroup species were from the genera *Kaloula* and *Glyphoglossus*. Both maximum parsimony and Bayesian methods were employed differing only in the placement of *Microhyla annamensis*. The validity of Genus *Micryletta* was upheld while the monophyly of Subgenera *Diplopelma* and *Microhyla* was not.

Introduction

Narrow-mouthed frogs of the genus *Microhyla* Tschudi 1838 occur in Asia from the Ryukyu Islands in Japan westward through China to India and Sri Lanka and southward through Southeast Asia to Borneo, Sumatra, Java, and Bali in Indonesia (Frost, 2007). The genus contains 28 species, of which 11 occur in Vietnam (Orlov et al., 2002; Frost, 2007). Much attention has been paid to their peculiar dietary specializations (Das 1995; Erfemeijer and Boeadi, 1991; Hirai and Matsui, 2000; Ziegler 2002), reproductive physiology (Matsui and Ota, 1984; Metter and Conaway, 1969), and other aspects of their natural history (Dey et al., 1989; Hiragond and Saidapur, 2001; Padhye and Ghate, 1988; Padhye and Ghate 1989; Ziegler, 2002). Narrow-mouthed frogs are specialized ant and termite eaters, with 77% (*Microhyla cf. annamensis*) to 98.4% (*Microhyla pulchra*) of their diet consisting of formicids and isopterans (Erfemeijer and Boeadi, 1991; Ziegler, 2002; Hirai and Matsui, 2000). They are small, ranging in SVL from 17mm to 34mm, the largest being *M. fowleri* (Manthey and Grossman, 1997; Ziegler, 2002; Liu, 1950). They are locally abundant (Watanabe et al., 2005) and broadly distributed (Lee et al., 2006; Lever, 2003; Liu, 1950; Maeda and Matsui, 1999). Several species are either habitat generalists or associated with disturbed habitats. They breed in roadside ditches, grasslands, buffalo troughs, and rice paddies and may be prone to human mediated dispersal via the transport of crops, potted plants and lumber (Lee et al., 2006; Ota, 1999; Ziegler, 2002). However, their phylogenetic relationships remain obscure.

Parker's (1934) review of *Microhyla* detailed morphological variation and distributional. Since then, 10 new species have been described (Andersson, 1942; Bain and Nguyen, 2004; Inger and Frogner, 1979; Frost, 2007; Tarkhnishvili, 1994). Dubois (1987) revised the taxonomy of *Microhyla* (*Mh.*), erecting a new genus, *Micryletta* (*Ml.*),

for *Mh. inornata*. He formed two subgenera, *Microhyla* and *Diplopelma*, and two species groups within Subgenus *Microhyla*. Unfortunately, this taxonomy was not based on an explicit phylogenetic hypothesis, but rather on grades of morphological distinctiveness. Consequently, it is possible that the taxonomy does not reflect the evolutionary history of the species.

The genus *Micryletta* has received mixed acceptance. Zhao and Adler (1993) did not recognize it in their treatment of Chinese herpetology, but Fei (1999) included it in his atlas of Chinese amphibians. Orlov et al. (2002) not only recognized the genus, they expanded it to include *Mh. erythropoda*, a morphologically similar animal. This generic arrangement was supported by Frost et al. (2006), who evaluated the higher-level relationships of the Anura; their single specimen of *Micryletta* was clearly separated from the one specimen of *Mh. heymonsi* plus one unidentified *Microhyla* obtained from a pet store. However, Frost et al. did not consider the inclusion of *Mh. erythropoda* to be adequately justified.

Recently, as part of a genealogical analysis of *Mh. ornata* from the Ryukyu archipelago, Matsui et al. (2005) uncovered evidence of cryptic species previously assigned to *Mh. ornata*. They hypothesized the phylogenetic relationships of five species of *Microhyla*, but their taxonomic sampling was insufficient to evaluate the validity of *Micryletta*. Thus, I undertook this study of the phylogenetic relationships of the genera *Microhyla* and *Micryletta*.

Materials and Methods

Specimens Examined

In total, 75 individuals of *Microhyla* and *Micryletta* from Southeast Asia were examined as ingroup taxa, including seven of the 14 species of Vietnamese species of *Microhyla*. The microhylid frogs *Kaloula pulchra*, *K. taprobanica*, and *K. conjuncta* served as outgroup taxa. Locality and voucher data for all specimens examined are presented in Table 3.1, and the localities are mapped in Figure 3.1. Voucher specimens are preserved in the Royal Ontario Museum (ROM) except as indicated in Table 3.1.

DNA gene selection

The relatively slowly evolving 16S ribosomal RNA gene (16S) is considered appropriate for resolving older divergences, perhaps as old as 150 Ma, and has been used extensively in anuran systematics at the species level (Che et al., 2007; Glaw and Vences, 2006; Matsui et al., 2005; Mindell and Honeycutt, 1990). The more rapidly evolving cytochrome *b* gene (Cyt *b*) is commonly used in both species and intraspecific levels to resolve more recent events including within species patterns of mitochondrial evolution (Glaw and Vences, 2006; Graybeal, 1993, 1994; Matsui et al., 2005; Zangari et al., 2006). These genes from the mitochondrial genome were selected to reconstruct the phylogeny of species.

DNA Amplification and Sequencing

Total genomic DNA was extracted from frozen or ethanol-preserved muscle or liver tissue samples by digestion with proteinase K for 7–12 hr, then purified three times with phenol-chloroform-isoamyl alcohol (PCI), and then once with chloroform-isoamyl

alcohol (CI). Double-stranded DNA fragments were amplified using the polymerase chain reaction (PCR; 92°C for 30 sec, 47–53°C for 45 sec 72°C for 90 sec) performed in 25 μ l reactions for 33 cycles. Annealing temperatures ranged from 47°C to 53°C as needed in order to improve primer binding and the quality of the PCR product. PCR reactions generally amplified the entire gene fragments from 16S3L to 16S2Hm for 16S, and MVZ15L to CytbB for Cyt b (Table 3.2). Occasionally, additional combinations of primers were required (Table 3.2). After amplification, a 2 μ l aliquot of PCR product was separated by electrophoresis on an agarose gel and stained with ethidium bromide to verify amplification. The remaining DNA was cleaned using QiaColumns (Qiagen) and eluted in Qiagen EB buffer. The cleaned double stranded DNA was sequenced directly using 8 μ l BigDye (Applied Biosystems International, ABI) fluorescent cycle sequencing reactions (96°C for 30 sec, 50°C for 15 sec 60°C for 4 min for 30 cycles). Sequencing product was ethanol-precipitated and resuspended in Hi-Di formamide (ABI). Resuspended DNA was then run out on either an ABI 377 automated sequencer using long plates or an ABI 3100 automated sequencer.

Sequence data for the outgroup taxa (*Glyphoglossus molossus*, *Kaloula pulchra*, *K. taprobanica* and *K. conjuncta*), and some additional ingroup samples (*Mh. ornata*, *Mh. rubra*, *Mh. okinavensis*, and *Mh. fissipes*) were obtained from GenBank (Table 3.1). Intraspecific maternal genealogies were hypothesized within species when multiple specimens were available.

DNA Sequence Analysis

Sequences were entered into BioEdit (ver. 7.0.5, Hall, 1999; 2005) for assembly, initially aligned with Clustal W (ver. 1.6, Thompson et al., 1994) and subsequently adjusted by eye. Sites and regions of ambiguous alignment were considered to have questionable homology and excluded (Hillis and Dixon, 1991). Redundant sequences were merged and potentially phylogenetically informative sites were retained for analysis.

Maximum Parsimony Evaluation

All characters were evaluated as unordered because there is no *a priori* reason to assume order of evolutionary change between nucleotide bases (Swofford et al., 1996). The data were analyzed using maximum parsimony (MP) as the criterion for selecting among all possible trees. The MP analysis was performed in PAUP* (ver. 4.0b10 Swofford, 2004) using an heuristic search with random addition sequence, 100 replicates, retaining minimal trees only, using tree bisection–reconnection branch swapping with steepest descent and collapsing zero length branches. Ratios of transitions to transversions were calculated in MacClade (ver. 4.0.5; Maddison and Maddison, 2002).

Nodal support was assessed by nonparametric bootstrap proportions (BSP; Felsenstein, 1985), using 10,000 replicates and “Fast” stepwise addition, and Decay Indices (DI; Bremer, 1988; Bremer, 1994), all calculated in PAUP*.

Because the specimens obtained from GenBank were sequenced using different primer sets, up to 40% of the aligned sites were missing. Consequently, an additional analysis was performed excluding any ingroup specimens missing $\geq 15\%$ of the aligned data. Nodal support values were also calculated for this second analysis.

Constraint trees were constructed for Dubois' (1987) subgenera and species groups. These trees were enforced in separate maximum parsimony analyses.

Bayesian Inference Analysis

Bayesian inference (BI) was also used to infer species relationships and maternal history within species (Huelsenbeck and Ronquist, 2001; Buckley et al., 2002; Nylander et al., 2004). Only specimens missing <15% of the total sequence data were included. The sequence data were partitioned by gene and the program MRMODELTEST (v2.2, Nylander, 2004) was used to select an evolutionary model that best fit the data for each gene using the Akaike information criterion (Akaike, 1974, 1979). Hierarchical likelihood ratio tests (Goldman, 1993) were also implemented using MRMODELTEST v2.2, comparing the log-likelihood scores of the 24 evolutionary models that can be implemented in MrBayes for each gene. A general time reversal model with invariant sites and a gamma distribution (GTR + I + G, Rodríguez et al., 1990; Gu et al., 1995; Waddell & Penny 1996) was selected for both genes and implemented for the combined dataset. BI was conducted using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). The analysis was initiated with random starting trees. Six Markov chains were used, and the dataset was run for 3×10^6 generations. Trees were sampled every 100 generations. Two independent analyses with different starting trees were run to avoid being trapped on local optima. The fluctuating likelihood values were graphically monitored (Huelsenbeck and Bollback, 2001) and stationarity was achieved when the log-likelihood values of the sample points reached a stable equilibrium value (Huelsenbeck and Ronquist, 2001). The analysis was a priori required to achieve a split frequency standard deviation of ≤ 0.02 .

After discarding the first 2000 sampled trees as burn-in, the remaining trees were used to generate a 50% majority rule consensus tree.

Results

Seventy-five specimens were sequenced for 16S and Cyt *b*. In total, 1392 sites were sequenced for 16S and 1012 for Cyt *b* for a combined total of 2404 aligned sites. Eighteen of these sites were ambiguously aligned and excluded from the analyses. Among the remaining sites, 850 were variable and 649 were potentially phylogenetically informative (Table 3.2). All sequences will be deposited in GenBank.

Parsimony evaluation

The 16S gene was evaluated separately using only the 59 specimens that contained ≥80% of the sequence data. Analysis of the 385 potentially informative sites yielded 14 most parsimonious trees (MPTs; Table 3.2) (length = 1382, CI = 0.45, RI = 0.86). Cyt *b*, with 427 potentially informative sites, yielded 1774 MPTs (Table 3.2; length = 2056, CI = 0.37, RI = 0.80). The topologies of both the strict and the 50% majority rule consensus trees from each gene were largely compatible. Differences occurred at terminal branches within species and the placement of *Mh. cf. fowleri*. *Microhyla cf. fowleri* was resolved with *Mh. fissipes* rather than *Mh. heymonsi* in some of the MPTs recovered by the *cyt b* analysis. Thus, two mitochondrial genes evolved in similar ways and I assume that this conciliation owes to genealogical history

Combining all mitochondrial gene sequence data into a single data set resulted in 850 potentially cladistically informative characters. Analysis of these data yielded 649 most parsimonious trees (4376 steps in length, CI=0.34, RI=0.80). The large number of trees owed to unresolved relationships within species. The genus *Micryletta* formed a monophyletic group separated from the remainder of the ingroup taxa. It was not possible to root the tree so that *Microhyla* was paraphyletic.

Within *Micryletta*, two distinct groups were resolved. One group contained specimens from the southern Vietnamese localities of Krong Pa and Tram Lap, as well as a specimen from Boulapha district, Khammouan province, Laos. The other clade contained a Laotian specimen from Nakai district, Khammouan province and specimens from Cat Tien National Park and Yok Don National Park in southern Vietnam.

Microhyla annamensis was resolved as the sister group of *Mh. butleri* plus the remaining species of *Microhyla*. A polytomy was obtained between the single southern Vietnamese specimen of *Mh. butleri*, the specimen from Bante Sre district, Siem Reap province, Cambodia, and a clade comprised of the Laotian specimen from Nakai district, Khammouan province plus northern Vietnamese frogs.

The two species from India and Bangladesh, *Mh. ornata* and *Mh. rubra*, formed a monophyletic group that was, in turn, the sister group of two clades (Figure 3.2: A and B): Clade A, included *Mh. berdmorei* and *Mh. pulchra*; Clade B contained *Mh. cf. fowleri*, *Mh. fissipes*, *Mh. okinavensis* and *Mh. heymonsi*.

Clade A had two subclades, each one consisting of a single species: *Mh. berdmorei* and *Mh. pulchra*. Within *Mh. berdmorei*, specimens from Laos formed the sister group to a clade comprised of the southern Vietnamese specimens. The clade of

Mh. pulchra, was partially unresolved. A polytomy contained the Cambodian and Laotian specimens, the southern Vietnamese specimens, animals from Con Cuong in north-central Vietnam, and a clade of northern Vietnamese plus frogs from Hainan Island.

Clade B had two major subclades, B1 and B2 (Figure 3.2). Clade B1 consisted of the two Southeast Asian and Japanese species recently removed from *Mh. ornata*: *Mh. okinavensis* and *Mh. fissipes*. These species were further resolved into geographical groups. Within *Mh. fissipes*, Vietnamese and Chinese specimens, from north of the Red River, were resolved as the sister group to the remaining specimens. The single Thai specimen was sister to an unresolved polytomy containing the two Laotian specimens, the two specimens from the coastal lowlands of north-central Vietnam, Vinh and Con Cuong, and specimens from southern Vietnam.

Clade B2 contained the single representative of *Mh. cf. fowleri* resolved as the sister group to *Mh. heymonsi*. Within *Mh. heymonsi*, the specimens from Yok Don National Park and Krong Pa in southern Vietnam formed the sister group to a clade of Laotian specimens plus the remaining specimens from northern Vietnam.

Bayesian Inference Evaluation

The BI analysis recovered a topology similar to the MP analysis, with the exception of the placement of *Mh. annamensis* (Figure 3.3). In the BI analysis, *Mh. annamensis* was resolved as the sister group to *Kaloula* plus *Micryletta*, as opposed to being the sister group to the remaining *Microhyla*.

Nodal Stability

Values of nodal support are indicated on the MP strict consensus tree (Figure 3.2). BSPs supported 36 nodes with a consistency greater than 70%. Bayesian posterior probabilities indicated relatively high support for most nodes, 37 of which were supported by Bayesian posterior probabilities of 100 percent, including several that were not supported by decay analyses or bootstrap proportions (DI<5, BSP<50%). The conflicting placement of *Mh. annamensis* was supported by a BSP of 100 in the MP analysis, and a BPP of 83 in the BI.

Discussion

Phylogenetic Hypotheses

All of the putative species examined were recovered as monophyletic groups. While the focus of this study is the phylogenetic relationships of the Vietnamese species of *Microhyla*, there is overlap with the study of Matsui et al. (2005). I expanded the analysis of Matsui et al. (2005) increasing the number of species as well as sampling additional populations and individuals within each species, more than doubling the number of specimens. The inclusion of *Mh. annamensis*, *Mh. berdmorei*, *Mh. cf. fowleri*, and *Micryletta* filled in the framework provided by Matsui et al. and supported the relationships that they recovered. The placement of *Mh. annamensis* is ambiguous, however, given the differing hypotheses recovered by the BI and MP analyses. The placement of *Mh. annamensis* as the sister group to the remaining *Microhyla*, as resolved in the MP analysis, was preferred here because it is compatible with the morphological characters that differentiate *Micryletta* and *Microhyla* (Dubois, 1987). *Microhyla*

berdmorei was found to be the sister group of *Microhyla pulchra*. Additionally, the sister group relationship of *Mh. heymonsi* and *Mh. fissipes* recovered by Matsui et al. (2005) was interrupted. *Microhyla cf. fowleri* was resolved as the sister species of *Mh. heymonsi*, separating it from *Mh. fissipes*. *Micryletta inornata* was more closely related to *Kaloula* than *Microhyla* where it was formerly placed.

Taxonomic Implications

Genus *Micryletta*, subgenera of *Microhyla*, and species groups

Dubois (1987) produced a new taxonomy for *Microhyla*. The monotypic genus *Micryletta* was created for *Ml. inornata*, and the remaining species were separated into two subgenera. In their analysis of higher-level anuran phylogeny, Frost et al. (2006) included a single representative of *Ml. inornata*, one specimen of *Mh. heymonsi* and one unknown *Microhyla*. As with this study, *Micryletta* was resolved as being distinct from, and not more closely related to any species of *Microhyla* than to other frogs. Although the validity of genus *Micryletta* was upheld in my assessment, the remaining arrangements of Dubois (1987) were not supported. Subgenus *Diplopelma* (resurrected from Günther 1859) nested in two separate clades within the subgenus *Microhyla* rendering both taxa paraphyletic. *Diplopelma* would require 38 more steps to be recovered as monophyletic and a monophyletic Subgenus *Microhyla* would require 72 additional steps. Furthermore, at least one of the species groups within Subgenus *Microhyla* was found to be nonmonophyletic. The *Mh. berdmorei* group was rendered paraphyletic by both the *Mh. achatina* group of Subgenus *Microhyla*, herein represented by *Mh. heymonsi*, and all representatives of *Diplopelma*. An additional 48 steps would be

required to resolve a monophyletic *Mh. berdmorei* group. Dubois hypothesized that the *achatina* species group (Subgenus *Microhyla*) gave rise to Subgenus *Diplopelma*. However, the members of Subgenus *Diplopelma* were distributed widely across the tree, with Indian representatives *Mh. ornata*, and *Mh. rubra* falling out together as the sister group to a clade containing the remaining members of the subgenus plus *Mh. berdmorei*, *Mh. cf. fowleri*, and *Mh. heymonsi* (Figure 3.2).

Dubois' Subgenus *Microhyla* is not monophyletic. Species from subgenus *Diplopelma* branched off in three places. The Indian species, *Mh. ornata* and *Mh. rubra*, fell between *Mh. butleri* and the remaining *Microhyla* except for *Mh. annamensis*. An additional 72 steps would be required to maintain a monophyletic Subgenus *Microhyla*.

Unfortunately, the monophyly of the *Mh. achatina* species group could not be evaluated because only one species was available in this analysis. Regardless, Dubois' hypothesis that the *Mh. achatina* species group gave rise to *Diplopelma* is also called into question. His approach is not compatible with cladistic theory where sister taxa share a common ancestor, rather than one persisting in time (Hennig, 1966). Furthermore, the only representative of the *Mh. achatina* species group, *Mh. heymonsi*, nestled within Subgenus *Diplopelma*. Thus, either *Mh. heymonsi* needs to be removed from the *Mh. achatina* species group and/or Dubois' hypothesis must be rejected. Further study including other representatives of the *Mh. achatina* species group will clarify the situation.

Microhyla okinavensis and *Mh. fissipes*

Okada (1931) placed *Mh. okinavensis* Stejneger 1901 in synonymy with *Mh. ornata*. The species was resurrected by Dubois (1987) and placed into Subgenus *Diplopelma*, but without discussion or comment. Matsui et al. (2005) demonstrated mtDNA distinctiveness of *Mh. okinavensis* from *Mh. ornata* and *Mh. fissipes*, which was upheld here. Similarly, the addition of 12 Vietnamese specimens did not change the recovery of *Mh. fissipes* Boulenger 1884 as a distinct clade, despite the species having been previously placed in the synonymy of *Mh. ornata* by Parker (1928).

A sister relationship between *Mh. okinavensis* and *Mh. fissipes*, as recovered by Matsui et al. (2005), was resolved. This, combined with the putative characters suggested by Matsui et al. (2005) and the acoustic characters discussed by Kuramoto and Joshy (2006), further supports the hypothesis that the two taxa are sister groups and valid species.

Biogeographical implications

The search for common patterns is central to looking for common processes. The overlapping distributions of the Vietnamese species of *Microhyla* provide a convenient comparative dataset within which to examine patterns or lack thereof. *Microhyla heymonsi* and *Mh. fissipes* show both structure and long branch-lengths below the species level, and the northern Vietnamese specimens of *Mh. butleri* are also set apart by a long internode. The monophyly of the northern Vietnamese specimens within each species is the only common pattern observed. Chinese specimens from Yunnan (*Mh. fissipes*) or Hainan (*Mh. pulchra*) formed the sister group to northern Vietnamese specimens.

Geographically, these tree-nodes were associated with the Red River, a pattern seen in other taxa (*Oligodon*, Marc Green, personal communication; *Acanthosaura*, Kalyabina-Hauf et al. 2004).

The patterns recovered for *Mh. heymonsi* and *Mh. fissipes* are not congruent with each other. Outside of the relationship between northern and southern Vietnamese populations, they show no common pattern. The relationships of populations from southern Vietnam, Cambodia, and Laos are unresolved in *Mh. butleri*, *Mh. pulchra* and *Mh. berdmorei*. This lack of correspondence may owe to more specific habitat requirements of some species and the ubiquitous, generalist, and vagile natures of others.

Due to their small size and association with disturbed habitat, *Microhyla* are ideal candidates for accidental human mediated dispersal via potted plants, crops, and lumber. The breeding habits of these frogs may also contribute to independent historical patterns. Their habitats are not restricted to permanent bodies of water or river systems, but rather they use any small body of standing water. Consequently, their breeding sites are often ephemeral. This could facilitate, if not encourage, the dispersal of individuals. In their initial work in the Central Highlands, Orlov and Darevsky rarely encountered *Microhyla*. Some 10 years later after the area had been significantly modified, *Microhyla* were common (Nikolai Orlov, personal communication). To test these hypotheses, further work needs to be done at the population level, perhaps using microsatellite DNA or anonymous nuclear loci to evaluate gene flow among populations. These data could reveal common patterns between species with similar habits, patterns that correspond to those seen in other taxa, geological processes like the Ailao Shan shear zone, mountain ranges, and river drainage systems, or even human trade routes.

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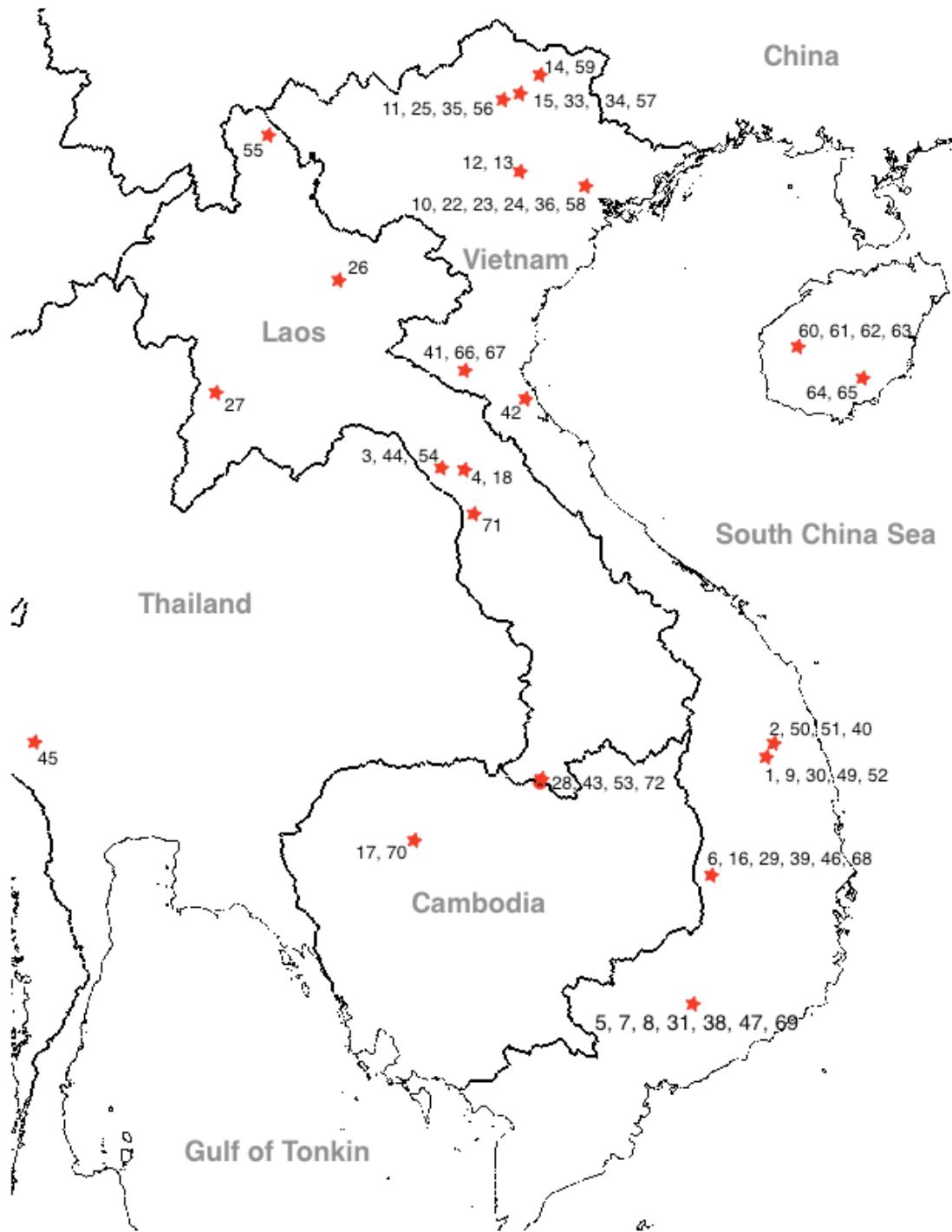


Figure 3.1. Map of *Microhyla* specimen localities. Numbers refer to the specimens in Table 3.1.

Figure 2.

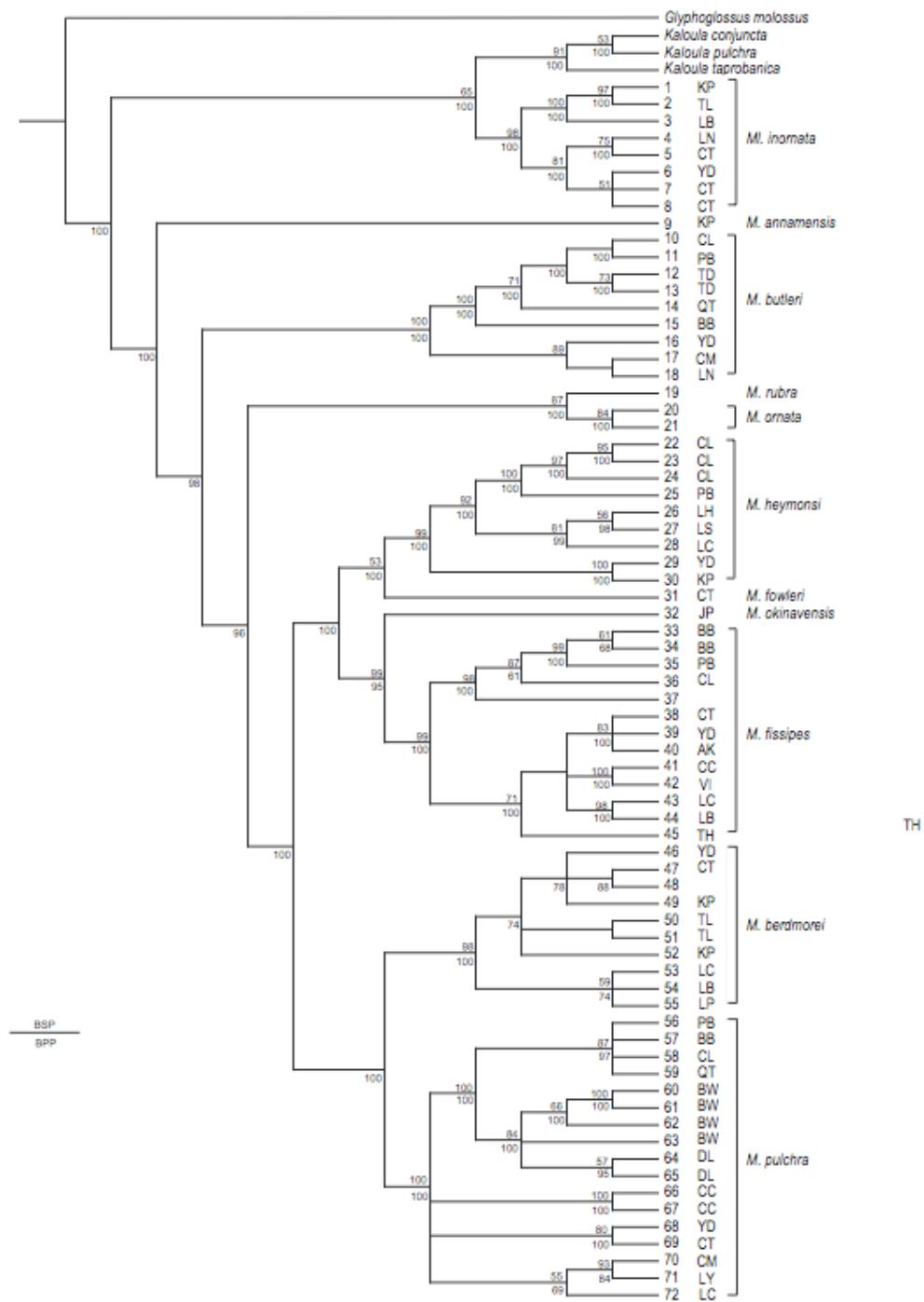


Figure 3.2. Maximum parsimony tree. Strict consensus of 576 most parsimonious trees.

Bootstrap proportions are above the branches and Bayesian posterior probabilities are

below. Outgroups have been pruned for clarity.

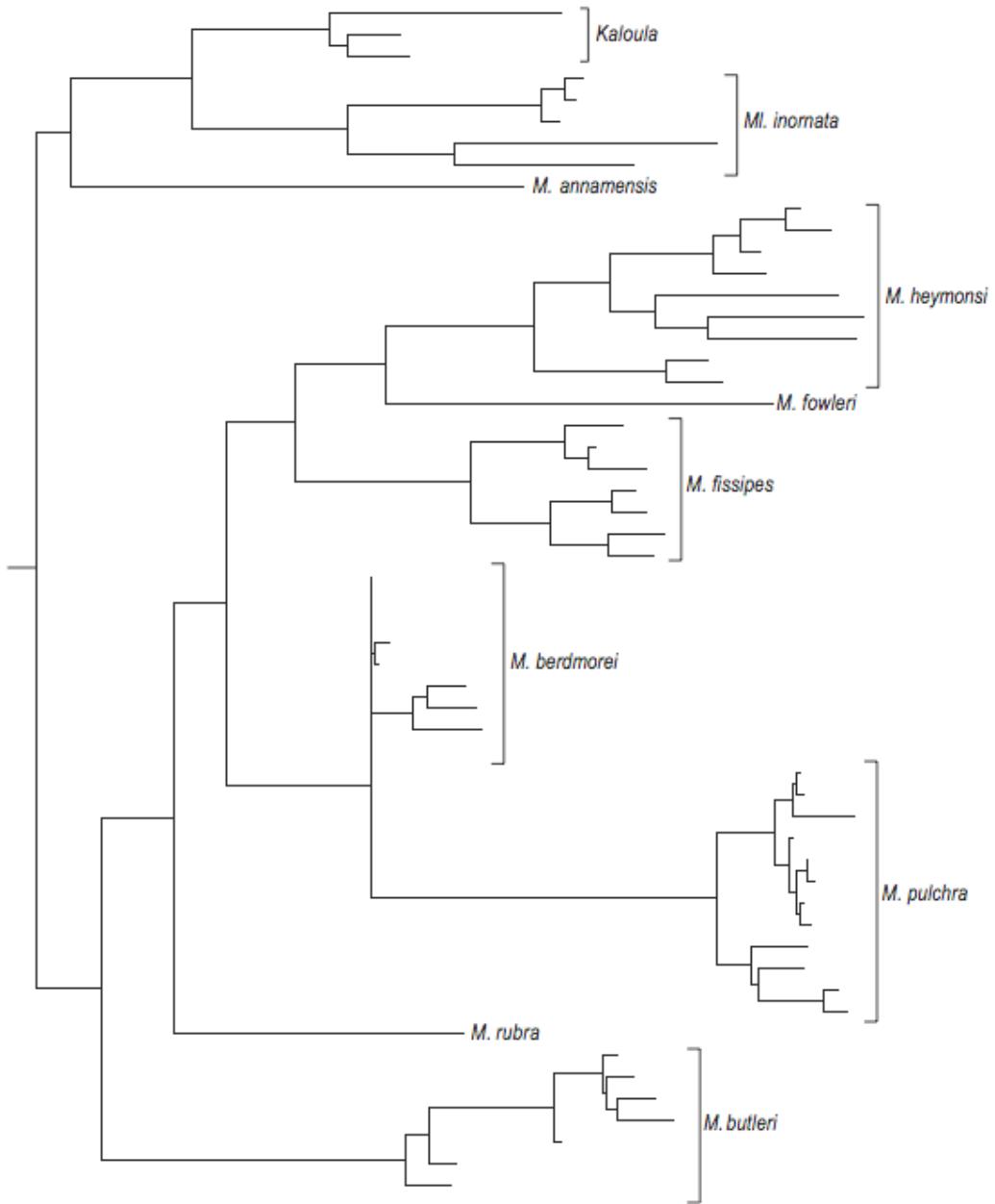


Figure 3.3. Bayesian 50% majority rule consensus tree showing relative branch lengths and species relationships. Outgroups have been pruned for clarity.

Table 3.1. Species of *Microhyla* (*Mh.*), *Micryletta* (*Ml.*) and *Kaloula* that were sequenced, voucher specimen catalog numbers, and collecting localities. Sequences for specimens marked with a dagger (†) were obtained from GenBank. The taxonomy follows Frost (2007).

Specimen #	Species	Locality	Voucher
1	<i>Ml. inornata</i>	Krong Pa, Gia Lai Prov., Vietnam	ROM 33407
2	<i>Ml. inornata</i>	Tram Lap, Gia Lai Prov., Vietnam	ROM 29194
3	<i>Ml. inornata</i>	Boulapha District, Khammouan Prov., Lao PDR	ROM 255121
4	<i>Ml. inornata</i>	Nakai District, Khammouan Prov., Lao PDR	ROM 255123
5	<i>Ml. inornata</i>	Cat Tien, Dong Nai Prov., Vietnam	ROM 261630
6	<i>Ml. inornata</i>	Yok Don, Dak Lak Prov., Vietnam	ROM 32697
7	<i>Ml. inornata</i>	Cat Tien, Lam Dong Prov., Vietnam	ROM 27147

8	<i>Ml. inornata</i>	Cat Tien, Dong Nai Prov., Vietnam	ROM 261629
9	<i>Mh. annamensis</i>	Krong Pa, Gia Lai Prov., Vietnam	ROM 33009
10	<i>Mh. butleri</i>	Chi Linh, Hia Duong Prov., Vietnam	ROM 36157
11	<i>Mh. butleri</i>	Pac Ban, Tuyen Quang Prov., Vietnam	ROM 6691
12	<i>Mh. butleri</i>	Tam Dao, Vinh Phu Prov., Vietnam	ROM 25874
13	<i>Mh. butleri</i>	Tam Dao, Vinh Phu Prov., Vietnam	ROM 31270
14	<i>Mh. butleri</i>	Quang Thanh, Cao Bang Prov., Vietnam	ROM 36160
15	<i>Mh. butleri</i>	Ba Be National Park, Cao Bang Prov., Vietnam	ROM 27051
16	<i>Mh. butleri</i>	Yok Don, Dak Lak Prov., Vietnam	ROM 33583

		Bante Sre District,	
17	<i>Mh. butleri</i>	Siem Reap Prov.,	ROM 257357
		Cambodia	
		Nakai District,	
18	<i>Mh. butleri</i>	Khammouan Prov.,	ROM 255225
		Lao PDR	
		released	
19	<i>Mh. rubra</i> [†]	Dharwad, Karnataka	GenBank
		State, India	AB201181
			AB201192
		ZSIK-A9119	
20	<i>Mh. ornata</i> [†]	Dharwad, Karnataka	GenBank AB201177
		State, India	AB201188
			AB201223
		DB-HI-FROG12005	
21	<i>Mh. ornata</i> [†]	Dinajpur, Parbatipur,	GenBank AB201176
		Bangladesh	AB201187
			AB201222
22	<i>Mh. heymonsi</i>	Chi Linh, Hia Duong Prov., Vietnam	ROM 36152

23	<i>Mh. heymonsi</i>	Chi Linh, Hia Duong Prov., Vietnam	ROM 36155
24	<i>Mh. heymonsi</i>	Chi Linh, Hia Duong Prov., Vietnam	ROM 25123
25	<i>Mh. heymonsi</i>	Pac Ban, Tuyen Quang Prov., Vietnam	ROM 29257
26	<i>Mh. heymonsi</i>	Vieng Tong District, Huaphahn Prov., Lao PDR	255086
27	<i>Mh. heymonsi</i>	Phiang District, Sayaboury Prov. Lao PDR	257985
28	<i>Mh. heymonsi</i>	Mounlapamok District, Champasak Prov., Lao PDR	255088
29	<i>Mh. heymonsi</i>	Yok Don, Dak Lak Prov., Vietnam	ROM 32873
30	<i>Mh. heymonsi</i>	Krong Pa, Gia Lai Prov., Vietnam	ROM 32798
31	<i>Mh. cf. fowleri</i>	Cat Tien, Lam Dong Prov., Vietnam	ROM 41169

		Amamioshima,	
32	<i>Mh. okinavensis</i> [†]	Ryukyu, Amami, Japan	KUHE12840
		Ba Be National Park, Cao Bang Prov., Vietnam	27048
33	<i>Mh. fissipes</i>	Ba Be National Park, Cao Bang Prov., Vietnam	27083
34	<i>Mh. fissipes</i>	Pac Ban, Tuyen Quang Prov., Vietnam	29193
35	<i>Mh. fissipes</i>	Chi Linh, Hia Duong Prov., Vietnam	36186
36	<i>Mh. fissipes</i>	Huangshan, Anhui Prov., China	KUHE32943
37	<i>Mh. fissipes</i> [†]	Cat Tien, Lam Dong Prov., Vietnam	ROM 41208
38	<i>Mh. fissipes</i>	Yok Don, Dak Lak Prov., Vietnam	ROM 33728
39	<i>Mh. fissipes</i>	Ankhe District, Gia Lai Prov., Vietnam	253169
40	<i>Mh. fissipes</i>		

41	<i>Mh. fissipes</i>	Con Cuong, Nghe An Prov., Vietnam	ROM 25827
42	<i>Mh. fissipes</i>	Vinh, Nghe An Prov., Vietnam	ROM 13417
43	<i>Mh. fissipes</i>	Mounlapamok District, Champasak Prov., Lao PDR	ROM 255131
44	<i>Mh. fissipes</i>	Boualapha District, Khammouan Prov., Lao PDR	ROM 255115
45	<i>Mh. fissipes</i> [†]	Kanchanaburi, Thong Pha Phum, Thailand	KUHE35165
46	<i>Mh. berdmorei</i>	Yok Don, Dak Lak Prov., Vietnam	ROM 33512
47	<i>Mh. berdmorei</i>	Cat Tien, Lam Dong Prov., Vietnam	ROM 41168
48	<i>Mh. berdmorei</i>		
49	<i>Mh. berdmorei</i>	Krong Pa, Gia Lai Prov., Vietnam	ROM 32704
50	<i>Mh. berdmorei</i>	Tram Lap, Gia Lai Prov., Vietnam	ROM 29200

51	<i>Mh. berdmorei</i>	Tram Lap, Gia Lai Prov., Vietnam	ROM 29196
52	<i>Mh. berdmorei</i>	Krong Pa, Gia Lai Prov., Vietnam	ROM 32699
53	<i>Mh. berdmorei</i>	Mounlapamok District, Champasak Prov., Lao PDR	255065
54	<i>Mh. berdmorei</i>	Boualapha District, Khammouan Prov., Lao PDR	255057
55	<i>Mh. berdmorei</i>	Phongsaly District, Phongsaly Prov., Lao PDR	257921
56	<i>Mh. pulchra</i>	Pac Ban, Tuyen Quang Prov., Vietnam	ROM 29175
57	<i>Mh. pulchra</i>	Ba Be National Park, Cao Bang Prov., Vietnam	ROM 27012
58	<i>Mh. pulchra</i>	Chi Linh, Hia Duong Prov., Vietnam	ROM 36217

59	<i>Mh. pulchra</i>	Quang Thanh, Cao Bang Prov., Vietnam	ROM 36163
60	<i>Mh. pulchra</i>	Bawang Ling Nature Reserve, Hainan, China	IOZCAS 1120
61	<i>Mh. pulchra</i>	Bawang Ling Nature Reserve, Hainan, China	IOZCAS 1121
62	<i>Mh. pulchra</i>	Bawang Ling Nature Reserve, Hainan, China	IOZCAS 1131
63	<i>Mh. pulchra</i>	Bawang Ling Nature Reserve, Hainan, China	IOZCAS 1130
64	<i>Mh. pulchra</i>	Diao Luo Shan Forest Park, Hainan, China	IOZCAS 1136
65	<i>Mh. pulchra</i>	Diao Luo Shan Forest Park, Hainan, China	IOZCAS 1137
66	<i>Mh. pulchra</i>	Con Cuong, Nghe An Prov., Vietnam	ROM 25879

67	<i>Mh. pulchra</i>	Con Cuong, Nghe An Prov., Vietnam	ROM 25878
68	<i>Mh. pulchra</i>	Yok Don, Dak Lak Prov., Vietnam	ROM 32750
69	<i>Mh. pulchra</i>	Cat Tien, Dong Nai Prov., Vietnam	ROM 261644
70	<i>Mh. pulchra</i>	Bante Sre District, Siem Reap Prov., Cambodia	ROM 257380
71	<i>Mh. pulchra</i>	Yommalat District, Khammouan Prov., Lao PDR	ROM 255092
72	<i>Mh. pulchra</i>	Mounlapamok District, Champasak Prov., Lao PDR	ROM 255094
73	<i>K. taprobanica</i> [†] (OG)	GenBank	AF249085
			AF249057
74	<i>K. conjunctiva</i> [†] (OG)	GenBank	AY326064

		KUHE35171
75	<i>K. pulchra</i> [†] (OG)	GenBank
		NC006405
		KUHE35182
76	<i>G. molossus</i>	GenBank
		AB201193
		AB201225

Table 3.2. Primers used for amplifying and sequencing fragments of genes from species of *Microhyla*, *Micryletta* and *Kaloula*. Sequence position indicates the starting position of the primer in the *Xenopus laevis* genome and is preceded by the amplification direction as indicated by (H) heavy or (L) light strand.

Name	Sequence 5' to 3'	<i>Sequence</i>	
		<i>position</i>	Reference
MVZ15L	GAACTAATGGCCCACACWWTACGNAA	L	Graybeal 1999
Cytb2.RC	TGAGGACAAATATCCTTCTGAGG	L16676	J.P. Dumbacher et al. 2003
Cytb B	CTTCTACTGGTTGTCCCTCCGATTCA	H17257	Bossuyt and Milinkovitch 2000
Cytb C	CTACTGGTTGTCCCTCCGATTCATG	H	Bossuyt and Milinkovitch 2000
16S3L	CCCGAAATCAAGTGAGCTAC	L3362	Fu (pers. comm.)
16S1H	GGCTATGTTTTGGTAAACAG	H3958	Modified from Palumbi

			(1996)
16S5H	CTACCTTGCACGGTTAGGATACCGCGGC	H4040	Fu (2000)
16S1M	CCGACTGTTACCAAAACAT	L3955	Fu (1998)
16S2H	CCGGATCCCCGGCCGGTCTGAACTCAGATCACG	H4552	Palumbi (1996)

Table 3.3. Summary of genes sequenced from the ingroup taxa, *Microhyla* and *Micryletta*, and the outgroup, *Kaloula*. TS = total number of homologous sites resolved; AS = number of ambiguous sites removed; NSR = number of homologous sites retained; NVS = number of variable sites; NPPIS = number of potentially phylogenetically informative sites; NMPTs = number of most parsimonious trees resolved; LMPTs = Length of most parsimonious solution; CI = consistency index; RI = retention index.

Gene	TS	Ts:Tv	AS	NSR	NVS	NPPIS	NMPTs	LMPTs	CI	RI
16S rRNA	1392	1:1.24	18	1358	911	385	14	1382	0.452	0.863
Cytochrome b	1012	1:1.65	0	1012	483	427	1774	2056	0.372	0.804
Combined	2404	1:1.44	18	2419	1433	850	649	4376	0.344	0.802

Chapter 4

Biogeographical Patterns of Maternal Lineages of *Microhyla heymonsi* Vogt 1911

Abstract

The diminutive, dark-sided chorus frog, *Microhyla heymonsi*, is widespread and locally abundant. Being a habitat generalist, it is often associated with disturbed habitats. Evidence from other species suggests that *Mh. heymonsi* could be a composite of several forms. However, in opposition to multiple species, there is a high potential for long distance dispersal in *Mh. heymonsi* with concomitant gene flow between maternal lineages. A total of 1688 aligned sites from 16S and 1012 for Cyt b gene regions of the mitochondrial DNA genome from 56 specimens were analyzed using both maximum parsimony and Bayesian methods and biogeographic patterns discussed. Two sympatric lineages were identified in Krong Pa, Vietnam and may represent different species.

Introduction

The diminutive, dark-sided chorus frog, *Microhyla heymonsi*, is found widely in Vietnam and other parts of Southeast and East Asia. They range from mainland India and the Nicobar and Adaman Islands eastward to Zhejiang province, China and the islands of

Taiwan and Hainan, and southward through Indochina to the Malay peninsula and Sumatra (Grosselet et al., 2004; Parker, 1934; Vogt, 1911; Zhao and Adler, 1993; Frost, 2007; Manthey and Grossman, 1997). The type locality, originally designated as “Formosa” (=Taiwan) by Vogt (1911), was subsequently narrowed to “Kosempo, Formosa” (=Jiaxian, Gaoxiong County, Taiwan) by Parker (1934).

These frogs range widely and are locally abundant (Ibrahim et al., 2006; Ziegler, 2004; Manthey and Grossman, 1997). Being habitat generalists, they are often associated with disturbed habitats, such as rice paddies and other agricultural areas, burned areas, grassy fields, and secondary growth (Ziegler, 2004; Manthey and Grossman, 1997; Inger and Iskandar, 2005). Inger and Voris (2001) note that the species is a non–forest commensal of man. Due to their small size, habit, and association with agriculture, they are possibly pulled up with crops, mud, and potted plants and subsequently translocated throughout the region.

In Chapter 3, the genus *Microhyla* in Vietnam is surveyed. Partially resolved relationships are reported for geographically defined groups of *Mh. heymonsi*. The lineages are set apart by relatively long branches, which might indicate the presence of multiple cryptic species. Evidence from other species suggests that *Mh. heymonsi* could be a composite of several forms. Stuart et al. (2006) questioned the concept of widespread amphibian species in Southeast Asia. Most of the putative “weedy,” widespread species in the region are actually complexes of species (Bain et al., 2003; Emerson et al., 2000; Chapter 3; Stuart et al., 2006; Toda et al., 1998), including another species of *Microhyla*. Matsui et al. (2005) documented two cryptic species and substantial geographical structure in the genealogical relationships in *M. ornata*.

However, in opposition to multiple species, there is a high potential for long distance dispersal in *Mh. heymonsi* with concomitant gene flow between maternal lineages. After all, they are often associated with humans.

To further investigate the patterns presented in Chapter 3, an additional 44 specimens from 12 additional localities were added, including a single specimen from GenBank. The GenBank specimen is notable because it is the sole representative of the species for which the entire mitochondrial genome has been sequenced. Additionally, this specimen may represent the type locality, Taiwan, although no locality information was provided.

Materials and Methods

Specimens Examined

In total, 53 individuals of *Mh. heymonsi* from 15 localities in Taiwan and Southeast Asia were examined as ingroup taxa. The microhylid frogs *M. berdmorei*, *M. pulchra*, and *M. cf. fowleri* served as outgroup taxa. Locality and voucher data for all specimens examined are presented in Table 4.1, and the localities are mapped in Figure 4.1. Voucher specimens are preserved in the herpetological collections of the Royal Ontario Museum (ROM) except as indicated in Table 4.1.

DNA gene selection

The relatively slowly evolving 16S ribosomal RNA gene (16S) has been considered appropriate for resolving older divergences, perhaps as old as 150 Ma, and has been used extensively in anuran systematics at the species level (Che et al., 2007;

Glaw and Vences 2006; Matsui et al., 2005; Mindell and Honeycutt, 1990). The more rapidly evolving cytochrome *b* gene (Cyt *b*) is commonly used in both species and intraspecific levels to resolve more recent events including within species patterns of mitochondrial evolution (Glaw and Vences 2006; Graybeal 1993, 1994; Matsui et al., 2005; Zangari et al., 2006). Consequently, these mitochondrial genes were selected to reconstruct the history of the maternal lineages within *Mh. heymonsi*.

DNA Amplification and Sequencing

Total genomic DNA was extracted from frozen or ethanol-preserved muscle or liver tissue samples by digestion with proteinase K for 7–12 hr, then purified three times with phenol-chloroform-isoamyl alcohol (PCI), and then once with chloroform-isoamyl alcohol (CI). Double-stranded DNA fragments were amplified using the polymerase chain reaction (PCR; 92°C for 30 sec, 50–53°C for 45 sec 72°C for 90 sec) performed in 25µl reactions for 33 cycles. Annealing temperatures ranged from 50°C to 53°C as needed in order to improve primer binding and the quality of the PCR product. PCR reactions generally amplified the entire gene fragments from 16S1L to 16S2Hm for 16S, and MVZ15L to CytbB for Cyt *b* (Table 4.2). Occasionally, additional combinations of primers were required (Table 4.2). After amplification, a 2µl aliquot of PCR product was separated by electrophoresis on an agarose gel and stained with ethidium bromide to verify amplification. The remaining DNA was cleaned using QiaColumns (Qiagen) and eluted in Qiagen EB buffer. The cleaned double stranded DNA was sequenced directly using 8µl BigDye (Applied Biosystems International, ABI) fluorescent cycle sequencing reactions (96°C for 30 sec, 50°C for 15 sec 60°C for 4 min for 30 cycles). Sequencing

product was ethanol-precipitated and resuspended in Hi-Di formamide (ABI). Resuspended DNA was then run out on either an ABI 377 automated sequencer using long plates or an ABI 3100 automated sequencer. Sequence data for the outgroup taxa and one ingroup specimen putatively from the type locality of Taiwan were obtained from GenBank (Table 4.1).

DNA Sequence Analysis

DNA sequences were entered into BioEdit (ver. 7.0.5, Hall, 2005) for assembly, initially aligned with Clustal W (ver. 1.6, Thompson et al., 1994) and subsequently adjusted by eye. Sites and regions of ambiguous alignment were considered to have questionable homology and excluded (Hillis and Dixon, 1991). Redundant sequences were merged and potentially phylogenetically informative sites were retained for analysis.

Maximum Parsimony Evaluation

All characters were evaluated as unordered because there is no *a priori* reason to assume order of evolutionary change between nucleotide bases (Swofford et al., 1996). The data were analyzed using maximum parsimony (MP) as the criterion for selecting among all possible trees. The MP analysis was performed in PAUP* (ver. 4.0b10 Swofford, 2004) using an heuristic search with random addition sequence, 100 replicates, retaining minimal trees only, using tree bisection-reconnection branch swapping with steepest descent and collapsing zero length branches. Ratios of transitions to transversions were calculated in MacClade (ver. 4.0.5; Maddison and Maddison, 2002).

Nodal support was assessed by nonparametric bootstrap proportions (BSP; Felsenstein, 1985), using 10,000 replicates and “Fast” stepwise addition calculated in PAUP*.

Bayesian Inference Analysis

Bayesian inference (BI) was also used to infer patterns maternal history within *Mh. heymonsi* (Huelsenbeck and Ronquist, 2001; Buckley et al., 2002; Nylander et al., 2004). The sequence data were partitioned by gene and the program MRMODELTEST v2.2 (Nylander, 2004) was used to select an evolutionary model that best fit the data for each gene using the Akaike information criterion (Akaike, 1974, 1979). Hierarchical likelihood ratio tests (Goldman, 1993) were also implemented using MRMODELTEST v2.2, comparing the log-likelihood scores of the 24 evolutionary models that can be implemented in MrBayes for each gene. A general time reversal model with invariant sites and a gamma distribution (GTR + I + G, Rodríguez et al., 1990, Gu et al., 1995, Waddell & Penny 1996) was selected for both genes and was implemented in the total evidence analysis. BI was conducted using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). The analysis was initiated with random starting trees. Six Markov chains were used, and the dataset was run for 1.7×10^6 generations. Trees were sampled every 100 generations. Two independent analyses with different starting trees were run to avoid being trapped on local optima. The fluctuating likelihood values were graphically monitored (Huelsenbeck and Bollback, 2001) and stationarity was achieved when the log-likelihood values of the sample points reached a stable equilibrium value (Huelsenbeck and Ronquist, 2001). The analysis was a priori required to achieve a split

frequency standard deviation of ≤ 0.02 . After discarding the first 2000 sampled trees as burn-in, the remaining trees were used to generate a 50% majority rule consensus tree.

Results

Fifty-six specimens were sequenced for 16S and Cyt *b*. In total, 676 sites were sequenced for 16S and 1012 for Cyt *b* for a combined total of 1688 aligned sites. Among these sites, 104 were ambiguously aligned and 373 were potentially phylogenetically informative (Table 4.2). All sequences will be deposited in GenBank.

Parsimony evaluation

The cyt**b** gene was evaluated separately. Analysis of the 308 potentially informative sites yielded 576 most parsimonious trees (MPTs; Table 4.2) (length = 1214, CI = 0.474, RI = 0.792). The 16s gene had only 26 sites that were potentially parsimony informative for the ingroup and so a separate analysis for this gene was not undertaken.

Combining all mitochondrial gene sequence data into a single data set resulted in 373 potentially cladistically informative characters. Analysis of these data yielded 576 MPTs (1214 steps in length, CI=0.47, RI=0.79). The ingroup formed a monophyletic group separated from the outgroup taxa.

A clade of specimens from Yok Don National Park and Krong Pa was resolved as the sister group to the remainder of the ingroup (Figure 4.2). A group containing a specimen each from Sayaboury and Champasak in Laos and Dong Nai in Vietnam was, in turn, the sister group to a single specimen from Huaphahn, Laos plus a group

containing two subclades containing both Chinese and Vietnamese specimens. The first subclade contained the Taiwanese specimen plus southern Vietnamese specimens from Cat Tien National Park, Tram Lap, and Krong Pa. Its sister clade was comprised of specimens from Diao Luo Shan on Hainan Island, China plus specimens from northern Vietnam.

Bayesian Inference Evaluation

The BI analysis recovered a topology similar to the MP analysis, with two exceptions. The specimen from Huaphahn, Laos was recovered in a polytomy with the other Laotian specimens and the specimen from nearby Cat Tien, Dong Nai, though there is poor bootstrap support (<50) and low Bayesian posterior probabilities (<60) at the involved nodes (Figure 4.3). The clade of specimens from Hainan was resolved as the sister group to the Taiwanese specimen plus specimens from Cat Tien, Tram Lap, and Krong Pa (Figure 4.3). The Bayesian posterior probabilities are high (>80) for this clade while the bootstrap proportion is less than 50 percent.

Nodal Stability

Eighteen nodes were supported by BSPs greater than 75, including the Yok Don–Krong Pa clade, which was well supported with a BSP of 100. Most nodes were also supported by BPP values of 80 or higher, with the majority of lower values associated with intrapopulation relationships (Figures 4.2 and 4.3).

Discussion

Taxonomic implications

The lineage containing specimens from Yok Don National Park and Krong Pa is distinguished from other lineages by a long branch and high uncorrected pairwise distances. However, it occurs in sympatry in Krong Pa with another lineage. This sympatry of distinct lineages suggests that they may, in fact, be different species (Stuart et al., 2006). As in the *Rana chalconota* and *Odorrana livida* complexes, the sympatric forms are not sister lineages (Stuart et al., 2006). This pattern is consistent with the concepts of reinforcement of species boundaries and population cohesion, which predict increased risk of competition and mating mistakes with sister taxa as opposed to more distantly related taxa. Separation of this lineage as a new species requires further work, including morphological and possibly molecular characters that can demonstrate gene flow or lack thereof. Stuart et al., (2006) noted that *a posteriori* analysis of the different lineages revealed at least one diagnostic morphological character for each group in all but two lineages evaluated, and this may prove to be true for *Mh. heymonsi*.

The presence of the two lineages at Krong Pa is the only occurrence of two sympatric lineages recovered in *Mh. heymonsi*. This singularity further highlights the distinctiveness of the lineage characteristic of Yok Don National Park and Krong Pa. To evaluate whether specimens belonging to the two lineages actually represent more than one species, further genetic work is needed in addition to morphological reexamination of the specimens. Nuclear markers such as microsatellites or anonymous nuclear loci can be used to document gene flow, or lack thereof, between the two lineages. Using a simple PCR technique, MAMA (Lindell and Murphy, 2007), individuals collected from the ranges of the two groups, including the area of overlap in Krong Pa, can be sorted by

lineage, thus ensuring adequate sampling for analysis. A record of the patterns of the distribution of the different mitochondrial lineages will allow for a more directed sampling approach as well as providing a way to potentially separate animals in the field

Biogeographic patterns

The patterns recovered are relevant to understanding the biology of *Mh. heymonsi*. The potential for long distance dispersal, association with disturbed habitat and agriculture, and the local abundance of these animals predict a mixture of haplotypes among populations. However, even if all of the examined ingroup specimens are considered the same species, there is genetic structure with respect to the Yok Don National Park and Krong Pa group and to a lesser extent with Laos. There are also longer internodes with apparent “cohesion” or lack of differentiation within the “terminal” clades. This structure implies a series of isolation events that interrupted gene flow. Long branches separate not only the population in Yok Don National Park and Krong Pa, but also the specimens from Laos and Dong Nai.

As in both clades of *Polypedates leucomystax* (Chapter 5) and *Microhyla fissipes* (Chapter 3), a clear split was resolved between specimens from northern and southern Vietnam. As in the other cases, this may be due to the geographical separation of these regions. The Central Highlands that separate them are drained by several distinct smaller river systems directly into the South China Sea. In contrast, the northern Vietnamese localities are drained by the Red River and the southern localities by the Mekong River. This effectively separates the north from the south, and likely also serves to isolate the populations occurring there. If true, then more extensive sampling in the Vietnamese

Central Highlands will likely reveal additional geographic structure and potentially even cryptic species.

Every locality sampled was resolved as being comprised of a single unique lineage with the exception of Krong Pa and Yok Don National Park. However, even at Krong Pa, the specimens within each lineage had a common maternal ancestor that they shared more closely with each other than with other specimens. Some of the specimens from Yok Don National Park shared a more recent common maternal ancestor with the specimens from Krong Pa than with other specimens from the same locality. This pattern fits a scenario of a recent invasion of Krong Pa from the lowland dipterocarp forest of Yok Don National Park. The scenario involving the other lineage is unclear, however, due to the limited sampling of Tram Lap and Cat Tien.

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tissues and preserved specimens were issued by Agriculture Canada. All fieldwork was conducted using approved Animal Use Protocols.

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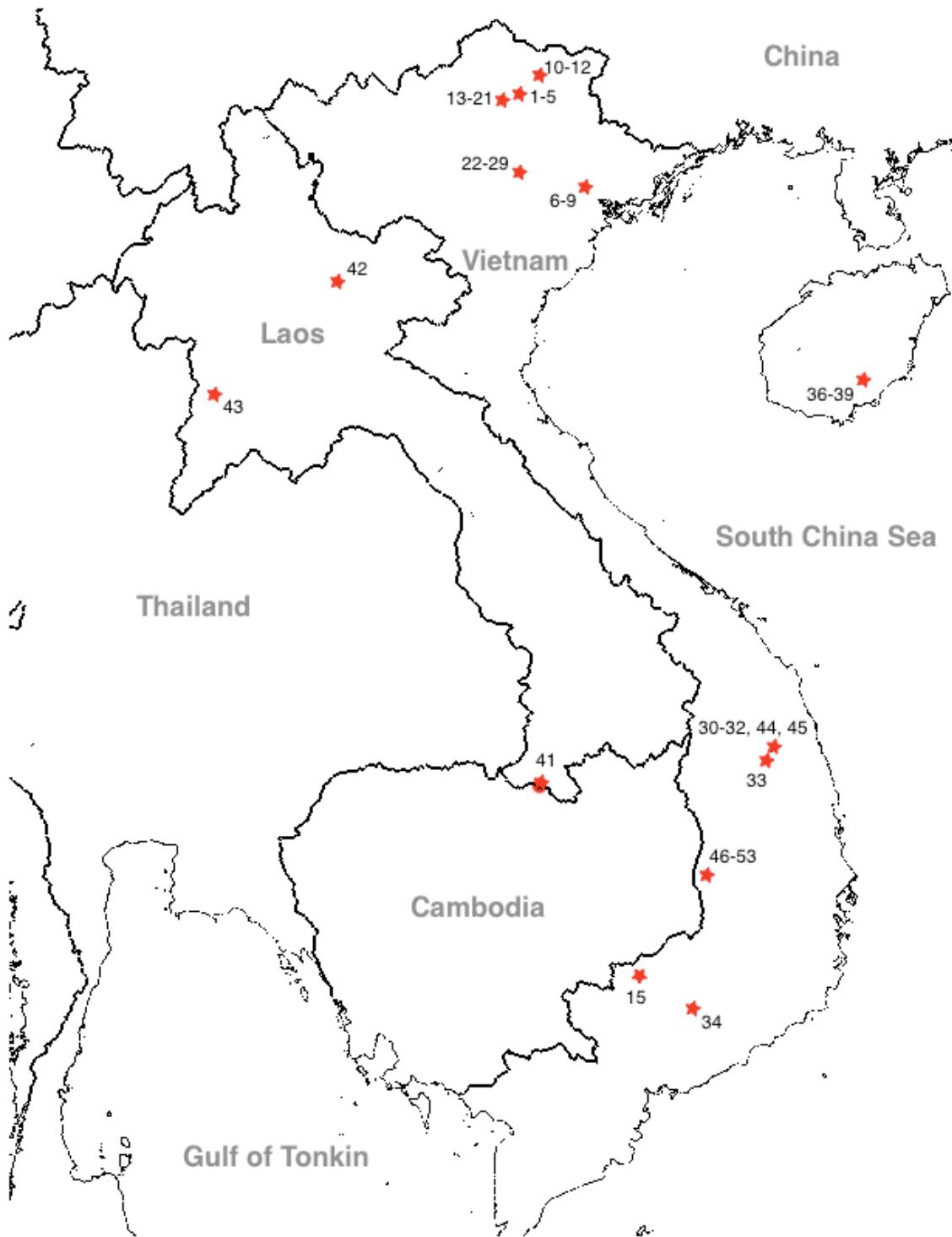


Figure 4.1. Map of *Microhyla heymonsi* specimen localities. Numbers refer to the specimens in Table 4.1.

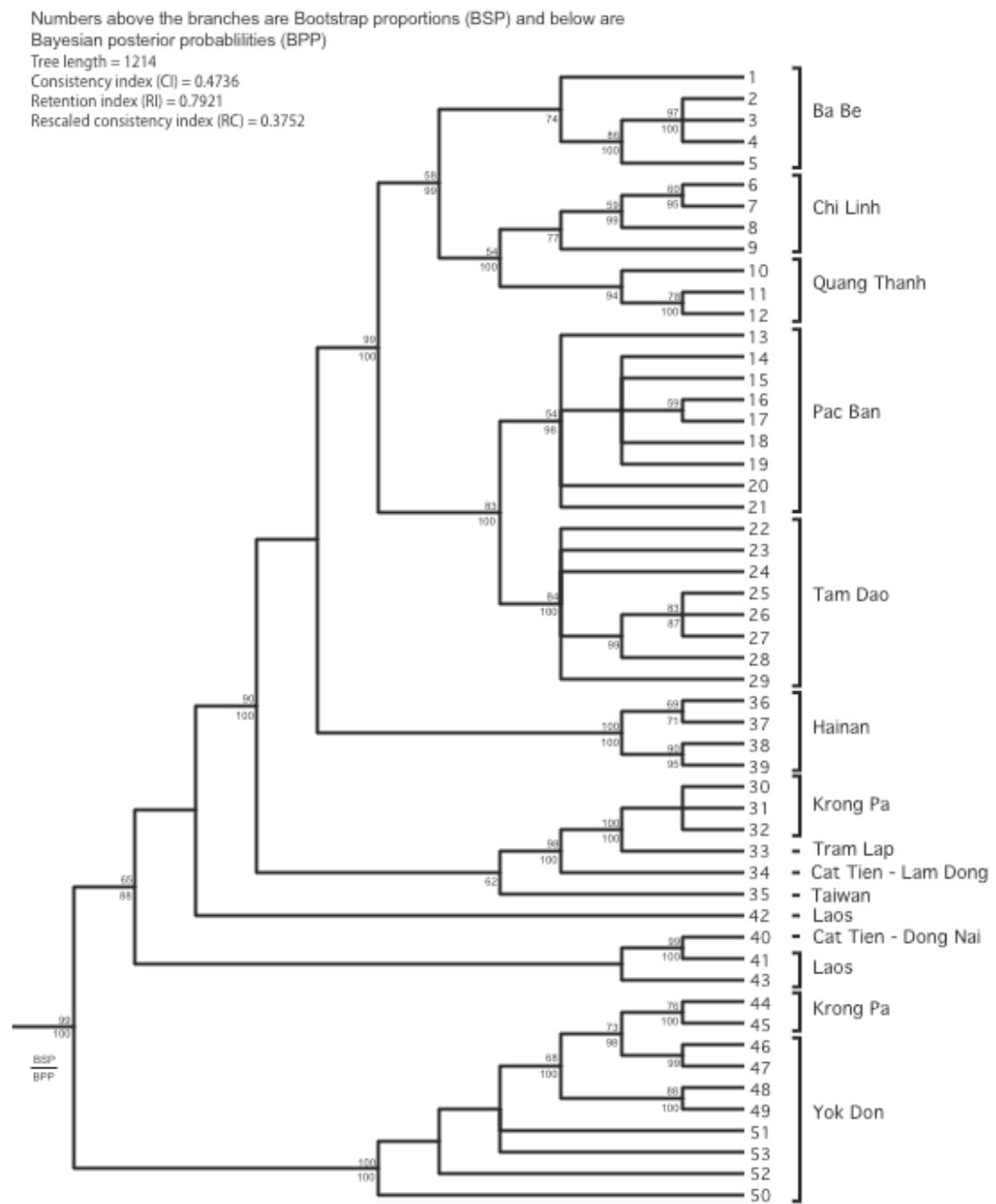


Figure 4.2. Maximum Parsimony tree. Strict consensus of 576 most parsimonious trees. Bootstrap proportions are above the branches and Bayesian posterior probabilities are below. Outgroups have been pruned for clarity.

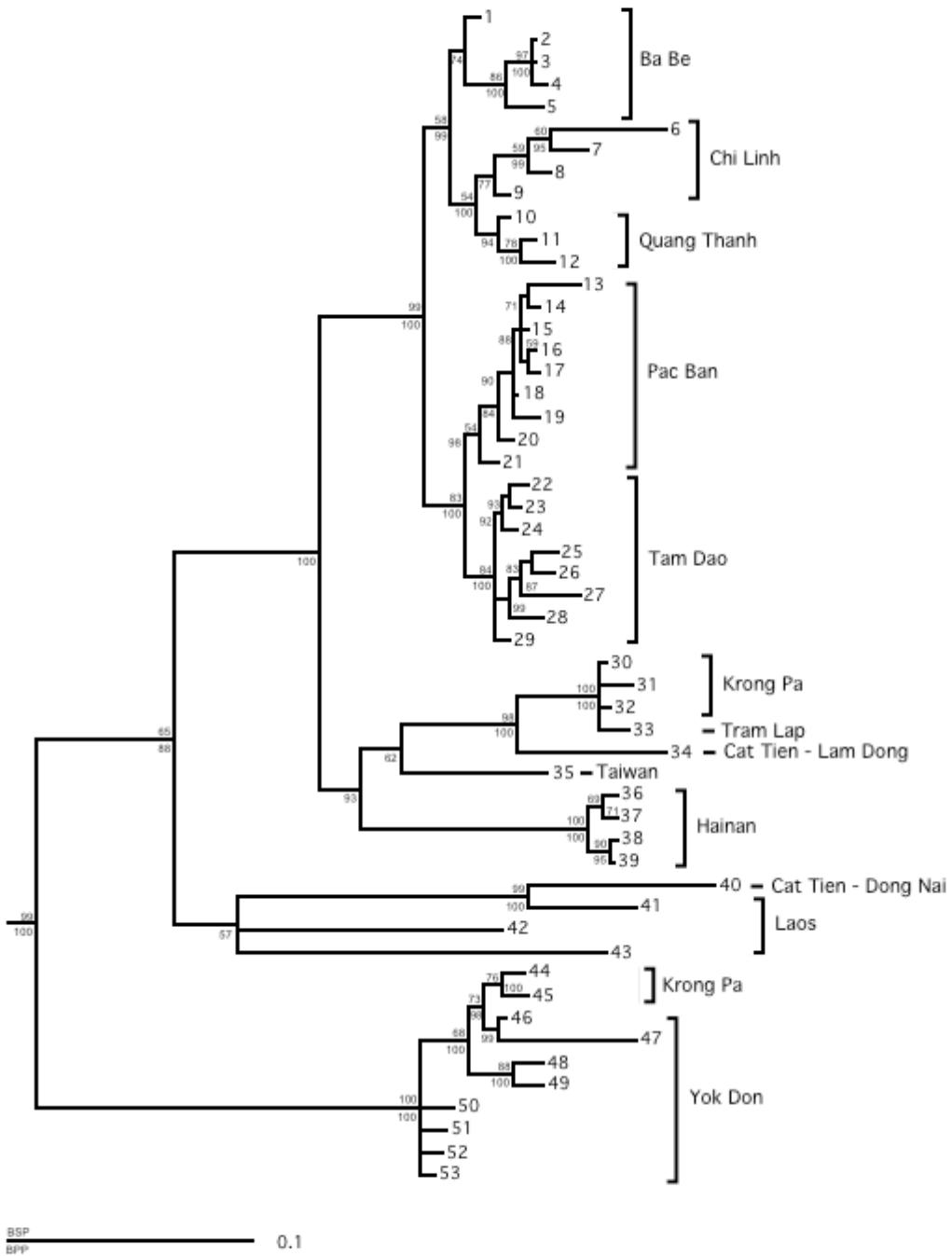


Figure 4.3. Bayesian 50% Majority Rule Consensus Tree showing relative branch lengths and species relationships. Bootstrap proportions are above the branches and Bayesian posterior probabilities are below. Outgroups have been pruned for clarity.

Table 4.1. Species sequenced, voucher specimen catalog numbers, and collecting locality.

The taxonomy follows Frost (2007).

Specimen #	Species	Locality	Voucher
1	<i>Mh. heymonsi</i>	Ba Be National Park, Cao Bang Province, Vietnam	ROM 19660
2	<i>Mh. heymonsi</i>	Ba Be National Park, Cao Bang Province, Vietnam	ROM 19809
3	<i>Mh. heymonsi</i>	Ba Be National Park, Cao Bang Province, Vietnam	ROM 19823
4	<i>Mh. heymonsi</i>	Ba Be National Park, Cao Bang Province, Vietnam	ROM 19221
5	<i>Mh. heymonsi</i>	Ba Be National Park, Cao Bang Province, Vietnam	ROM 19817
6	<i>Mh. heymonsi</i>	Chi Linh, Hia Duong Province, Vietnam	ROM 25191
7	<i>Mh. heymonsi</i>	Chi Linh, Hia Duong Province, Vietnam	ROM 25392
8	<i>Mh. heymonsi</i>	Chi Linh, Hia Duong	ROM 25192

		Province, Vietnam	
9	<i>Mh. heymonsi</i>	Chi Linh, Hia Duong Province, Vietnam	ROM 25123
10	<i>Mh. heymonsi</i>	Quang Thanh, Cao Bang Province, Vietnam	ROM 26198
11	<i>Mh. heymonsi</i>	Quang Thanh, Cao Bang Province, Vietnam	ROM 26199
12	<i>Mh. heymonsi</i>	Quang Thanh, Cao Bang Province, Vietnam	ROM 26197
13	<i>Mh. heymonsi</i>	Pac Ban, Tuyen Quang Province, Vietnam	ROM 6530
14	<i>Mh. heymonsi</i>	Pac Ban, Tuyen Quang Province, Vietnam	ROM 6710
15	<i>Mh. heymonsi</i>	Pac Ban, Tuyen Quang Province, Vietnam	ROM 6623
16	<i>Mh. heymonsi</i>	Pac Ban, Tuyen Quang Province, Vietnam	ROM 6730
17	<i>Mh. heymonsi</i>	Pac Ban, Tuyen Quang Province, Vietnam	ROM 6731
18	<i>Mh. heymonsi</i>	Pac Ban, Tuyen Quang	ROM 6747

		Province, Vietnam	
19	<i>Mh. heymonsi</i>	Pac Ban, Tuyen Quang Province, Vietnam	ROM 6503
20	<i>Mh. heymonsi</i>	Pac Ban, Tuyen Quang Province, Vietnam	ROM 6732
21	<i>Mh. heymonsi</i>	Pac Ban, Tuyen Quang Province, Vietnam	ROM 6796
22	<i>Mh. heymonsi</i>	Tam Dao, Vinh Phu Province, Vietnam	ROM 16239
23	<i>Mh. heymonsi</i>	Tam Dao, Vinh Phu Province, Vietnam	ROM 16240
24	<i>Mh. heymonsi</i>	Tam Dao, Vinh Phu Province, Vietnam	ROM 16955
25	<i>Mh. heymonsi</i>	Tam Dao, Vinh Phu Province, Vietnam	ROM 16241
26	<i>Mh. heymonsi</i>	Tam Dao, Vinh Phu Province, Vietnam	ROM 16544
27	<i>Mh. heymonsi</i>	Tam Dao, Vinh Phu Province, Vietnam	ROM 16243
28	<i>Mh. heymonsi</i>	Tam Dao, Vinh Phu Province, Vietnam	ROM 16865

29	<i>Mh. heymonsi</i>	Tam Dao, Vinh Phu Province, Vietnam	ROM 16956
30	<i>Mh. heymonsi</i>	Krong Pa, Gia Lai Province, Vietnam	ROM 23630
31	<i>Mh. heymonsi</i>	Krong Pa, Gia Lai Province, Vietnam	ROM 23631
32	<i>Mh. heymonsi</i>	Krong Pa, Gia Lai Province, Vietnam	ROM 23632
33	<i>Mh. heymonsi</i>	Tram Lap, Gia Lai Province, Vietnam	ROM 7154
34	<i>Mh. heymonsi</i>	Cat Tien, Lam Dong Province, Vietnam	ROM 27494
35	<i>Mh. heymonsi</i> [†]	Unreported	GenBank AY458596
36	<i>Mh. heymonsi</i>	Diao Luo Shan, Hainan Island, China	IOZCAS 1038
37	<i>Mh. heymonsi</i>	Diao Luo Shan, Hainan Island, China	IOZCAS 1023
38	<i>Mh. heymonsi</i>	Diao Luo Shan, Hainan Island, China	IOZCAS 1024
39	<i>Mh. heymonsi</i>	Diao Luo Shan, Hainan Island, China	IOZCAS 1080

40	<i>Mh. heymonsi</i>	Cat Tien, Dong Nai Province, Vietnam	17117
41	<i>Mh. heymonsi</i>	Mounlapamok District, Champasak Province, Lao PDR	HKV 63469
42	<i>Mh. heymonsi</i>	Vieng Tong District, Huaphahn Province, Lao PDR	HKV 63329
43	<i>Mh. heymonsi</i>	Phiang District, Sayaboury Province, Lao PDR	HKV 64119
44	<i>Mh. heymonsi</i>	Krong Pa, Gia Lai Province, Vietnam	ROM 23633
45	<i>Mh. heymonsi</i>	Krong Pa, Gia Lai Province, Vietnam	ROM 23432
46	<i>Mh. heymonsi</i>	Yok Don, Dak Lak Province, Vietnam	ROM 22212
47	<i>Mh. heymonsi</i>	Yok Don, Dak Lak Province, Vietnam	ROM 23115
48	<i>Mh. heymonsi</i>	Yok Don, Dak Lak Province, Vietnam	ROM 22202
49	<i>Mh. heymonsi</i>	Yok Don, Dak Lak Province, Vietnam	ROM 22196

50	<i>Mh. heymonsi</i>	Yok Don, Dak Lak Province, Vietnam	ROM 22124
51	<i>Mh. heymonsi</i>	Yok Don, Dak Lak Province, Vietnam	ROM 22200
52	<i>Mh. heymonsi</i>	Yok Don, Dak Lak Province, Vietnam	ROM 22201
53	<i>Mh. heymonsi</i>	Yok Don, Dak Lak Province, Vietnam	ROM 22199
54	<i>Mh. cf. fowleri</i>	Cat Tien, Lam Dong Province, Vietnam	ROM 41169
55	<i>Mh. berdmorei</i>	Mounlapamok District, Champasak Province,	HKV 63495
		Lao PDR	
		Boualapha District,	
56	<i>Mh. berdmorei</i>	Khammouan Province,	HKV 63179
		Lao PDR	
57	<i>Mh. pulchra</i>	Pac Ban, Tuyen Quang Province, Vietnam	ROM 6531

Table 4.2. Primers used for amplifying and sequencing fragments of genes in this study. Sequence position indicates the starting position of the primer in the *Xenopus laevis* genome and is preceded by the amplification direction as indicated by (H) heavy or (L) light strand.

Name	Sequence 5' to 3'	<i>Sequence</i>	
		<i>position</i>	Reference
MVZ15L	GAACTAATGGCCCACACWWTACGNAA	L	Graybeal 1999
Cytb2.RC	TGAGGACAAATATCCTTCTGAGG	L16676	J.P. Dumbacher et al. 2003
Cytb B	CTTCTACTGGTTGTCCTCCGATTCA	H17257	Bossuyt and Milinkovitch 2000
Cytb C	CTACTGGTTGTCCTCCGATTCATG	H	Bossuyt and Milinkovitch 2000
16S3L	CCCGAAATCAAGTGAGCTAC	L3362	Fu (pers. comm.)
16S1H	GGCTATGTTTTGGTAAACAG	H3958	Modified from Palumbi (1996)
16S5H	CTACCTTGCACGGTTAGGATACCGCG GC	H4040	Fu (2000)
16S1M	CCGACTGTTACCAAAAACAT	L3955	Fu (1998)

16S2H	CCGGATCCCCGGCCGGTCTGAACTCAGAT CACG	H4552	Palumbi (1996)
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Table 4.3. Summary of genes sequenced from the ingroup and outgroup taxa. TS = total number of homologous sites resolved; AS = number of ambiguous sites removed; NSR = number of homologous sites retained; NVS = number of variable sites; NPPIS = number of potentially phylogenetically informative sites; NMPTs = number of most parsimonious trees resolved; LMPTs = Length of most parsimonious solution; Ts:Tv = transition to transversion ratio; CI = consistency index; RI = retention index.

Gene	TS	AS	NSR	NVS	NPPIS	NMPTs	LMPTs	Ts:Tv	CI	RI
Cytochrome b	1392	0	1012	483	427	1774	2056	1:1.65	0.372	0.804
16S rRNA	1012	18	1358	911	26	n/a	n/a	n/a	n/a	n/a
Combined	2404	18	2419	1433	850	649	4376	1:1.44	0.344	0.802

Chapter 5

Are There Any Widespread Forest Amphibian Species in Asia: Cryptic Species and the *Polypedates leucomystax* Complex.

Abstract

The Asian treefrogs allied to the Javan whipping frog, *Polypedates leucomystax* (Gravenhorst 1829), are a widespread complex of species. Often multiple forms can be found at a single site, clouding the picture. Recent work has separated this group into two species, *P. leucomystax* and *P. megacephalus*, based on call differences, though definitive assignment of names is lacking. Specimens from across the range in Vietnam, as well as from southwestern China were sequenced. The mtDNA tree elucidated patterns of maternal lineage dispersal and differentiation in the region. Two major clades with similar patterns were recovered, several cryptic species were identified, and a phylogenetic component to broad geographic distribution noted.

Introduction

The Asian treefrogs allied to the Javan whipping frog, *Polypedates leucomystax*

(Gravenhorst 1829), are a widespread complex of species. These rhacophorid frogs range from India and Nepal eastward through Indochina and the Sunda shelf, and northward into mainland China, Taiwan, and Japan (Brown and Alcala, 1994; Frost, 2007; Manthey and Grossman, 1997; Matsui et al., 1986; Zhao and Adler, 1993). Not only are these frogs geographically wide-ranging, they are also locally abundant and found in a variety of habitats, including ponds in primary forests, on the edges of secondary growth, and even in human settlements (Liu 1950; Manthey and Grossman, 1997; Pope 1930; Pope and Boring, 1940; Ziegler, 2004). They occur in major metropolitan areas including Hanoi and Ho Chi Minh City, Vietnam. Their broad geographic range, combined with their apparently weedy nature and morphological polymorphism, has lead to significant controversy regarding their identity and taxonomic status (Frost, 2007; Matsui et al., 1986; Narins et al., 1998; Trépanier et al., 1999). This concern is echoed in their colorful synonymy, with different groups being designated as subspecies, full species, returned to synonymy, and resurrected (Annandale, 1912; Ao et al., 2003; Frost, 2007; Gravenhorst, 1829; Tschudi, 1838).

The taxonomic confusion is at least partly due to the extent of color pattern diversity exhibited by these animals. Populations of these frogs have either four clear longitudinal lines on the dorsum, six lines, an “X-shaped” marking on the dorsum, random flecking, a triangle pattern behind the head, a patternless dorsum, or a combination of these (Boulenger, 1890, 1912; Bourret, 1942; Church, 1963; Flower, 1896; Kirtisinghe, 1957). Easily observable morphometric differences, documented call differences, and discrete oviposition sites compound the confusion (Liu, 1950; Matsui et al., 1986; Narins et al., 1998; Trépanier et al., 1999). While these differences can be so

prevalent between populations that general impressions may be formed, often multiple forms can be found at a single site, thereby clouding the picture. Individual *P. leucomystax* can change their color patterns in life, at least between striped and patternless forms (Church, 1963; Flower, 1896; Liu, 1950). These changes can be retained at death after preservation in ethanol (Flower, 1896).

Church (1963) collected four clutches of eggs from foam nests in a disturbed habitat and found two distinct pattern groups, a spotted form and a broken stripe or striped form. He also found that most dorsal pattern combinations could arise from the same set of parents, although an interorbital crossbar sometimes seen in the spotted forms was not found in the striped group. Church hypothesized that X-shaped dorsal marking and its components represented a hybrid form, possibly due to hybridization with *P. cruciger* with subsequent introgression of genes controlling the dorsal pattern.

Upon comparing karyotypes, adult morphology, and advertisement calls of *P. leucomystax* from Taiwan and Borneo, Matsui et al. (1986) found two distinct forms. The Bornean population was retained in *P. leucomystax* because it was geographically closer to the type locality, Java. The population from Taiwan was assigned to *P. megacephalus* Hallowell 1861 because it was the oldest available name for Chinese specimens.

When separating *P. megacephalus* from *P. leucomystax*, Matsui et al. (1986) suggested that the Chinese forms previously ascribed to *P. leucomystax* were probably *P. megacephalus* and that the name *P. leucomystax* was most appropriately applied to animals from Borneo and Java. Mainland Chinese populations, particularly the one from Anhui province, were possibly another species and warranted further examination. In the interim, they recommended that all mainland specimens be ascribed to *P. megacephalus*.

Zhao and Adler (1993) followed this suggestion, asserting that *P. leucomystax* does not occur in China and that all specimens were *P. megacephalus*. The implication of this was that the Indochinese specimens also belonged to *P. megacephalus* because the region is contiguous with and shares more of its fauna with China than with Borneo.

Subsequently, Trépanier et al. (1999) examined frog calls from two sites in northern Vietnam, Ba Be National Park and Pac Ban in Na Hang Nature Reserve, both within 125km of Yunnan province, China. The analyzed calls were closer in both dominant frequency and number of notes per call to the Bornean population of *P. leucomystax* than to the Taiwanese *P. megacephalus*.

Currently, at least two species are recognized: *P. leucomystax* and *P. megacephalus* (Frost, 2007; Matsui et al., 1986; Narins et al., 1998). Given the recent discoveries in other putative Southeast Asian species of anurans with similar distributions, such as the *Odorrana livida* complex (Bain, 1998; Bain et al., 2003; Stuart et al., 2006), the *Limnonectes limnocharis* complex (Emerson et al., 2000; Toda et al., 1998), *Microhyla ornata* (Matsui et al., 2005) and the *Rana chalconota* complex (Stuart et al., 2006), it is not unlikely that *P. leucomystax* is also a complex of cryptic species.

Acoustic analyses have revealed cryptic species within *P. leucomystax*. Narins et al. (1998) found two distinct types of mating calls in sympatry in Malaysia, which they were also able to tie to genetic and morphological characters implying sympatric species. Their evidence included nuclear genes (allozymes), call differences, and morphological characters. Unfortunately, the color pattern characters cannot serve to diagnose the species because they are ephemeral (Flower, 1896). However, the other morphological and acoustic characters are sufficient to diagnose the species at this site.

Several studies compare vocalization patterns of *P. leucomystax* to other sympatric species of frogs to characterize the areas acoustically (Heyer, 1971; Kuramoto, 1986; Márquez and Xavier, 2006; Matsui, 1982; Sanchez-Herraiz et al. 1995). However, except for the split of *P. leucomystax* and *P. megacephalus* by Matsui et al. (1986), these differences have not been tied to voucher specimens, genetic differentiation or taxonomic rearrangements.

Specimens of *P. leucomystax* and *P. megacephalus* from across their range in Vietnam were sequenced, extending the coverage provided by both Trépanier et al. (1999) and Matsui et al. (1986), as well as specimens from southwestern China. Because this approach more clearly delineated the boundaries of these species, it facilitated an investigation into possible cryptic species. The mtDNA tree elucidated patterns of maternal lineage dispersal and differentiation in the region.

In order to further delineate species in this complex, nuclear markers, such as microsatellites or anonymous nuclear loci, can be used to document gene flow or lack thereof. The broad range of these animals, the sympatric distribution of multiple lineages, the extent of morphological variation, and the likelihood that these are cryptic species make appropriate sampling logically and financially complicated. In order to insure that enough specimens of each lineage are sampled across the range several thousand animals would need to be collected in the absence of a clear framework such as the one this study provides. This record of the patterns of distribution of the different mitochondrial lineages will allow for a more directed sampling approach as well as providing a way to potentially separate animals in the field using a simple PCR technique, MAMA (Lindell and Murphy, 2007).

Materials and Methods

Specimens examined

In total, 172 individuals from the *P. leucomystax* complex from Southeast Asia were examined as ingroup taxa. The survey included 23 localities throughout Vietnam and southern China as well as specimens from 12 localities in Laos, Cambodia, Thailand, Sabah/Borneo, and the Philippines. The rhacophorid and pipid frogs *Buergeria buergeri* (AB127977) and *Xenopus laevis* (AY789013) served as outgroup taxa. Locality and voucher data for all specimens examined are presented in Table 5.1, and mapped in Figure 5.1. Tissue samples used for DNA analysis were either frozen or ethanol-preserved heart, skeletal muscle, or liver. Voucher specimens are preserved in the Royal Ontario Museum (ROM) except as indicated in Table 5.1.

DNA amplification and sequencing

Segments of the NADH subunit 1 (ND1) and cytochrome *b* genes (Cyt *b*) were selected to reconstruct the history of the mtDNA genome, which was assumed to reflect the phylogeny of species. Total genomic DNA was extracted from frozen or alcohol-preserved muscle or liver tissue samples by digestion with proteinase K for 7–12 hr, then purified three times with phenol–chloroform–isoamyl alcohol (PCI), and then once with chloroform–isoamyl alcohol (CI). Double-stranded DNA fragments were amplified using the polymerase chain reaction (PCR; 92°C for 30 sec, 47–53°C for 40 sec 72°C for 1.0 min) performed in 25µl reactions for 37 cycles. Annealing temperatures ranged from

47°C to 53°C as needed in order to improve primer binding and the quality of the PCR product. PCR reactions generally amplified the entire gene fragments from ND1-4863L to ND1-5800H for (ND1) and 16430L or MVZ15L to CytbB for Cyt *b* (Table 5.2). In the event that amplification of the larger fragment was not possible, additional combinations of primers were used, occasionally amplifying from the neighboring tRNA^{Glu} gene into the NADH subunit 1 gene region (Table 5.2). After amplification, a 2µl aliquot of PCR product was separated by electrophoresis on an agarose gel and stained with ethidium bromide to verify amplification and to determine relative DNA concentration. The remaining DNA was cleaned using QiaColumns (Qiagen) and eluted in Qiagen EB buffer. The cleaned double stranded DNA was sequenced directly using 8µl BigDye® (Applied Biosystems International, ABI) fluorescent cycle sequencing reactions (92°C for 30 sec, 47–53°C for 30 sec 72°C for 1.5 min). Sequencing product was ethanol precipitated and resuspended in Hi-Di® formamide (ABI). Resuspended DNA was then run out on either an ABI 377 automated sequencer using long plates or an ABI 3100 automated sequencer. Sequence data for the outgroup specimens *Buergeria* and *Xenopus*, as well as for the putative representative of *P. megacephalus* from Hong Kong were obtained from GenBank (Table 5.1).

DNA sequence alignment

Sequences were entered into BioEdit (ver. 7.0.5, Hall, 2005) for assembly and the identities of the specimens were confirmed using a BLASTn search (Altschul et al., 1990). They were then aligned using Clustal W (ver. 1.6, Thompson et al., 1994) and subsequently adjusted by eye.

Maximum parsimony evaluation

All characters were evaluated as unordered because there is no *a priori* reason to assume order of evolutionary change between nucleotide bases (Swofford et al., 1996). Maximum parsimony (MP) was used as the criterion for selecting among all possible trees. The MP analysis using PAUP* (ver. 4.0b10, Swofford, 2004) employed an heuristic search, with random addition sequence, 1000 replicates, retaining minimal trees only, using tree bisection–reconnection branch and collapsing zero length branches. This analysis was performed on a subset of the data corresponding to each gene as well as on the concatenated dataset for a total evidence analysis (Kluge, 1998). Nodal support was assessed via bootstrap proportions (BSP, Felsenstein, 1985) using 10,000 replicates and “Fast” stepwise addition calculated in PAUP*.

Bayesian inference evaluation

Bayesian inference (BI; Buckley et al., 2002; Huelsenbeck and Ronquist, 2001; Nylander et al., 2004) was also used to infer species relationships and maternal history within species. The data were partitioned by gene. The program MRMODELTEST (ver. 2.2, Nylander, 2004) was used to select an evolutionary model that best fit the data for each gene using the Akaike information criterion (Akaike, 1974, 1979). Hierarchical likelihood ratio tests (Goldman, 1993) were also implemented using MRMODELTEST v2.2, comparing the log–likelihood scores of the 24 evolutionary models that can be implemented in MrBayes for each gene.

The same model was selected by both criteria for both genes. BI was conducted implementing a general time reversal model with invariant sites and a gamma distribution using MrBayes (ver. 3.1.2, Huelsenbeck and Ronquist, 2001). The analysis was initiated with random starting trees, six Markov chains were used, five cold chains and one heated, and the dataset was run for 4.5×10^6 generations. Trees were sampled every 100 generations. Two independent analyses with different starting trees were run to avoid being trapped on local optima. The fluctuating likelihood values were graphically monitored (Huelsenbeck and Bollback, 2001) and it was determined that stationarity was achieved when the log-likelihood values of the sample points reached a stable equilibrium value (Huelsenbeck and Ronquist, 2001). After discarding 25% of the sampled trees as burn-in, the remaining trees were used to generate a 50% majority rule consensus tree. Nodal support, in the form of Bayesian posterior probabilities (BPP), was mapped on to the tree.

Results

One hundred and seventy one ingroup specimens were sequenced for ND1 and Cyt *b*. Sequences from another ingroup member and two outgroup specimens were obtained from GenBank. In total, 913 sites were sequenced for ND1 and 801 for Cyt *b* for a combined total of 1714 aligned sites. Among these sites, 818 were variable and 600 were potentially phylogenetically informative. All sequences will be deposited in GenBank.

Parsimony evaluation

The two genes were evaluated separately and combined. Analysis of ND1 based on 304 potentially informative sites yielded 9900 most parsimonious trees with a tree length of 849 steps (MPTs; Table 5.3) ($CI = 0.63$, $RI = 0.96$). Analysis of Cyt b, with 296 potentially informative sites, produced 62346 MPTs with a tree length of 910 steps. (Table 5.3; length = 910, $CI = 0.54$, $RI = 0.96$). The topologies of both the strict and the 50% majority rule consensus trees from each gene were congruent. Differences occurred at terminal branches within species. Thus, two mitochondrial genes were assumed to have evolved in similar ways and that this conciliation owed to genealogical history.

Analysis of the combined data yielded 40796 most parsimonious trees (1780 steps in length, $CI=0.59$, $RI=0.96$). A phylogram of the strict consensus of these is presented in Figure 5.2.

Branching patterns

The ingroup was resolved as monophyletic with respect to the outgroup taxa. Within the ingroup, two distinct groups were recovered: Clade A and Clade B (Figure 5.2). Within Clade A, two subclades were resolved (Clade A1 and Clade A2). Clade A1 was composed of specimens from Sa Pa on Fan Si Pan Mountain and Simao in southwestern Yunnan province, China. Clade A2 had two subclades. Clade A2a contained specimens from Diao Luo Shan and Wu Zhi Shan in Hainan Island, China. Clade A2b included specimens from southern and northern Vietnam, a Laotian specimen from Champasak, and Chinese animals from Chuangfou, Yunnan province.

Clade B contained two subclades. Clade B1 included specimens from Simao,

Nanxianhe, and Nanwenhe in Yunnan province, China, Quang Thanh in northeastern Vietnam, Phongsaly, Ventienne, Sayaboury, and Huaphahn in Laos, Siem Reap province in Cambodia, and Luzon Island in the Philippines. Clade B2 contained two subclades. Clade B2a contained specimens from Huaphahn and Khammouan (Laos), Myanmar, and a single specimen each from Cat Tien National Park (Vietnam) and Yunnan province (China). These were resolved as the sister group to Clade B2b.

Within Clade B2b, two distinctive clades were recovered: B2bi and B2bii. Clade B2bi contained a single specimen from Con Cuong, Vietnam, which was recovered as the sister group to specimens from Hong Kong, China. This clade also contained specimens from Chi Linh, Tam Dao National Park, and Ba Be National Park in northern Vietnam. Its sister group, Clade B2bii, was composed of two animals from Hong Kong and a clade of animals from Bawang Ling Nature Reserve and Haikou in Hainan Island, China. In turn, this Chinese clade was the sister group to a clade from Ngoc Linh mountain in the Central Highlands of Vietnam plus a group of specimens from Ventienne, Champasak, and Phongsaly provinces in Laos, Sabah on the island of Borneo, Thailand, the Philippines, Cat Tien National Park, Yok Don National Park, Krong Pa, and Tram Lap in southern Vietnam.

Bayesian inference evaluation

The BI analysis recovered a topology virtually identical to the MP analysis with the exception of two shifts and the resolution of terminal branches. Clade B2a, containing specimens from Laos, Yunnan, and Myanmar was resolved as the sister group to Clade B1 which also contained specimens from Laos and Yunnan. The Bayesian posterior

probability for this node was 56 in comparison to a BSP of 73 for the MP arrangement. There was also a difference of clade associations within Clade A2B. The clade containing five specimens from Con Cuong (33, 34, 35, 36, and 37) and a single specimen from Champasak, Laos (32) was resolved as the sister clade to the clade containing specimens from Chuangfou, Yunnan, rather than the clade of specimens from Tam Dao (27 and 28) and Chi Linh (30 and 31). Neither the MP nor the BI analysis had support values greater than 50% for this conflicting node.

Nodal stability

BSPs and BPPs are indicated on the strict consensus phylogram (Figure 5.2). BSPs supported 36 nodes with a consistency greater than 70%. Bayesian posterior probabilities indicated relatively high support for most nodes, 32 of which were supported by Bayesian posterior probabilities of 100%, including four that were not well supported by bootstrap proportions (BSP<70%). Nodal support for the nodes conflicting in the BI and MP analyses was generally low; only the differing node in Clade A had a BSP or BPP of above 70%.

Discussion

The clear delineation of monophyletic units within the *P. leucomystax* species complex is necessary to fully understand the potential threats to and impacts on these frogs. The perception that *P. leucomystax* is a widespread “junk” frog may lead to it being overlooked in ecological risk assessments or dismissed as not being in need of

protection due to its ubiquity. However, such sweeping assessments may be grievous errors. A stable phylogeny and taxonomy are critical to extrapolating recent discoveries on one population to new environmental circumstances or different populations. This extrapolation is necessary for effective management and conservation since different ecological traits and tendencies are often associated with different lineages through phylogenetic inertia (Brooks and McLennan, 2002; Funk et al., 2002).

Phylogeny and patterns

The phylogenetic assessment discovered interesting patterns, and the lack thereof. The two primary lineages were similar in their overall distributions, yet they also shared some common branching patterns. Clades A and B had a basal split involving specimens from Simao, Yunnan, a clade from Hainan Island and a clear separation between southern and northern Vietnamese localities, except for Sa Pa, Vietnam (Figures 5.2b and c). Clade A also involved specimens from Sa Pa and Clade B contained specimens from the Philippines, Laos, other Yunnan localities, and Quang Thanh in northwestern Vietnam (Figure 5.2b).

Notwithstanding the similarities, several differences occurred. Within Clade A, the lineage on Hainan Island was resolved as the sister group to the clade containing both northern and southern Vietnamese specimens (Clade A2b); in Clade B, Hainan Island fell out between the northern and southern Vietnamese clades (Clade B2bi and the remaining members of Clade B2bii). Another key difference between the two clades was the prevalence of Laotian, Philippine, Bornean, and Thai localities across Clade B. This increased geographical breadth was distributed throughout subclades B1 and B2 rather than being restricted to any particular subclade. The relatively low level of sequence

differentiation of the majority of these specimens suggests a relatively recent dispersal. In support of this scenario, *P. leucomystax* (*sensu lato*) has been introduced to Japan and Western Papua New Guinea in recent times (Maeda and Matsui, 1999), and a specimen has been found hitchhiking on an airplane to Guam (Christy et al., 2007; Wiles, 2000). It is possible that Clade B corresponds to a lineage typified by increased vagility or a preference for disturbed habitat and, therefore, an increased likelihood of human-mediated translocation. This hypothesis is supported by the occurrence of specimens across Laos in the Me Kong River drainage. Here, there is a continuous corridor and the Troung Son mountain range cannot restrict gene flow. This hypothesis could be tested by sampling broadly across the range of these animals while using rapidly evolving nuclear gene markers to assess gene flow via population structure assessments (e.g. STRUCTURE, Pritchard et al., 2000; BAPS, Corander et al., 2004; PARTITION, Dawson and Belkhir, 2001) and assignment tests (WHICHRUN, Banks and Eichert, 2000).

One possible explanation for the similarities in branching patterns between Clades A and B is the perpetuation of ancestral polymorphism combined with differential extinction. However, several lines of evidence preclude this explanation. In terms of branching patterns, if the genetic variation was ancestral and represented only variation within breeding units, then identical branching patterns in Clade A and Clade B would be expected. The sequence of isolation events would be expected to have mirrored one another. In contrast, the geographic histories of Clade A and Clade B are largely independent. Also, given the acoustic data, this finding is best explained by the presence of cryptic species.

Sympatric maternal lineages

Not only were two major lineages recovered in this analysis, they often occurred in sympatry. Populations of “*P. leucomystax*” contained descendants of two lineages at 15 localities (Table 5.4). Curiously, no site contained representatives from more than two distinctive lineages. Hong Kong, Laos (Phongsaly and Ventienne) and the Philippines contained representatives from the major Clade B (Table 5.4). No locality contained sympatric species belonging to Clade A. Each of the sympatric lineages at Quang Thanh, Hainan Island, Hong Kong, and the Philippines had different geographic associations. For example, specimens from one lineage (Clade A) in Quang Thanh had a sister group relationship with northern Vietnamese specimens (Clade A2N) while sympatric specimens from Clade B had a sister group relationship with Yunnan, Laos, Cambodia and the Philippines (Clade B1). Similarly, specimens from Hong Kong were resolved in two separate places within Clade B, with Hainan Island (Hainan Clade B) and with northern Vietnamese specimens (Clade B2bi).

Taxonomic implications

The genealogical analysis revealed two very distinctive maternal lineages within what is currently recognized as *P. leucomystax*. The long internodal branch lengths and short terminal branches further distinguish ten unique groups (Figure 5.2; Table 5.5). The deep levels of cladogenic divergence combined with different sequences of cladogenic events, and the geographic distribution of these groups, suggests that *P. leucomystax* is a complex of cryptic species. A list of these undescribed species, labeled *Polypedates sp.* a-

j, along with clade names and localities is presented in Table 5.5.

Support for the recognition of these lineages as species is bolstered by acoustic evidence for multiple species within the group. Narins et al. (1998) found evidence for two sympatric species of *Polypedates* in peninsular Malaysia. These may correspond to one or more of the lineages recovered herein. Unfortunately, mtDNA samples are not available for the animals evaluated by Narins et al. and thus cannot be added to this assignment. However, the existence of only two sympatric lineages implies that a similar situation likely occurs in other parts of the range. Behavioral and morphological characters that will easily diagnose these cryptic species remain to be identified.

The description of species is complicated by problems of identifying *P. leucomystax* and *P. megacephalus*. Call analyses are not available from the type locality in Java, and given that two species commonly co-occur sympatrically, it is possible that Java, like Hong Kong, may have multiple species. The identification of *P. megacephalus* is even more problematic. Pope (1931) recognized *P. l. megacephalus* as a subspecies, but later decided that it could not be diagnosed as different from specimens on Java, the type locality for *P. leucomystax*. Consequently, he reduced it to synonymy. Pope's (1931) confusion may have owed to the presence of two lineages in Hong Kong, one of which is more closely related to some individuals from Borneo and the Philippines. His assessment of variation within the complex involved at least two cryptic species.

Hallowell (1861) described *P. megacephalus* based on a single specimen from Hong Kong. Unfortunately, the description is meager, the type specimen has since been lost and no neotype has been designated (Frost, 2007). Both Clade A and Clade B occur in Hong Kong making the identity of *P. megacephalus* unknowable. One specimen from

Na Hang Nature Reserve, (ROM 6514) Vietnam was diagnosed by Trépanier et al. (2000) as *P. leucomystax* on the basis of having similar mating calls to a putative population of *P. leucomystax* from Borneo. This designation is tentative for two reasons. First, the Bornean specimens belong to a different lineage than Trépanier et al.'s specimen (Clade B2bii versus B2Bi). Second, it is possible that representatives of both Clade A and Clade B occur in Java. Regardless, it will be necessary to designate a neotype for *P. megacephalus* in order to maintain taxonomic stability.

Biogeography

The reciprocal exclusivity of localities such as Diao Lo Shan and Bawang Ling Nature Reserve in Hainan Island suggest multiple colonizations of the island by different lineages. Despite the fact that the localities containing the sister groups to the two clades on Hainan Island contain both lineages in sympatry neither Diao Lo Shan nor Bawang Ling Nature Reserve has both. This exclusivity requires that either there was reciprocal extinction at both sites or that, by chance, both groups of populations just happened to be founded by individuals of the opposite lineage.

These differences in lineage distribution are significant because they require either separate invasions of each locality or complicated patterns of lineage sorting. The more parsimonious explanation of separate invasions serves as evidence that the different lineages had different evolutionary and biogeographical histories with different patterns of dispersal and vicariance.

Stuart et al. (2006) raised the question of whether widespread amphibian species exist in Southeast Asia. Detailed evaluations have revealed that most widespread species

of anurans are complexes of cryptic species. These assessments owe more to recent advances in the ability to detect morphologically similar species than to a lack of widespread species, a point made particularly clear in the case of the *P. leucomystax* species group. While the group is several distinct lineages or cryptic species, several of these lineages, particularly within Clade B, remain geographically widespread, some ranging from Thailand and Laos through Vietnam to Borneo and the Philippines (Figure 5.2c; B1, *P. sp. d*; B2a, *P. sp. e*; B2bii, *P. sp. i*). While it is possible that additional sampling will lead to further splitting of these lineages, the low number of changes separating the different haplotypes within these groups suggests otherwise.

The clear separation of lineages in northern and southern Vietnam may be ascribed to the physiographic separation of the two regions. The intervening Vietnamese Central Highlands are not part of either the Red River drainage system, which connects the localities in northern Vietnam, or the Me Kong River drainage system, which drains the southern localities. The Central Highlands drain directly into the South China Sea by several, distinct, smaller river systems. This drainage pattern effectively separates the north from the south, and likely also serves to isolate the populations of amphibians occurring there. If true, then more extensive sampling in the Vietnamese Central Highlands would likely reveal additional cryptic species.

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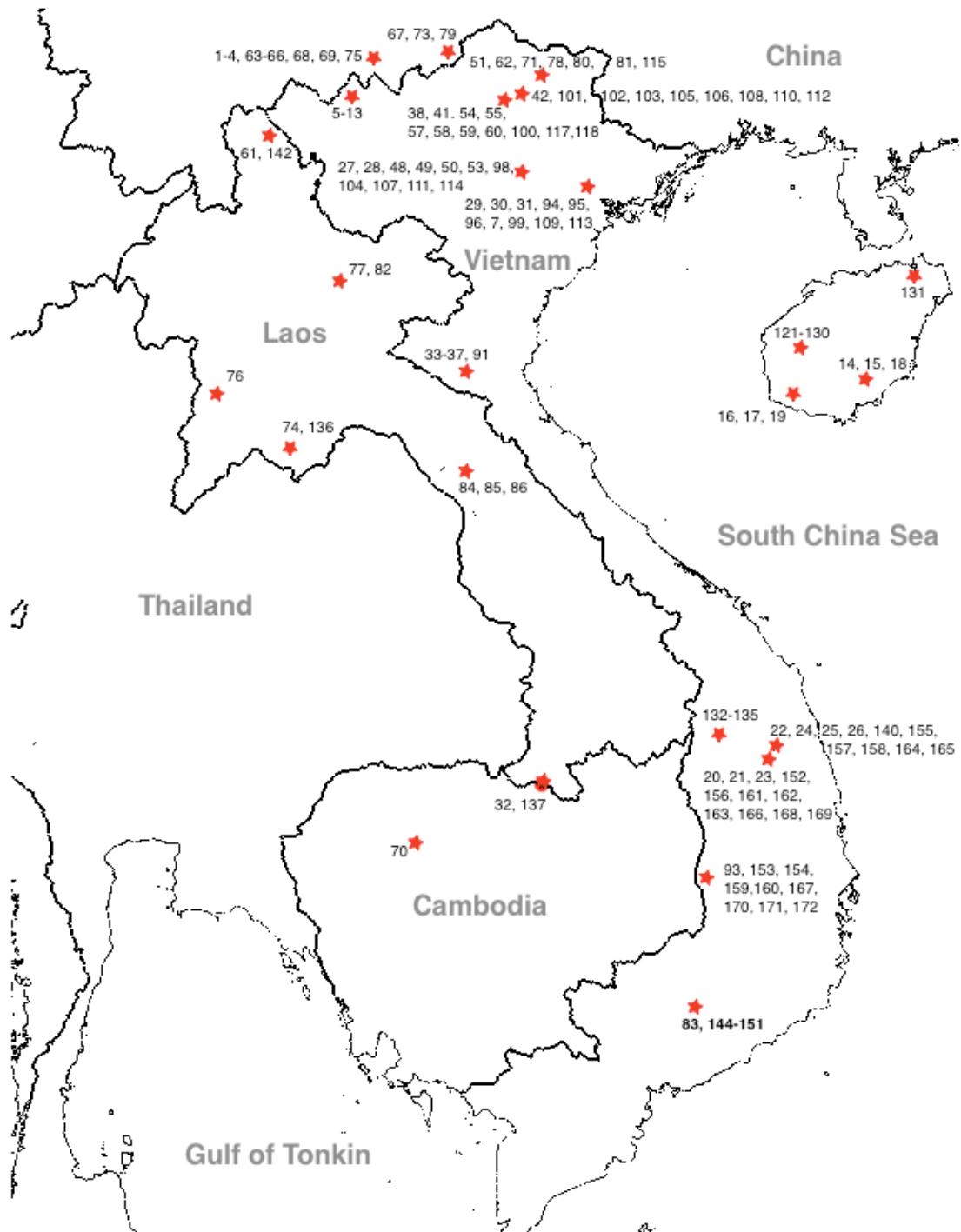
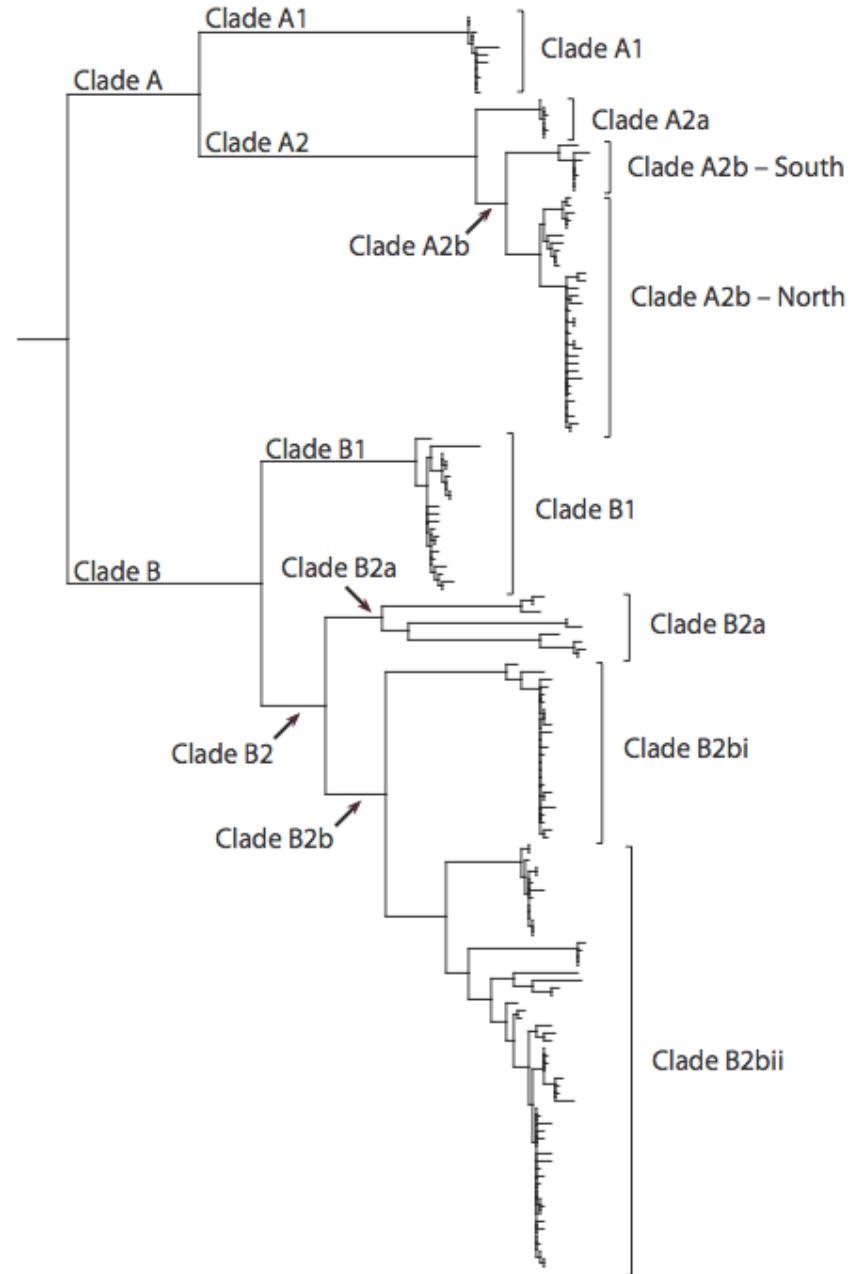


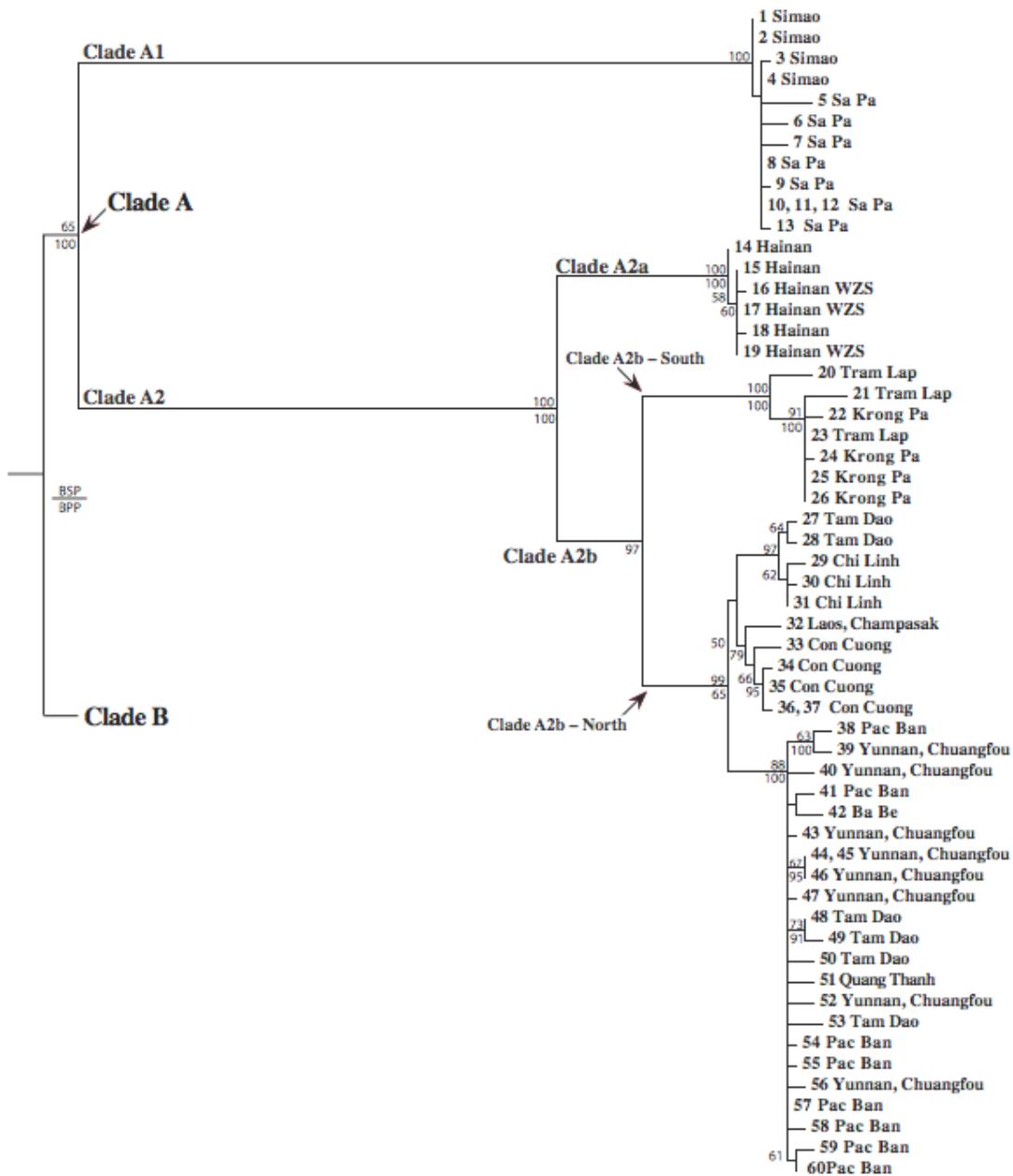
Figure 5.1. Map of Indochinese specimen localities. Numbers refer to the specimens in Table 5.1.

Figure 5.2. **a)** Phylogram of the relationships of ingroup specimens labeled with the clade names used in the text and figures. **b)** Phylogram of the relationships of the specimens in Clade A. Bootstrap proportions above 50% are placed above the branches and Bayesian posterior probabilities above 50% are below. **c)** Phylogram of the relationships of the specimens in Clade B. Bootstrap proportions above 50% are placed above the branches and Bayesian posterior probabilities above 50% are below.

Figure 2a. Clade names used in the text and figures.



b)



c)

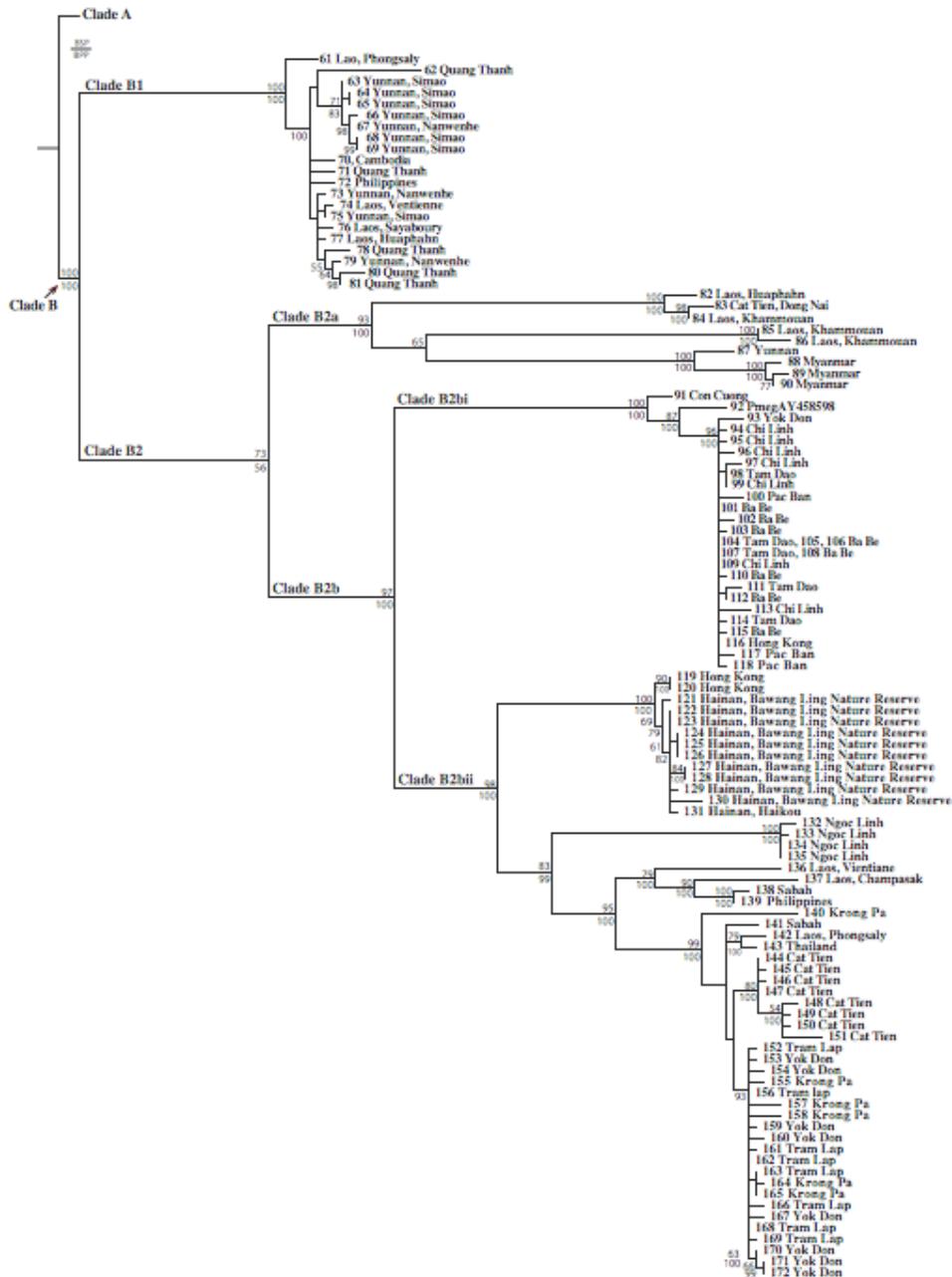


Table 5.1. Specimens used, voucher specimen catalog numbers, and collecting locality.

Sequences for specimens marked with a dagger (†) were obtained from GenBank.

Specimen No.	Country	Province	Locality	Voucher No.
1	China	Yunnan	12.3 km SE Simao	ROM 35914
2	China	Yunnan	12.3 km SE Simao	ROM 35953
3	China	Yunnan	12.3 km SE Simao	ROM 35915
4	China	Yunnan	12.3 km SE Simao	ROM 35949
5	Vietnam	Lao Cai	Sa Pa Vicinity	ROM 28323
6	Vietnam	Lao Cai	Sa Pa Vicinity	ROM 30860
7	Vietnam	Lao Cai	Sa Pa Vicinity	ROM 30843
8	Vietnam	Lao Cai	Sa Pa Vicinity	ROM 30668
9	Vietnam	Lao Cai	Sa Pa Vicinity	ROM 28151
10	Vietnam	Lao Cai	Sa Pa Vicinity	ROM 28090

11	Vietnam	Lao Cai	Sa Pa Vicinity	ROM 28109
12	Vietnam	Lao Cai	Sa Pa Vicinity	ROM 28254
13	Vietnam	Lao Cai	Sa Pa Vicinity	ROM 30407
14	China	Hainan	Diao Luo Shan Forest Park	IZOCAS
15	China	Hainan	Diao Luo Shan Forest Park	
16	China	Hainan	Wu Zhi Shan	
17	China	Hainan	Wu Zhi Shan	
18	China	Hainan	Diao Luo Shan Forest Park	
19	China	Hainan	Wu Zhi Shan	
20	Vietnam	Gia Lai	Tram Lap	ROM 7606
21	Vietnam	Gia Lai	Tram Lap	ROM 7661
22	Vietnam	Gia Lai	Krong Pa	ROM 23194

23	Vietnam	Gia Lai	Tram Lap	ROM 7660
24	Vietnam	Gia Lai	Krong Pa	ROM 23193
25	Vietnam	Gia Lai	Krong Pa	ROM 23195
26	Vietnam	Gia Lai	Krong Pa	ROM 23196
27	Vietnam	Vinh Phu	Tam Dao National Park	ROM 18039
28	Vietnam	Vinh Phu	Tam Dao National Park	ROM 14851
29	Vietnam	Hia Duong	Chi Linh	ROM 25103
30	Vietnam	Hia Duong	Chi Linh	ROM 24275
31	Vietnam	Hia Duong	Chi Linh	ROM 26101
32	Laos	Champasak Province	Khong District	FMNH 257891
33	Vietnam	Nghe An	24 km W of Con Cuong	ROM 14677
34	Vietnam	Nghe An	24 km W of Con Cuong	ROM 14674

35	Vietnam	Nghe An	24 km W of Con Cuong	ROM 14629
36	Vietnam	Nghe An	24 km W of Con Cuong	ROM 14675
37	Vietnam	Nghe An	24 km W of Con Cuong	ROM 14676
38	Vietnam	Tuyen Quang	Pac Ban, Na Hang Nature Reserve	ROM 6744
39	China	Yunnan	5 km NW Chuangfou	ROM 35511
40	China	Yunnan	5 km NW Chuangfou	ROM 35507
41	Vietnam	Tuyen Quang	Pac Ban, Na Hang Nature Reserve	ROM 7012
42	Vietnam	Cao Bang	Ba Be National Park	ROM 19814
43	China	Yunnan	5 km NW Chuangfou	ROM 35523
44	China	Yunnan	5 km NW Chuangfou	ROM 35508
45	China	Yunnan	5 km NW Chuangfou	ROM 35509
46	China	Yunnan	5 km NW Chuangfou	ROM 35525

47	China	Yunnan	5 km NW Chuangfou	ROM 35526
48	Vietnam	Vinh Phu	Tam Dao National Park	ROM 14833
49	Vietnam	Vinh Phu	Tam Dao National Park	ROM 16188
50	Vietnam	Vinh Phu	Tam Dao National Park	ROM 14834
51	Vietnam	Cao Bang	Quang Thanh	ROM 26162
52	China	Yunnan	5 km NW Chuangfou	ROM 35510
53	Vietnam	Vinh Phu	Tam Dao National Park	ROM 16633
54	Vietnam	Tuyen Quang	Pac Ban, Na Hang Nature Reserve	ROM 6510
55	Vietnam	Tuyen Quang	Pac Ban, Na Hang Nature Reserve	ROM 6726
56	China	Yunnan	5 km NW Chuangfou	ROM 35527
57	Vietnam	Tuyen Quang	Pac Ban, Na Hang Nature Reserve	ROM 6523
58	Vietnam	Tuyen Quang	Pac Ban, Na Hang Nature Reserve	ROM 6741

59	Vietnam	Tuyen Quang	Pac Ban, Na Hang Nature Reserve	ROM 7010
60	Vietnam	Cao Bang	Ba Be National Park	ROM 19739
61	Laos	Phongsaly Province	Phongsaly District	FMNH 257885
62	Vietnam	Cao Bang	Quang Thanh	ROM 26160
63	China	Yunnan	8.8 km W Simao	ROM 35706
64	China	Yunnan	8.8 km W Simao	ROM 35704
65	China	Yunnan	8.8 km W Simao	ROM 35707
66	China	Yunnan	12 km SSW Simao	ROM 35973
67	China	Yunnan	Nanxianhe	ROM 35474
68	China	Yunnan	12 km SSW Simao	ROM 35971
69	China	Yunnan	12 km SSW Simao	ROM 35972
70	Cambodia	Siem Reap Province	Siem Reap District	FMNH 257341

71	Vietnam	Cao Bang	Quang Thanh	ROM 26165
72	Philippines	Luzon	Kalinga Province	FMNH 259464
73	China	Yunnan	5 km N Nanwenhe	ROM 35578
74	Laos	Vientiane Municipalit y	Kasi District	FMNH 257883
75	China	Yunnan	8.8 km W Simao	ROM 35705
76	Laos	Sayaboury Province	Phiang District	FMNH 257884
77	Laos	Huaphahn Province	Vieng Tong District	FMNH 255297
78	Vietnam	Cao Bang	Quang Thanh	ROM 26166
79	China	Yunnan	5 km N Nanwenhe	ROM 35579
80	Vietnam	Cao Bang	Quang Thanh	ROM 26161
82	Laos	Huaphahn Province	Vieng Tong District	FMNH 255308
82	Vietnam	Cao Bang	Quang Thanh	ROM 26167

83	Vietnam	Dong Nai	Cat Tien National Park Headquarters	FMNH 261687
84	Laos	Bolikhampasay Province	Khamkeut District	FMNH 254649
85	Laos	Khammouan Province	Boualapha District	FMNH 255292
86	Laos	Khammouan Province	Boualapha District	FMNH 255296
87	China	Yunnan		ROM 35640
88	Myanmar			USNM 520536
89	Myanmar			JBS 6448
90	Myanmar			USNM 520535
91	Vietnam	Nghe An	24 km W of Con Cuong	ROM 13339
92[†]	China	Hong Kong		AY458598
93	Vietnam	Dak Lak	Yok Don National Park	ROM 23086
94	Vietnam	Hia Duong	Chi Linh	ROM 25019

95	Vietnam	Hia Duong	Chi Linh	ROM 25319
96	Vietnam	Hia Duong	Chi Linh	ROM 24194
97	Vietnam	Hia Duong	Chi Linh	ROM 25905
98	Vietnam	Hia Duong	Chi Linh	ROM 24231
99	Vietnam	Vinh Phu	Tam Dao National Park	ROM 16703
100	Vietnam	Tuyen Quang	Pac Ban, Na Hang Nature Reserve	ROM 6512
101	Vietnam	Cao Bang	Ba Be National Park	ROM 19586
102	Vietnam	Cao Bang	Ba Be National Park	ROM 19259
103	Vietnam	Cao Bang	Ba Be National Park	ROM 19179
104	Vietnam	Vinh Phu	Tam Dao National Park	ROM 16558
105	Vietnam	Cao Bang	Ba Be National Park	ROM 19260
106	Vietnam	Cao Bang	Ba Be National Park	ROM 19632

107	Vietnam	Vinh Phu	Tam Dao National Park	ROM 16542
108	Vietnam	Cao Bang	Ba Be National Park	ROM 19178
109	Vietnam	Hia Duong	Chi Linh	ROM 25022
110	Vietnam	Cao Bang	Ba Be National Park	ROM 19156
111	Vietnam	Vinh Phu	Tam Dao National Park	ROM 16704
112	Vietnam	Cao Bang	Ba Be National Park	ROM 19155
113	Vietnam	Hia Duong	Chi Linh	ROM 25021
114	Vietnam	Vinh Phu	Tam Dao National Park	ROM 14852
115	Vietnam	Cao Bang	Ba Be National Park	ROM 19191
116	China	Hong Kong	Hong Kong	ROM 16749
117	Vietnam	Tuyen Quang	Pac Ban, Na Hang Nature Reserve	ROM 6511
118	Vietnam	Tuyen Quang	Pac Ban, Na Hang Nature Reserve	ROM 6514

119	China	Hong Kong	Hong Kong	ROM 16303
120	China	Hong Kong	Hong Kong	ROM 16304
121	China	Hainan Island	Bawang Ling Nature Reserve	IOZCAS 1134
122	China	Hainan Island	Bawang Ling Nature Reserve	IOZCAS 1124
123	China	Hainan Island	Bawang Ling Nature Reserve	IOZCAS 1125
124	China	Hainan Island	Bawang Ling Nature Reserve	IOZCAS 1129
125	China	Hainan Island	Bawang Ling Nature Reserve	IOZCAS 1123
126	China	Hainan Island	Bawang Ling Nature Reserve	IOZCAS 1126
127	China	Hainan Island	Bawang Ling Nature Reserve	IOZCAS 1127
128	China	Hainan Island	Bawang Ling Nature Reserve	IOZCAS 1132
129	China	Hainan Island	Bawang Ling Nature Reserve	IOZCAS 1133
130	China	Hainan Island	Bawang Ling Nature Reserve	IOZCAS 1128

131	China	Hainan Island	Haikou	IOZCAS 1143
132	Vietnam	Kon Tum	Ngoc Linh	ROM 27428
133	Vietnam	Kon Tum	Ngoc Linh	ROM 27429
134	Vietnam	Kon Tum	Ngoc Linh	ROM 27471
135	Vietnam	Kon Tum	Ngoc Linh	ROM 27526
136	Laos	Vientiane Municipalit y	Kasi Dist	FMNH 257882
137	Cambodia	Siem Reap Province	Siem Reap Dist	FMNH 257340
138	Malaysia	Sabah	Tenom Dist	FMNH 239162
139	Philippines	Luzon	Kalinga Prov.	FMNH 259463
140	Vietnam	Gia Lai	Krong Pa	ROM 23201
141	Malaysia	Sabah	Tenom Dist	FMNH 239159
142	Laos	Phongsaly Province	Phongsaly District	FMNH 258462

143	Thailand			FMNH 257935
144	Vietnam	Dong Nai	Cat Tien National Park	ROM 24963
145	Vietnam	Dong Nai	Cat Tien National Park	ROM 24913
146	Vietnam	Dong Nai	Cat Tien National Park	ROM 24815
147	Vietnam	Dong Nai	Cat Tien National Park	ROM 24932
148	Vietnam	Dong Nai	Cat Tien National Park	ROM 24927
149	Vietnam	Dong Nai	Cat Tien National Park	ROM 24905
150	Vietnam	Dong Nai	Cat Tien National Park	ROM 24821
151	Vietnam	Dong Nai	Cat Tien National Park	ROM 24926
152	Vietnam	Gia Lai	Tram Lap	ROM 7120
153	Vietnam	Dak Lak	Yok Don National Park	ROM 22890
154	Vietnam	Dak Lak	Yok Don National Park	ROM 22300

155	Vietnam	Gia Lai	Krong Pa	ROM 23200
156	Vietnam	Gia Lai	Tram Lap	ROM 7118
158	Vietnam	Gia Lai	Krong Pa	ROM 23203
160	Vietnam	Dak Lak	Yok Don National Park	ROM 22036
161	Vietnam	Gia Lai	Tram Lap	ROM 7179
162	Vietnam	Gia Lai	Tram Lap	ROM 7122
163	Vietnam	Gia Lai	Tram Lap	ROM 7238
164	Vietnam	Gia Lai	Krong Pa	ROM 23204
165	Vietnam	Gia Lai	Krong Pa	ROM 23202
166	Vietnam	Gia Lai	Tram Lap	ROM 7119
167	Vietnam	Dak Lak	Yok Don National Park	ROM 23087
167	Vietnam	Gia Lai	Krong Pa	ROM 23688

168	Vietnam	Gia Lai	Tram Lap	ROM 7178
169	Vietnam	Gia Lai	Tram Lap	ROM 7121
170	Vietnam	Dak Lak	Yok Don National Park	ROM 23108
171	Vietnam	Dak Lak	Yok Don National Park	ROM 22852
172	Vietnam	Dak Lak	Yok Don National Park	ROM 23109
173	Vietnam	Dong Nai	Cat Tien National Park	ROM 24816
174[†]	N/A	N/A	(Outgroup <i>Xenopus laevis</i>)	GenBank
175[†]	N/A	N/A	(Outgroup <i>Buergeria sp</i>)	GenBank

Table 5.2. Primers used for amplifying and sequencing fragments of the Cytochrome b and ND1 genes in this study. Sequence position indicates the starting position of the primer on the *Xenopus laevis* mitochondrial genome and is preceded by the direction of amplification (H=heavy / L=light strand).

Name	Sequence 5'-3'	Sequence position	Reference
MVZ15L	GAACTAATGGCCCACACWWTACGNAA	L	Graybeal 1999
GLU-cytb	TATTCAACTACAAAAACCTTATG	L	This study
B2L	TGAGGACAAATATCCTTCTGAGG	L16676	J.P. Dumbacher et al. 2003
Cytb B	CTTCTACTGGTTGTCCCTCCGATTCA	H17257	Bossuyt and Milinkovitch 2000
Cytb C	CTACTGGTTGTCCCTCCGATTCATG	H	Bossuyt and Milinkovitch 2000
Cytb-16430L	CACTACACAGCAGACACATC	L16430	This study
Cytb-17071H	TGCATAGGCAAAGAGGAA	H17071	This study
ND1-4861L	T GCC CCA ATT CTG CTT GC	L4861	This study
ND1-4967L	CAACCAATTGCAGACG	L4967	This study
ND1-5800H	CATATTATCCTCCCTATCA	H5800	This study
NDH-D	GGTATGGGCCAAAAGCT T	H	Hoegg et al.

Table 5.3. Summary of genes sequenced from the ingroup and outgroup taxa. TS = total number of homologous sites resolved; NVS = number of variable sites; NPPIS = number of potentially phylogenetically informative sites; NMPTs = number of most parsimonious trees resolved; LMPTs = length of most parsimonious solution excluding uninformative characters; CI = consistency index; RI = retention index; RC = rescaled consistency index.

Gene	TS	NVS	NPPIS	NMPTs	LMPTs	CI	RI	RC
NADH subunit 1	913	437 (47.9%)	304 (32.9%)	9900	849	0.63	0.96	0.60
Cytochrome <i>b</i>	801	381 (47.6%)	296 (37.0%)	62346	910	0.54	0.96	0.51
Combined	1714	818 (52.6%)	600 (38.4%)	40796	1780	0.59	0.96	0.56

Table 5.4. Localities containing sympatric representatives of distinct lineages and the clades represented.

Localities with both Clade A and B

Country	Province / region	Locality	Clades present
China			
	Yunnan	Simao	A1, B1
Vietnam			
	Northern Vietnam	Ba Be	A2bN, B2bi
		Pac Ban	A2bN, B2bi
		Con Cuong	A2bN, B2bi
		Tam Dao	A2bN, B2bi
		Chi Linh	A2bN, B2bi
		Quang Thanh	A2bN, B1
	Southern Vietnam	Krong Pa	A2bS, B2bii
		Tram Lap	A2bS, B2bii
Laos			
	Champasak		A2bN, B2bii

Localities with more than one lineage from Clade B

China			
	Hong Kong	Hong Kong	B2bi, B2bii
Laos			
	Phongsaly		B1, B2bii
	Ventienne		B1, B2bii
Philippines			
	Luzon		B1, B2bii

Table 5.5. Unnamed potential species of the *Polypedates leucomystax* species complex.

Species a-j	Clade and Comments
<i>Polypedates sp. a</i>	A1
<i>Polypedates sp. b</i>	A2a
<i>Polypedates sp. c</i>	A2b
<i>Polypedates sp. d</i>	B1
<i>Polypedates sp. e</i>	B2a
<i>Polypedates sp. f/j</i>	B2bi (This may be 2 species if the Con Cuong population is found to be distinct.)
<i>Polypedates sp. g</i>	B2bii Hong Kong and Hainan
<i>Polypedates sp. h</i>	B2bii Ngoc Linh
<i>Polypedates sp. i</i>	B2bii remaining areas

Chapter 6

Summary

Introduction

In this chapter I summarize and synthesize the main findings of my doctoral research. When combined, the individual studies allow for the search for common patterns of genetic differentiation. They provide insight into the effects of major geographic features such as river systems, watersheds, and mountain ranges, on the biogeography of Indochina in general, as well as that of Vietnam in particular.

The significance of this dissertation falls into three primary areas: the effects of river systems and mountain ranges, the existence of widespread forest species, and common patterns. I discuss my results in the context of current research, and suggest some opportunities for further investigations.

Geographic Barriers and Conduits

The complex topology of Vietnam, with mountain ridges separated by valleys and river systems, highland plateaus, and broad deltas, predicts complicated patterns of relationships among its flora and fauna. Wallace (1852) noted that the faunas on opposite

sides of major rivers often differed and he postulated that the rivers somehow acted as barriers to dispersal. This observation has since been developed into the riverine barrier hypothesis, which states that rivers impede gene flow between populations on opposing banks thereby increasing the possibility of allopatric speciation (Capparella, 1987; 1991; Ressem and Parker, 1983; Salo et al., 1986; Sick, 1967). Further study and the application of genetic techniques have revealed similar patterns around major Neotropical rivers such as the Amazon, Negro, and Madiera rivers (Ayers et al., 1992; Capparella, 1987; 1991; Sick, 1967). However, there have been notable exceptions. Hass and Hedges (1991) and Gascon et al. (1996, 1998) used morphology and allozymes to study groups of amphibians separated by the Jurua River and found no differences between the populations.

While larger rivers, such as the Amazon and the Rio Negro, may impede gene flow across them by virtue of their size, not all river systems may have that effect on all species. Because rivers flow downhill, often draining broad areas, potentially including multiple hills and mountains, they may serve as conduits to gene flow along their length. In this scenario, rivers could connect populations that are bounded by other geographical features, such as mountain ridges. This alternative hypothesis also leads to testable predictions.

Reciprocal monophyly of populations on either side of rivers and mountain ridges would be expected if these geographic features serve as barriers to dispersal and gene flow. However, if river systems serve as conduits for migration, and thus gene flow, then populations along their length should be more closely related to each other than to populations in nearby drainage systems. Using mtDNA genealogical patterns as a

surrogate for dispersal and gene flow, several tests of these alternative hypotheses occur in each chapter of this dissertation. The inclusion of five species of *Microhyla* (*Mh.*), one currently recognized species of *Micryletta* (*Ml.*), and the widely ranging *Polypedates leucomystax* complex, serve to test the isolation and connectivity effects of the mountain ranges and river systems on frogs in Vietnam.

Rivers as Conduits

Broad sampling throughout Vietnam allows for the comparison of seven river drainages as conduits of gene flow. The potential conduits involve the following riverine systems: 1) Ky Cung / Bay Giang river drainage, 2) Red River drainage, 3) Thai Binh River drainage, 4) Ca River drainage, 5) Thu Bon River drainage, 6) Dong Nai River drainage, and 7) Mekong River drainage. In general, populations in each of these river drainages are monophyletic lineages, even when some populations are geographically closer to populations in other river drainages. However, specimens from two localities form notable exceptions to this pattern: Quang Thanh and Sa Pa. While Quang Thanh is drained by the Ky Cung / Bay Giang river system, its specimens of *Mh. butleri*, *Mh. heymonsi*, and *Mh. pulchra* are resolved among frogs in the Thai Binh and Red Rivers. This discovery is not repeated in all anurans. For example, specimens of the *P. leucomystax* complex from Quang Thanh are not resolved with Vietnamese populations in the Red River drainage. Similarly, specimens of the *P. leucomystax* complex from Sa Pa, in the Red River drainage of Vietnam fall out with animals from Simao, China rather than with populations along the same drainage, as seen the species of *Microhyla*.

The Red River and the Thai Binh River systems form a complex network of

waterways that anastomose and drain together in a broad delta. Among the species of *Microhyla* examined, including *Mh. butleri*, *Mh. fissipes*, *Mh. heymonsi*, and *Mh. pulchra*, each serves to test the hypothesis that this system acts as a conduit for gene flow. Gene flow along a river connects populations along its length to each other more than to those in other systems. Again, some lineages of *Mh. butleri*, *Mh. heymonsi*, and *Mh. pulchra* from the Red River drainage are more closely related to those from the Ky Cung / Bay Giang river system than they are to other specimens in their own drainage. While populations in the Red River are resolved as the monophyletic sister group to the sole representative of the Thai Binh river drainage in *Mh. fissipes*, no specimens of this species were available from the Ky Cung / Bay Giang river system. In *P. leucomystax*, specimens from the Red River and Thai Binh River drainages are resolved as a monophyletic group in Clade B. However, the picture is more complicated in Clade A (Figure 5.2). In Clade A, specimens from Sa Pa associate with those from Simao, China, and clade containing specimens from Con Cuong separates the remaining northeastern Vietnamese specimens into two clades.

Populations from Con Cuong and Vinh occur in the Ca River system. *Microhyla fissipes*, *Mh. pulchra*, and Clade A and Clade B of the *P. leucomystax* complex contains specimens from this drainage. Only *Mh. fissipes* includes animals from both Con Cuong and Vinh, both on opposite banks of the Ca River. This pattern provides a powerful test of the effects of this river on dispersal. For all species examined, populations along this drainage were resolved as monophyletic in each group examined suggesting that the Ca river does act as a conduit for dispersal and thus gene flow for these species.

In the central region of Vietnam, along the Thu Bon River drainage, all specimens

of *P. leucomystax* from Ngoc Linh fell out together in Clade B. These specimens are separated from the other Vietnamese specimens by long branches and intervening clades from China, Laos, Borneo, and the Philippines. No specimens from this river drainage were available for inclusion in any of the *Microhyla* or *Micryletta* analyses, thus no multi-species comparisons were possible.

Further south, the picture is slightly more complicated. Two separate river drainages, the Mekong River drainage and the Ba River drainage, are examined. The Mekong River drainage is of particular note because the southern Vietnamese populations along this river are not resolved with the upstream Laotian populations. Rather, these Vietnamese specimens are associated with other southern Vietnamese populations along the Ba River drainage. Populations in the Mekong and Ba River drainages are resolved as reciprocally monophyletic only in *Ml. inornata*. Populations along these two drainages are not consistently resolved as reciprocally monophyletic in the other species; in all other cases, either 1) only one of these two drainages is represented (*Mh. butleri*, *Mh. pulchra*, and Clade A of the *P. leucomystax* complex), 2) they are resolved together, but relationships among the populations are uncertain (*Mh. fissipes*), or 3) they are resolved together, but not as reciprocally monophyletic groups (*Mh. heymonsi*, *Mh. berdmorei*, and Clade B of the *P. leucomystax* complex). The analysis of *Mh. heymonsi* is of further note. Specimens from both the Mekong and Ba River drainages are resolved together in two separate places on the tree. One of these clades, however, forms the sister group to the remaining specimens of *Mh. heymonsi* and is separated by a relatively high sequence divergence. This discovery indicates a possible cryptic species.

Rivers as Barriers

The question of whether or not rivers serve as barriers to dispersal, and therefore gene flow, is tested herein on many levels. In northeastern Vietnam, the Red River drainage system is comprised of several large tributaries (e.g., the Chay, Da, Gam, and Lo rivers), all of which join the Red River at various points along its length. These rivers, combined with those comprising the Thai Binh River drainage with which it anastomoses, serve to separate the different collecting sites. This pattern of isolation provides a series of tests along a range of river sizes and taxa.

Along the Red River drainage, the *P. leucomystax* complex and several species of *Microhyla* serve to test the barrier effects of rivers. Individual haplotypes in the *P. leucomystax* complex from northeastern Vietnam are not found to be more closely related to others from their own collection sites but rather are mixed. This discovery implies that the tributaries of the Red River do not act as barriers to dispersal. In most cases, the lineages mix across the tributaries. There were two exceptions to this generality. Specimens in the *P. leucomystax* complex from Sa Pa are resolved with those from Simao, Yunnan, rather than ones from Ba Be National Park, Na Hang Nature Reserve, Tam Dao National Park, or other nearby localities from which it was separated by rivers. Specimens from Chi Linh in Clade A also appear as a monophyletic group. In contrast, specimens from Chi Linh are not resolved as a monophyletic group in Clade B. In the analyses of the species of *Microhyla*, the picture is equally unclear. Because most species did not have multiple representatives from each locality, the criterion of reciprocal monophyly is not testable. Every locality represented by multiple specimens within a

species is recovered as monophyletic. However, there are no common groupings that correspond to river separations between any of the species of *Microhyla*.

Other river systems do not provide stringent tests of the riverine barrier hypothesis due the limited number of collection localities and the geographic distance between them. Geographic distance renders any correlation of differentiation across the rivers to riverine effects questionable because any number of other factors may be present in the intervening areas. Another major complication in elucidating the effects of river systems is their association with mountains. This association is especially prevalent with respect to the Mekong River, which is separated from most of the Vietnamese collecting sites by the Annamite Mountain Range. Consequently, it was not possible to attribute the separation of the Laotian specimens from those in Vietnam to barrier effects from either the Mekong or the Annamite Mountain range. Additionally, in northeastern Vietnam, most of the major rivers are associated with mountain arcs and massifs that separate them along at least part of their lengths (Figure 6.1).

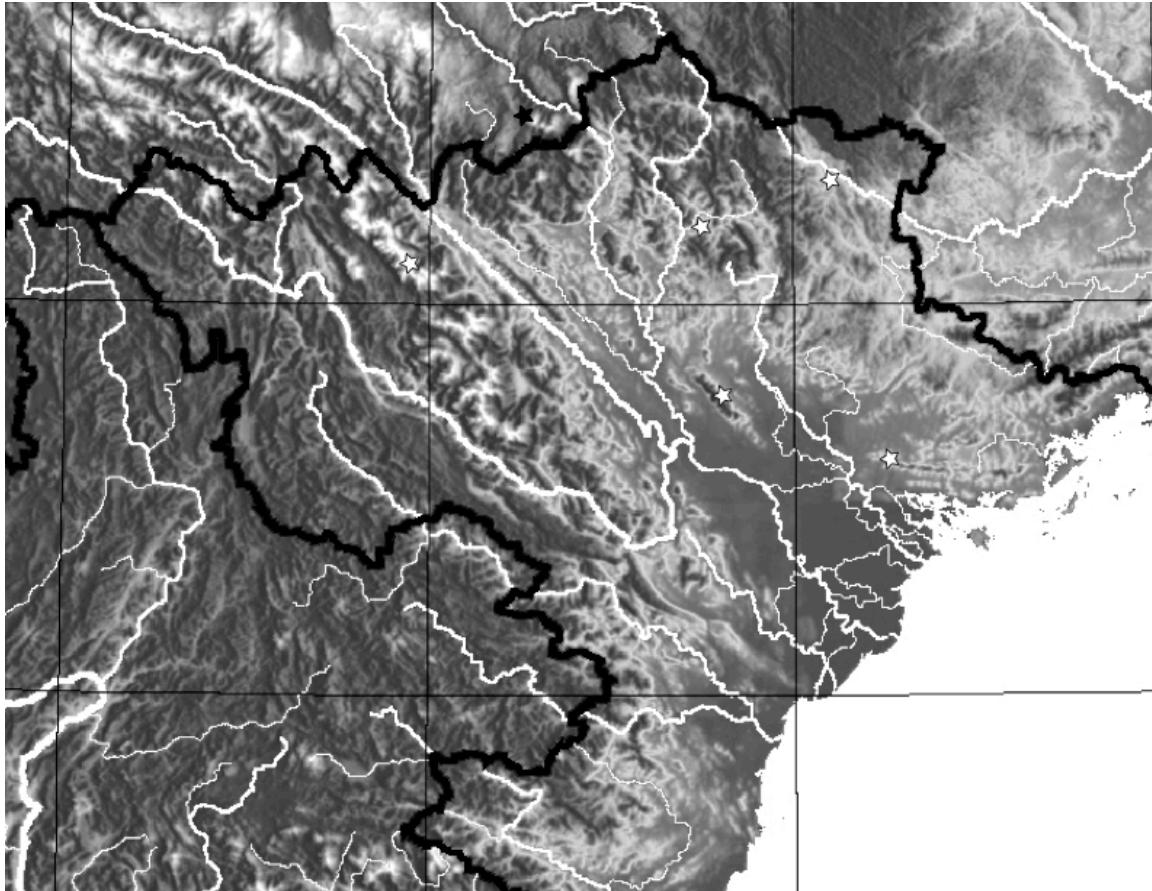


Figure 6.1. Relief map of northern Vietnam, showing river systems and associated mountains, massifs, and deltas.

As a result of their close association and intertwined nature, mountains, rivers, and geographic distance need to be looked at in concert to appreciate the full picture. For example, some of the populations were geographically closer to those in other river drainages than to samples in their own drainage. They are on opposite sides of mountain ranges. This connection between drainage systems and mountain ranges obscures the extent of the effects of each individually. Multiple factors may be involved, and these possibly work synergistically in the generation and maintenance of genetic diversity.

Widespread Forest Species

Indochina, and Vietnam in particular, has been the focus of recent surveys and species descriptions (Bain et al., 2003, 2004, 2006; Lathrop et al., 1998; Orlov et al., 2002; Orlov, 2005; Stuart et al., 2005). As a result of this focus, between 1997 and 2005, 53 new amphibian species were described from Indochina (Bain et al., 2007). Many of what were once considered widespread species of anurans in Southeast Asia have been found to be complexes of cryptic species (Bain, 1998; Bain et al., 2003; Emerson et al., 2000; Matsui et al., 2006; Stuart et al., 2006; Toda et al., 1998). As a result, Stuart et al. (2006) raised the question of whether widespread amphibian species exist in Southeast Asia or not. They suggested that this trend of uncovering cryptic species in putatively widespread forest anuran species will only continue, until we find that they are all, in fact, complexes of species. This trend probably owes more to recent advances in molecular techniques and therefore the ability to separate morphologically similar species, than to an actual lack of widespread species. Stuart et al., (2006), for example, identified lineages of *Odorrana* using genetic techniques, then relied on *a posteriori* analyses of the different lineages to reveal diagnostic morphological characters for each group. Further, most of the studies of widespread species in Southeast Asia have focused on stream breeders, such as the *Odorrana livida* complex (Bain et al., 2003; Stuart et al., 2006), *Limnonectes* (Emerson et al., 2000; Evans et al., 2003; Gillespie et al., 2004; Toda et al., 1998), and *Rana chalconota* (Gillespie et al., 2004; Stuart et al., 2006). These animals would be expected to be tied to drainages and, therefore, show greater genetic structuring and more likely represent a complex of species.

My analyses of *Microhyla ornata* and *Mh. heymonsi* in chapters 3 and 4 provide

examples of single widely distributed species. Their range in Vietnam spans nearly 15 degrees in latitude from Quang Thanh in the north to Cat Tien National Park in the south. Given the findings of Matsui et al. 2005, both of these will likely be separated into multiple species across their range following closer examination and genetic evaluations. The relatively short branch lengths and low sequence divergence of the Vietnamese populations of both *Mh. heymonsi* and *Mh. ornata* suggest that they do not represent more than one species. Therefore, even after splitting the group, at least one widely ranging form each, of what was previously recognized as *Mh. heymonsi* and *Mh. ornata*, will have to be recognized.

The whipping frogs examined in chapter 5 also serve to test for the existence of widespread species. These frogs are broadly distributed across Asia, from India and Nepal eastward through Indochina and the Sunda shelf, and northward into mainland China, Taiwan, and Japan (Brown and Alcala, 1994; Frost, 2007; Manthey and Grossman, 1997; Matsui et al., 1986; Zhao and Adler, 1993). They occur primarily in forest habitats, though they are also found in disturbed habitats and even urban settings. The fact that at least eleven different species were recovered might seem to support Stuart et al.'s (2006) claim that there are no widespread forest dwelling anuran species, and that they are all simply complexes of more restricted species. However, despite the fact that the group contains several distinct lineages or cryptic species, several of these lineages remain geographically widespread, some ranging from Thailand and Laos through Vietnam to Borneo and the Philippines (Figure 6.2). The Vietnamese specimens are generally restricted to their own subclades within these broad geographical clades and it is possible that additional sampling might lead to further splitting of these lineages.

However, the low number of changes separating the different haplotypes within these groups suggests otherwise.

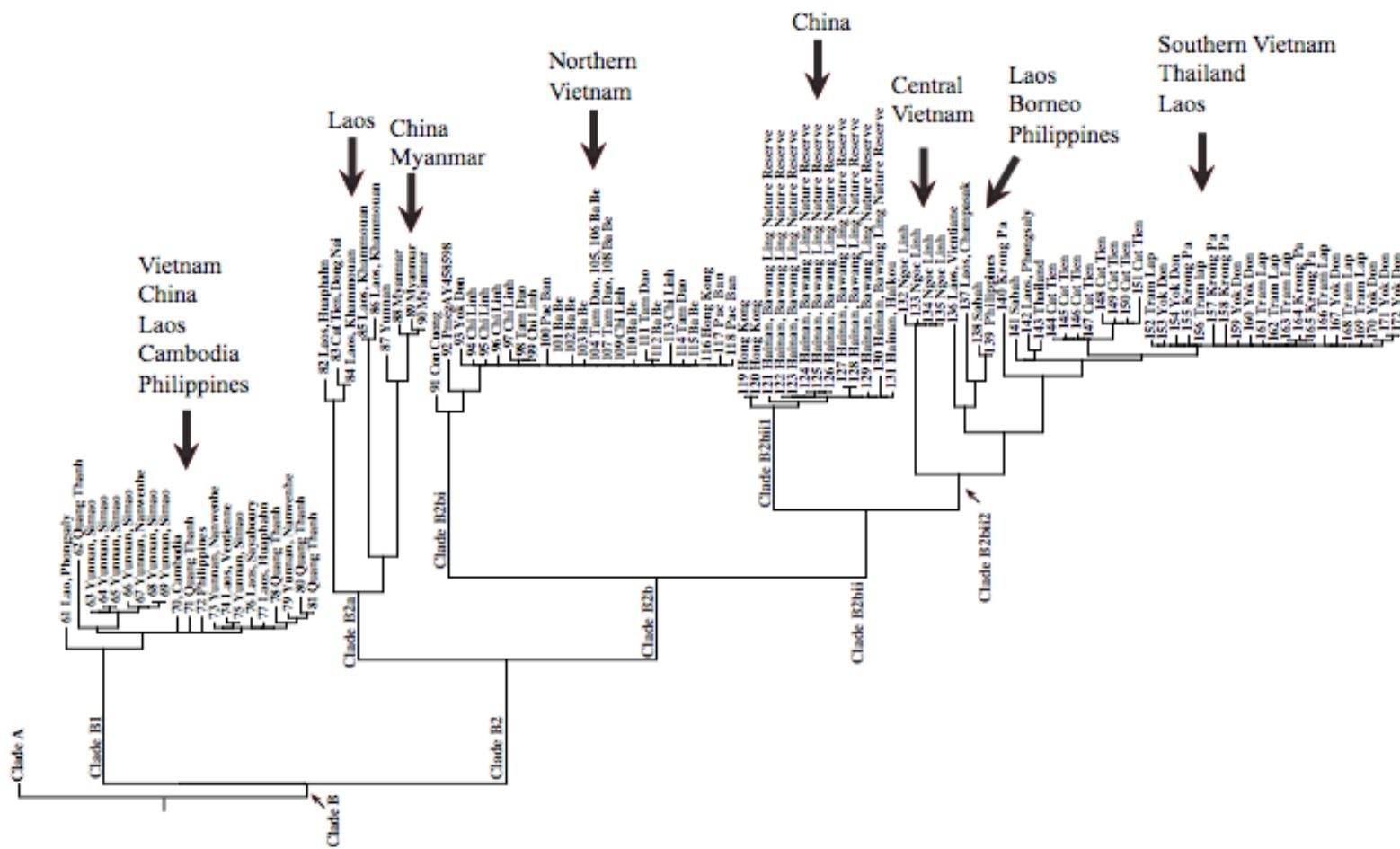


Figure 6.2. Maximum parsimony tree of Clade B of the *Polypedates leucomystax* complex, showing the geographic breadth of the subclades.

There appears to be a phylogenetic component to being a widespread species. Groups from Clade A of the *P. leucomystax* complex are restricted to Vietnam, adjacent Southern China (Yunnan and Hainan provinces) and nearby Laos (Chamapasak province). Its sister group, Clade B, however, contains all of the widespread lineages recovered in this analysis. The low sequence divergence between the Vietnamese and Philippine specimens in Clade B1 and the Bornean, Thai, and Vietnamese specimens in Clade B2bii2 suggest that these specimens are relatively recently separated from each other. In support of this, *P. leucomystax* (*sensu lato*) has been introduced to Japan and Western Papua New Guinea in recent times (Maeda and Matsui, 1999), and a specimen has been found hitchhiking on an airplane to Guam (Christy et al., 2007; Wiles, 2000). This further implies increased dispersal ability tied to clade B. The specific factor that is responsible for this is still unknown. It is possible that this tendency corresponds to increased vagility or a preference for disturbed habitat and, therefore, an increased likelihood of human-mediated translocation. Further ecological and fine-scaled genetic analyses might help to elucidate the processes involved in these biogeographic patterns and provide insight into the question of why some groups are more speciose than others.

The historical biogeography of Vietnam: a clouded picture

While multiple patterns are clear in another peninsula, Baja California (Hafner and Riddle, 1997; Zink et al., 1997; Riddle et al. 2000; Lawlor et al., 2002; Murphy and Aguirre-León, 2002; Lindell et al., 2006), Vietnam is a topographically complex region rife with rivers, streams, hills, and mountains. This complexity adds another layer to the picture, as different species would be expected to respond differently to different

geographic features based on their particular ecologies. Furthermore, the glacial cycles, alternately raising and lowering sea levels, complicate things further. Fluctuating water levels have alternately rendered the area essentially a long peninsula and a large land mass, connected to the islands of Java, Sumatra, Taiwan, Hainan, Borneo, and the Philippines (Figure 1.1; Voris et al., 2000; Inger and Voris, 2001). This repeated expansion and contraction of potential habitat might have alternately isolated lineages, leading to localized differentiation, and merged them, allowing the lineages to mix.

A few groupings appear as common patterns among multiple taxa despite the occasional exception. The first grouping consists of the populations in the northeastern region of Vietnam drained by the Red River and the Thai Binh River that meet in a broad delta before emptying into the South China Sea as well as from Quang Thanh on the northeastern border of Vietnam. Another involves specimens from along the Ca River drainage in north-central Vietnam. All taxa exhibited an association of specimens from central to southern portion of Vietnam to the exclusion of all other localities. Finally, specimens along the Mekong River to the west of the Annamite Mountain Range were resolved as separate from those along the same drainage in Vietnam.

While these common patterns do exist with respect to the monophyletic grouping of organisms along some river systems and the reciprocal monophyly of populations on opposite sides of mountain ridges and the Red River, the biogeographical relationships of these areas generally differs among taxa. The biogeographic relationships of populations on Taiwan and Hainan Island provide an example of this. As with much of the region, both Taiwan and Hainan Island were connected to Vietnam during parts of the Pleistocene (Figure 1.1; Voris, 2000), and isolated from it during others. As a result, there were several opportunities for invasion of these islands. Three different geographical associations were found for populations on frogs on Hainan Island. In *Mh. heymonsi*,

both Taiwan and Hainan were associated with populations from southern Vietnam. In *Mh. pulchra* and Clade B of *P. leucomystax (sensu lato)* populations from Hainan Island were more closely related to those from northern Vietnam. In contrast, populations of Clade A of *P. leucomystax* complex from Hainan Island had a sister group relationship to all remaining specimens from Vietnam and China, with the exception of those from Sa Pa and Simao. Similarly, the specimens from the Ca River drainage were resolved in different places in the different groups, and the specific relationships of the northern specimens to the others varied.

These differences in relationships among the range of taxa examined serve to highlight several facts. The geological and historical complexities of the region alone complicate the search for common patterns. The complex topography of Vietnam provides many different mountain ranges, massifs, river systems, and even habitats (Chapter 1). Different organisms would be expected to respond differently to the different features, with rivers acting as barriers to some and conduits for dispersal to others. Additionally, several of these factors are often associated with each other, further complicating the picture. Similarly, by allowing ranges to expand, contract, and potentially mingle, the glacial/interglacial cycles may have obscured much of the common pattern that may have existed prior.

Possibly the most important things underscored by the findings in this thesis are the exceptions to the patterns and both the answers and questions provided by them. There appears to be an inherent human tendency to pigeon-hole things and to look for common patterns. While common patterns can suggest common processes and thereby provide insight into those processes, nature is not always that simple. To nearly every rule there exist exceptions, and these exceptions help clarify the extent of the rules and prompt new questions. In the case of this thesis, river systems were shown to serve as

conduits for dispersal in some groups yet not in others. This leads to the questions: what factor or factors are responsible for the differences observed between the groups and what, specifically, is involved? Identifying these factors will then allow us to better understand and predict patterns of differentiation and diversity in different groups in otherwise understudied areas, permitting informed conservation decisions. The differences in the phylogenetic distribution of cryptic and widespread species also raise questions. Despite similar distributions in Vietnam to the species of *Microhyla* examined, the *P. leucomystax* complex exhibits greater genetic differentiation and cryptic species, though the reason for this remains unclear and yet another avenue for future research. There also appears to be a phylogenetic component to being widely ranging exhibited in this group, with lineages within Clade B being consistently more widespread than the equivalent groups in either Clade A or in the other species examined. This allows for future comparisons between these groups to identify the specific trait or traits responsible. Identification of these traits will provide greater understanding of animal distribution in general, biodiversity patterns, and speciation processes.

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Appendix 1

Taxonomic Chaos In Asian Ranid Frogs: An Initial Phylogenetic Resolution

Introduction

For many years following the major revisions of Boulenger (1882, 1918, 1920), the taxonomy of ranid frogs was stable. Now it is in a state of chaos.

Numerous generic and subgeneric shifts have been proposed, usually without an examination of phylogenetic relationships. Dubois (1986 (1987)) recognized six tribes within the Raninae (=Ranidae by most authorities). Among ranids, his Ranini included the genera *Altirana*, *Amolops*, *Batrachylodes*, *Micrixalus*, *Nanorana*, *Staurois*, and *Rana*, with the subgenera *Amietia*, *Hylarana*, *Paa*, and *Strongylopus*, and *Rana*. Dubois' Tomopternini only included the genus *Tomopterna*. The Tribe Ptychadenini had *Ptychadena* and *Hildebrandtia*. His Dicroglossini contained *Ceratobatrachus*, *Conraua*, *Discodeles*, *Limnonectes* (with five subgenera: *Limnonectes*, *Bourretia*, *Fejervarya*, *Hoplobatrachus*, and *Taylorana*), *Occidozyga* (with two subgenera: *Occidozyga* and *Euphlyctis*), *Palmatorappia*, *Phrynobatrachus*, *Platymantis*, and *Ingerana* (with *Ingerana* and *Liurana* as subgenera). The fifth tribe, Pyxicephalini, included *Pyxicephalus*. Finally, Tribe Ranixalini had *Ranixalus*, *Nannophrys*, and *Nyctibatrachus*.

Higher taxonomy has continued to change. Dubois (1992) raised the Tribe Dicroglossini to the subfamily level, Dicroglossinae. He recognized four tribes in this

subfamily: (1) Ceratobatrachini (*Ceratobatrachus*, *Discodeles*, *Ingerana*, *Palmatorappia*, *Platymantis*, and *Taylorana*), (2) Conrauaini (*Conraua*), (3) Dicroglossini (*Euphlyctis*, *Occidozyga*, and *Phrynobatrachus*), and (4) Limnonectini (*Hoplobatrachus* and *Limnonectes*). These arrangements have been controversial. Inger (1996) noted that Tribe Limnonectini was demonstrably paraphyletic with respect to the Ceratobatrachini, Conrauaini, and Dicroglossini. No evidence supported the monophyly of Limnonectini. It may be paraphyletic with respect to the Mantellinae and through the mantellines to the Rhacophoridae. Laurent (1951, 1979) and Ford (1993) questioned the monophyly of the Ranidae (sensu stricto) with respect to the family Rhacophoridae.

Recently, Chinese authorities have proposed numerous other changes, in particular generic reallocations. Fei et al. (1990) described or erected a number of new generic combinations for many Chinese species. Further generic changes were made by Ye et al. (1993) and Fei (1999). These changes were made in the absence of a phylogenetic evaluation. Thus, we undertook an investigation of ranid relationships, particularly for representative Southeast Asian genera and species. When we initiated this study, no phylogenetic evaluation of the group had been attempted at a higher taxonomic level, although one distance-based evaluation had been made (Wallace et al., 1973). Subsequently, four other phylogenetic studies have reported on the relationships of ranid frogs, as discussed below.

Materials and methods

Specimens Examined

Forty-five individuals, most of them Southeast Asian ranines, were sequenced for three mitochondrial DNA genes. Additional sequence data from GenBank were used for

the following species: *Rana pipiens* (X86247, X86318), *R. catesbeiana* (M57572), *R. temporaria* (Y11977), and *Xenopus laevis* (M10217). We used *X. laevis* as our initial outgroup taxon, and included an Asian treefrog (Family Rhacophoridae, Subfamily Rhacophorinae), *Polypedates megacephalus* (AF026350, AF026367), and an African mantelline (Family Rhacophoridae, Subfamily Mantellinae), *Laliostoma labrosum* (AF026354, AF026374), in our study to evaluate the monophyly of the family Ranidae as questioned by Laurent (1951, 1979) and Ford (1993). GenBank accession numbers, collection locality and voucher data for specimens sequenced in this study are given in an electronic Appendix downloadable from the Journal's website (<http://biology.bangor.ac.uk/~bss166/HJ/>). These tissue samples and most voucher specimens are preserved in the Royal Ontario Museum (ROM), or in the tissue collections of Jinzhong Fu (JF) and James P. Bogart (JPB) (Department of Zoology, University of Guelph). We also incorporated sequences from an analysis of fanged ranids (Emerson and Ward, 1998). These species include *Limnonectes acanthi* (U66120–21), several populations of *L. blythii* (U55262–3, U55269–70, U66114–15, U66126–27, U66130–31, U66134–37), *L. grunniens* (U66124–25), *L. ibanorum* (U66122–23), *L. ingeri* (U55268, U55275), *L. limnocharis* (U55265, U55272), *L. macrocephala* (66116–17), *L. macrodon* (U66132–33), *L. magna* (U66118–19), *L. paramacrodon* (U55267, U55274), *Limnonectes* sp. (“duboisii,” a nomen nudum; Dubois, 1999) (U66112–13), and *Occidozyga laevis* (U66138–39). Taxonomic assignment of examined species generally follows Frost (2004).

DNA Amplification and Sequencing

Three ribosomal RNA genes, 12S, 16S, and tRNA^{Val} from the mitochondrial genome were selected to reconstruct the phylogeny. Total genomic DNA was extracted

from frozen or alcohol preserved tissue samples of muscle or liver by digestion with proteinase K for 5–12 hr, and purified three times with phenol–chloroform–isoamyl alcohol (PCI), and then once with chloroform–isoamyl alcohol (CI). The mtDNA region of 12S through 16S was sequenced using the following method. Double-stranded fragments were amplified in 33 cycles of the polymerase chain reaction (PCR; 92°C for 30 sec, 45–55°C for 30 sec 72°C for 1.5 min) performed in 25µl reactions. Annealing temperatures were changed from 45°C to 55°C as needed in order to improve the quality of PCR products. Usually, PCR reactions amplified the entire fragment from 12S1L to 16S2H. Subsequently, several internal primers were used for sequencing. Infrequently, amplification of the larger fragment was not possible and thus the following primers were used: 12S1L, 12S2H, 16S3H, 16S3L, 16S5H, 16SML and 16S2H (Table 1). After amplification, the 25µl product was separated by electrophoresis on an agarose gel and stained with ethidium bromide. The bands containing DNA were excised and the DNA was eluted using Gene Clean II kit (Bio101) and suspended in distilled, deionized water. The cleaned DNA was sequenced directly with Thermo Sequenase ³³P-labeled terminator cycle sequencing kit (Amersham). Locations of the primers are shown in Figure 1.

The products of the sequence reactions were resolved in a polyacrylamide–7M urea gel that was then dried and visualized on autoradiograph films (Kodak) within 24–48 hr. A few sequences were resolved using an ABI 377 automated DNA sequencer using the manufacturer's protocols.

DNA Sequence Analysis

Sequences were initially aligned using ClustalW (Thompson et al., 1994) with gap-open and gap-extension penalties set to 5. Subsequently, minor adjustments to the

computer alignments were made by eye in BioEdit (ver. 5.0.9; Hall, 2001) and MacClade (ver. 4.0.5; Maddison and Maddison, 2002). Sites with ambiguous alignment were excluded from the phylogenetic analysis because the homology cannot be confidently assumed (Hillis, 1991). Only potentially cladistically informative sites were maintained for the analysis in PAUP* (ver. 4.0b8a; Swofford, 2001).

All multistate characters were evaluated as unordered because there is no a priori reason to assume order of evolutionary change between nucleotide bases adenine (a), cytosine (c), guanine (g), or thymine (t) (Swofford et al., 1996). The phylogenetic analysis using PAUP* employed an heuristic search, with random addition sequence, 500 replicates, retaining minimal trees only, using tree bisection–reconnection branch swapping with steepest descent and collapsing zero length branches. The two genes were initially analyzed separately, because different genes may experience different evolutionary pathways. Second, a combined data analysis was conducted. Ratios of transitions to transversions were calculated in MacClade.

Nodal support was assessed for the combined data sets. Bootstrap proportions (BSP; Felsenstein, 1985) used 1000 replicates calculated in PAUP*. We also performed decay analyses (DI; Bremer, 1988) using AutoDecay (ver 4.0.2; Eriksson, 1999).

Results

Forty-five specimens were sequenced for 12S through 16S RNA genes. In total, 546 sites were sequenced for 12S, 72 for tRNA^{Val}, and 1509 for 16S for a total of 2127 aligned sites. Among these sites, 56 were ambiguously aligned and 1012 were potentially

phylogenetically informative (Table 2). All sequences were deposited in GenBank (12S = AF206072–AF206116; tRNA^{Val} = AF206117–206161; 16S rRNA = 206453–206497).

Parsimony evaluation

For 12S, analysis of the 236 potentially informative sites yielded six most parsimonious trees (MPTs, Table 2). We did not attempt a separate phylogenetic analysis of the tRNA^{Val} gene because there were too few potentially phylogenetically informative sites for a meaningful analysis. For 16S, 728 potentially informative sites resulted in 16 cladograms, the differences constrained to one subclade. Because of similarities in nucleotide proportions and levels of site divergence, all RNA gene sequence data were combined for a total evidence analysis.

Combining all RNA gene sequence data into a single data set resulted in 1012 potentially cladistically informative characters. Analysis of these data yielded three most parsimonious trees (8512 steps in length, CI=0.24, RI=0.53). *Ptychadena* was resolved as the sister group of *Pyxicephalus* plus two major speciose clades (A and B) of ranids (Figure 2): Clade A, included *Amolops*, *Hylarana*, *Nidirana*, *Odorrana*, and *Rana*; Clade B was composed of *Chaparana*, *Hoplobatrachus*, *Limnonectes*, *Nanorana*, *Occidozyga*, and *Paa*, plus a mantelline, *Laliostoma*, and rhacophorine, *Polypedates*.

Clade A was treated as having two major subclades, A1 and A2. *Nidirana chapaensis* was resolved as the sister group to all other members of clade A1, including specimens of *Odorrana* and *Rana*. *Rana (Pantherana) pipiens* was resolved as the sister species of *R. (Aquarana) catesbeiana*. Their sister group contained *R. (Pseudorana) johnsi* plus *R. (Rana) temporaria*. The clade containing *Rana* formed the sister group to a clade composed of *Amolops (Huia) nasica* and a paraphyletic *Odorrana*.

In Clade A2, a monophyletic *Amolops* (*Amolops*) was the sister group to a clade of *Rana* including subgenera *Hylarana* and *Pelophylax*, and a paraphyletic subgenus *Sylvirana*.

Clade B was treated as having four major subclades, B1–B4. In Clade B1, the mantelline, *Laliostoma labrosum*, and the rhacophorine, *Polyypedates megacephalus*, were resolved as sister taxa. Together they formed the sister group of the three species of *Occidozyga*. Clade B1, in turn, was the sister group of clades B2–B4. Clade B2 consisted of *Hoplobatrachus crassus*, *H. rugulosus*, *Limnonectes cancrivorus*, and a paraphyletic *L. limnocharis*. It was the sister group of clades B3 and B4. Clade B3 contained *Paa* (as a paraphyletic taxon), *Chaparana* and *Nanorana*. The sister group of Clade B3 was B4, which consisted of *Limnonectes blythii* and its relatives, with *L. blythii* being resolved as paraphyletic (Figure 2.)

Assessing Nodal Stability

Values of nodal support are indicated on the tree (Figure 2). Bootstrapping (BS) trials supported 43 nodes with a consistency greater than 70%. Decay analyses revealed that many nodes required a considerable number of additional steps to collapse, except those not generally supported by high BS proportions.

Discussion

Because different portions of the mtDNA genome evolve at different rates, cladograms from different genes for the same set of organisms may differ. The relatively

slowly evolving 12S and 16S rRNA genes seem appropriate for resolving older divergences, perhaps as old as 150 Ma (Mindell and Honeycutt, 1990).

The two mitochondrial genes evolved in similar ways. We assume that this conciliation owes to their phylogenetic history. Our phylogenetic analysis of the combined data resulted in three MPTs. Independent analyses for each gene revealed compatible branching patterns.

Previous studies

The phylogenetic relationships of ranid frogs have been investigated in three recent molecular studies. Marmayou et al. (2000) evaluated a short, 305 bp segment of mtDNA 12S for 28 species of ranid and rhacophorid frogs using maximum parsimony, transversion weighting, and phenetic neighbor joining. Their unweighted parsimony evaluation resolved *Occidozyga* and *Phrynobatrachus* as sister taxa, which together formed the sister group of all other ranids plus rhacophorids. The remaining taxa clustered into four groups whose relationships to each other were not resolved. Representative rhacophorids, including species of *Buergeria*, *Philautus*, *Polypedates* and *Chirixalus*, formed one monophyletic group. *Amolops* and *Rana chalconota* formed another group. The genera *Limnonectes*, *Fejervarya*, *Hoplobatrachus*, *Sphaerotilis*, and *Taylorana* formed a third clade and several species of *Rana* formed the fourth cluster. In this taxonomy, *Rana* was paraphyletic. Transversion weighting and the phenetic evaluation resolved paraphyly in *Philautus*, *Limnonectes*, and an additional example of paraphyly with respect to *Rana*. Given the small numbers of characters analyzed, it is not surprising that most nodes received low levels of branch support.

Bossuyt and Milinkovich (2000) evaluated 2,692 bp of mitochondrial and nuclear homologous DNA sequence sites, excluding third position codon sites for cytochrome *b*.

They constructed trees using maximum likelihood and BS consensus methods based on maximum parsimony. Because the initial outgroup was very divergent it was dropped from the analysis and Madagascan ranids and rhacophorids were used to root the network of Asian ranids and rhacophorids combined, and vice versa. Unfortunately, bootstrapping is problematic (Kluge and Wolf, 1993) and consensus methods themselves have long been known to be suspicious (Miyamoto, 1985; Miyamoto and Fitch, 1995). Maximum likelihood analyses are philosophically problematic (Kluge, 1997; Siddall and Kluge, 1997). This puzzle is exemplified, in part, by “Brooks’ conundrum” (D. R. Brooks, Univ. of Toronto, pers. comm., 2002): “Do you believe that evolution occurs in a most parsimonious manner? If not (which is demonstrably true—homoplasy exists), then why try to force a model of maximum parsimony on the analysis of your data, which is exactly what maximum likelihood does?” Maximum parsimony should be used as a criterion for selecting among all possible trees, and not as a model of evolution.

Regardless, the basal relationships in the bootstrap consensus tree of Bossuyt and Milinkovich were unresolved. Asian treefrogs were monophyletic, as was a clade containing representative species of *Fejervarya*, *Hoplobatrachus*, *Nanophrys*, *Euphlyctis*, Asian *Tomopterna* (*Sphaeroteca*) and some *Limnonectes*.

Kosuch et al. (2001) investigated the monophyly of tiger frogs, *Hoplobatrachus*, which occur in both Asia and Africa. They evaluated 34 ranids using a total of 903 homologous nucleotide sites from 16SrRNA and 12SrRNA with 281 sites being potentially phylogenetically informative. Though their focus was on the biogeographical relationships of Asian and African *Hoplobatrachus*, representatives of *Fejervarya*, *Limnonectes*, *Nannophrys*, *Occidozyga*, *Phrynobatrachus*, *Ptychadenidae* and several species of *Rana* were also included. Support was found for a monophyletic *Hoplobatrachus*,

which was resolved as the sister group to *Fejervarya*. Subfamily Dicroglossinae was not resolved as a monophyletic grouping in either of the two trees presented.

More recently, Roelants et al. (2004) evaluated DNA sequences of several groups of ranid frogs, though their focus was on the biogeography of these frogs rather than taxonomy. The taxonomic implications of their study are summarized below.

Patterns of relationships

Although the relationships we resolved among the putative subfamilies of ranid frogs were not entirely consistent with previous taxonomies, lower taxonomic groupings were congruent in a number of ways with those proposed by Dubois (1986[1987], 1992). However, our analysis discovered several problematic associations. For example, the genus *Rana* was not resolved as a monophyletic taxon and *Limnonectes limnocharis* appears to be paraphyletic with respect to *L. cancrivorus*.

Monophyly of the Ranidae and relationships among Subfamilies

Family Ranidae was resolved as a paraphyletic taxon with respect to rhacophorids. Therefore, recognizing Family Rhacophoridae as a subfamily within Family Ranidae, as suggested by Dubois (1992) and Blommers-Schlösser (1993), provides an acceptable solution. Alternatively, in order to avoid having an extremely speciose Ranidae, multiple families could be recognized. The problem requires further investigation using sequences from more conserved genes and a broader array of taxa, especially African ranids and rhacophorids.

Raninae, Clade A

Clade A consisted of five potential genera of ranid frogs: *Amolops*, *Hylarana*, *Nidirana*, *Odorrana*, and *Rana*, although group membership did not mirror current taxonomy. These genera were distributed amongst two subclades (A1 and A2).

Clade A1

Genus *Rana* (part), Subgenus *Nidirana*: One species, *R. (Nidirana) chapaensis*, was used to represent this subgenus of *Rana*. It was resolved as the sister taxon to the following two subclades of Clade A1:

Genus *Rana* (part), Subgenera *Aquarana*, *Pantherana*, *Rana*, and *Pseudorana*: One species each was used to represent four relatively speciose subgenera of *Rana*. The two North American species, *R. (Pantherana) pipiens* and *R. (Aquarana) catesbeiana*, were resolved as sister taxa. Their sister group contained the Asian species, *R. (Pseudorana) johnsi*, and its sister group represented by the European *R. (Rana) temporaria*.

Genus *Rana*, Subgenus *Odorrana*: The group containing *Odorrana* and *Amolops (Huia)* forms the sister group to the clade containing *Rana catesbeiana* and *R. pipiens* plus *R. johnsi* and *R. temporaria*.

The large, odoriferous ranids sometimes referred to Genus *Odorrana* Fei Ye and Huang 1990 formed a paraphyletic lineage with respect to *Amolops (Huia) nasica*. *Amolops (Huia)* was resolved within a group of *Odorrana*, and not with other *Amolops* with which it is usually associated (Yang, 1991). *Amolops (Huia)* differs from *Odorrana* by its non-glandular skin and the absence of enlarged toe discs.

Our data also support the finding that *Odorrana chloronota* is a species complex (Murphy et al., 1997; Bain et al., 2003). As cryptic species are identified, the number of species of *Odorrana* will likely increase significantly.

In some regions, like the Khe Moi River, Nghe An Province, Vietnam, three large species of this clade occur in sympatry (Bain et al., 2003). Some sympatric species are derived from distant lineages, such as the co-occurrence of *O. chloronota* and a similar species, *O. bacboensis*. However, other sympatric species appear to be much more closely related, such as *O. chloronota* and *O. morafkai*. This pattern of sympatry repeats in most other areas in Vietnam, although the species composition changes.

Clade A2

Genus *Amolops*, Subgenus *Amolops*: The sampled species are monophyletic, and a larger survey of species is currently underway. The two most anatomically similar species included in this analysis, *A. ricketti* and *A. cremnobatus*, formed a terminal sister relationship, followed basely by *A. loloensis*, and the geographically more distant, but anatomically similar *A. hongkongensis*. *Amolops spinapectoralis* was resolved as the sister group of these species. *Amolops* formed the sister group of the remaining subclade containing *Rana maosonensis* and *R. erythraea*.

Genus *Rana*, Subgenera *Hylarana*, *Pelophylax*, and *Sylvirana*: This clade includes a paraphyletic assemblage of subgenera within the Genus *Rana*. The association of subgenera is as follows: (*Sylvirana*((*Sylvirana*, *Pelophylax*)(*Hylarana*))).

Raninae/Rhacophoridae, Clade B

The second major group of ranines contains relatively stocky, largely edible Asian frogs. Frogs within this clade belong to several genera, possibly reflecting, in part, their

economic significance (and, hence, greater attention) and a greater amount of anatomical divergence. The frogs within clade B clustered into four serially arranged clades as follows: (B1, (B2, (B3, and B4))).

Clade B1

Genera *Occidozyga*, *Polypedates*, and *Laliostoma*: The two representative rhacophorids, *Polypedates megacephalus*, a rhacophorine, and *Laliostoma labrosum*, a mantelline, were resolved as sister taxa. These taxa formed the sister group to a monophyletic *Occidozyga*. The sister group to this clade contains the dicroglossine frogs of the Genera *Fejervarya*, *Hoplobatrachus*, and *Limnonectes*, separated by the ranine frogs *Chaparana*, *Nanorana*, and *Paa* (Figure 2).

Clade B2

Genera *Hoplobatrachus*, *Limnonectes* (part) and *Fejervarya*: This subclade, sometimes considered to be three genera, has been particularly problematic. Kosuch et al. (2001) examined the biogeographic relationships of *Hoplobatrachus*, and found a monophyletic *Hoplobatrachus* to be the sister group to *Fejervarya*. We also found a monophyletic *Hoplobatrachus*, with *H. crassus* plus *H. rugulosus* being the sister group to the remainder of the clade.

The rice frog, *L. limnocharis*, is resolved as paraphyletic with respect to *L. cancrivorus*. A considerable amount of allozyme work in other parts of its extensive range suggests that it is a composite of many cryptic species (e.g., Dubois, 1984; Toda et al., 1998a, 1998b, 1994). Our data and cladogram support this conclusion.

Clade B3

Genera *Paa*, *Chaparana*, and *Nanorana*: This clade is a paraphyletic assemblage of genera. *Paa spinosa* is resolved as the sister group of a clade containing two other species of *Paa* plus *Chaparana fansipani*, *Nanorana parkeri* and *N. pleskei*. Thus, the genus *Paa* is paraphyletic with respect to *Nanorana* and *C. fansipani*. The association of these species is particularly interesting, given that, though *Chaparana* and *Nanorana* are heavy-set, they are not large frogs like *Paa*. This association does not appear to be spurious since all nodes within this clade received substantial support. This clade, in turn, is resolved as the sister group to the remaining ranine clade.

Clade B4

Genus *Limnonectes* (part): The third subclade of Asian edible frogs includes species placed in this genus. Within this clade, paraphyly is the rule rather than exception. Populations of *L. blythii* are variously associated with *L. macrodon*, *L. ingeri*, and *L. paramacrodon*. The clades have a greater correspondence to geographic location than taxonomy. Sister taxa co-occur on a single island. Some species appear to be large complexes of morphologically similar species. For example, Inger et al. (1999) noted several anatomical differences between *L. blythii* from the Malay Peninsula and southern Vietnam. Thus, as with *L. limnocharis*, the taxonomy of this group needs to be revised as it undoubtedly represents far more species than previously thought. Our arrangement differs from that of Roelants et al. (2004) who resolved this group as the sister of clade B2+B3. However, both studies found weak support at the conflicting nodes. Whereas we included 19 specimens, Roelants et al. (2004) sequenced two representatives.

Taxonomic Implications

Type species of *Rana* Linnaeus 1758: Before undertaking revisions, it is first necessary to establish the relationships of the type species of the genus *Rana*. Fleming (1822) designated *Rana temporaria* Linnaeus, 1758 as the type species of *Rana*. This species is the name–bearer of the genus, subgenus, tribe, subfamily, and family. Genus *Rana* sensu Frost (2004) has more than 240 species divided into 22 subgenera. It is one of the most speciose groups of vertebrates and contains many independent lineages. Taxonomically, recognition of these major lineages as genera would better summarize their phylogenetic history.

Taxonomic chaos: At virtually every hierarchical level, taxonomic problems exist. For example, Tribe Ranini is a paraphyletic assemblage of genera with respect to the genus *Rana* and at the subfamilial level with the genus *Nanorana*.

The taxonomy of these frogs has been unstable. Not exhaustive, Table 3 briefly summarizes some of the changes for Asian groups from 1985 onward for many of the species included in this study. For most species, placement in one group or another has remained relatively stable, but the taxonomic rank accorded to the groups has been quite unstable. For example, the crab–eating frog, *Limnonectes cancrivorus*, was placed in Genus *Rana*, Subgenus *Euphlyctis* by Frost (1985), then into Genus *Limnonectes*, Subgenus *Hoplobatrachus* by Dubois (1986 [1987]). Subsequently, it was assigned to Genus *Euphlyctis* by Fei et al. (1990), then to Genus *Hoplobatrachus*, Subgenus *Fejervarya* by Dubois (1992). Most recently, the species was placed in Genus *Fejervarya* (Fei, 1999). Yet others (e.g., Inger, 1996; Nguyêñ and Ho, 1996; Zhao, 1994; Zhao and Adler, 1993) have left the species in the genus *Rana*. Much of this taxonomic instability owes to the absence of a reasonable phylogeny upon which to identify membership within particular clades.

A phylogenetically based taxonomy reflects the greatest amount of information within a hierarchical system (Farris, 1967; Wiley, 1980; Brooks and McLennan, 1991, 2002). Below, we review the taxonomy of these frogs and make taxonomic changes that directly reflect phylogenetic history, albeit conservatively.

Subfamily Dicroglossinae, Tribe Dicroglossini: This group was represented by three of 12 species from the genus *Occidozyga*: *O. laevis* and *O. lima*, and *O. martensii*. *Occidozyga laevis* and *O. martensii* have been placed in the genus *Phrynobatrachus* by many authorities (e.g., Peters, 1867; Smith and Chasen, 1931; Taylor, 1962; Dubois, 1986 [1987]). Our data do not refute this placement but recognition of *Phrynobatrachus* could result in a paraphyletic *Occidozyga*.

Subfamily Raninae: The subgenus *Nidirana* Dubois 1992 contains seven species: *R. (Nidirana) adenopleura*, *R. (N.) caldwelli*, *R. (N.) chapaensis*, *R. (N.) daunchina*, *R. (N.) lini*, *R. (N.) pleuraden*, and *R. (N.) psaltes*. It has been resolved as the sister taxon to the clade containing *Rana temporaria* plus *Odorrana*. Within the genus *Rana*, *R. johnsi* forms the sister group of the type species, *R. temporaria* and the two North American representatives of the subgenera *Pantherana* and *Aquarana*. Our data neither refute recognition of Fei's genus *Pseudorana* nor support it. Given the lack of additional specimens from this group and arguments for its rejection (Tanaka-Ueno et al., 1998), we believe it preferable not to recognize *Pseudorana* until sufficient evidence exists.

Genus *Nidirana*:

In order to maintain recognition of Genus *Odorrana* and not render Genus *Rana* paraphyletic, Subgenus *Nidirana* Dubois 1992 must be elevated to generic status for *N. adenopleura*, *N. caldwelli*, *N. chapaensis*, *N. daunchina*, *N. lini*, *N. pleuraden*, and *N. psaltes*.

Genus *Hylarana*:

The genus *Rana* is paraphyletic with respect to *Amolops*. In order to maintain the genus *Amolops*, another ranine genus must be recognized. The group of ranids that form the sister group of *Amolops* contains the subgenera *Hylarana* Tschudi 1838, *Pelophylax* Fitzinger 1843, *Sylvirana* Dubois 1992, and *Tenuirana* Fei, Ye, and Huang 1990. On the basis of priority, we recognize Genus *Hylarana* Tschudi, 1838. It contains those species associated with the subgenera *Hylarana*, *Sylvirana*, *Tenuirana*, and *Pelophylax*. The type species of *Hylarana*, *H. erythraea*, was included in our evaluation.

Recognition of the subgenera within *Hylarana* requires a phylogeny and the current taxonomy results in paraphyletic groupings (Figure 2). For example, Dubois (1992) included *H. guentheri*, *H. maosonensis* and *H. milleti* in Genus *Rana*, Subgenus *Sylvirana*. However, whereas *H. guentheri* is the sister group of Subgenus *Pelophylax*, *R. maosonensis* plus *R. milleti* is the sister group of the clade containing *R. guentheri* (Subgenus *Sylvirana* in part), Subgenus *Hylarana*, and Subgenus *Pelophylax*.

The subgenus *Tenuirana* Fei, Ye, and Huang 1990 is also a puzzle. *Tenuirana* contains only *R. taipehensis* and *R. macrodactyla*. Although these two species are sister taxa, recognition of this subgenus results in the paraphyly of other subgenera. Thus, *Tenuirana* should not be elevated to generic status as it leaves *Hylarana* a paraphyletic taxon.

Given the large number of species in *Hylarana*, the apparent polyphyly within the subgenus *Sylvirana*, and the problems surrounding the recognition of *Tenuirana*, recognition of these or any other subgenera or genera is premature in the absence of a more complete phylogeny.

Genus *Rana*:

Rana temporaria is a member of the clade consisting of *R. johnsi* and the American frogs, *R. catesbeiana* and *R. pipiens*. Dubois (1992) included *R. johnsi* (as *R. sauteri*) in the subgenus *Pseudorana*, and *R. pipiens* in Subgenus *Pantherana*. He placed *R. temporaria* in Subgenus *Rana*, and *R. catesbeiana* in Subgenus *Aquarana*. This subgeneric arrangement is phylogenetically acceptable from the perspective of our data. Taxonomically, these species have been closely associated with one another.

Genera *Odorrana* and *Huia*:

The usually large, odoriferous frogs referred to the genus *Odorrana* Fei, Ye and Huang 1990 are the sister group to *Rana*. The type species for the genus *Odorrana* is *Rana margaretae* Liu, 1950 by original designation. Unfortunately, we did not have tissue samples from this species and no sequences exist in GenBank. Nevertheless, for the moment, we recognize Genus *Odorrana* and include within it *O. bacboensis*, *O. banaorum*, *O. chloronota*, *O. daorum*, *O. hmongorum*, *O. megatypanum*, *O. morafkai*, and *O. nasica*. This list of species is not exclusive and at least 13 additional species could belong to the genus, including: *O. andersonii*, *O. anlungensis*, *O. exiliversabilis*, *O. grahami*, *O. hainanensis*, *O. jingdongensis*, *O. huangwuensis*, *O. livida*, *O. lungshengensis*, *O. margaretae*, *O. nasuta*, *O. schmackeri*, and *O. swinhoana*.

Genus *Amolops*:

Few have questioned the validity or membership of Genus *Amolops*, though our data reveal that *Amolops (Huia) nasica* occurs within the clade containing *Odorrana chloronota*. Consequently, membership in one genus or another may be uncertain for many of the larger species referred to as either *Amolops (Huia)* or *Odorrana* (see above).

Subfamily Limnonectinae (new content/combination): Dubois placed Genus *Paa* in Subfamily Raninae, Tribe Paini. However, Subfamily Raninae is a paraphyletic group. Consequently, Tribe Paini must be moved from Subfamily Raninae and placed in Subfamily Dicroglossinae, Tribe Limnonectini along with the genera *Hoplobatrachus* and *Limnonectes*. However, doing so still leaves Subfamily Dicroglossinae a paraphyletic group with respect to the Rhacophorinae and Mantellinae. Thus, to avoid paraphyly, Tribe Limnonectini must be elevated to Subfamily Limnonectinae. Recognition of the families Rhacophoridae and Mantellidae will necessitate recognition of the family Limnonectidae.

Limnonectinae has three distinctive lineages (Figure 3). One lineage contains Genus *Hoplobatrachus* and some species of Genus *Limnonectes* referred to Genus *Fejervarya* by Fei (1999). These frogs are placed in the Tribe Hoplobatrachini (new combination). Another lineage, Tribe Paini, contains the genera *Chaparana*, *Nanorana* and *Paa* (but see below). Finally, Tribe Limnonectini contains Genus *Limnonectes* excluding those species previously referred to *Fejervarya*.

Genus *Hoplobatrachus*:

This genus was represented by the species *H. crassus* and *H. rugulosus*. Kosuch et al. (2001) found this genus to be monophyletic. Our data support their conclusion.

Genus *Fejervarya*:

Fejervarya Bolkay, 1915 is represented, in this clade, by the two species *F. limnocharis* and *F. cancrivora*. However, *F. limnocharis* is paraphyletic with respect to *F. cancrivora*. Though both species are generally assigned to Genus *Limnonectes*, Fei (1999) included both species in the genus *Fejervarya*. Our data and cladogram support this conclusion.

Genera *Chaparana*, *Nanorana*, and *Paa*:

The subclade containing *Paa* also contains members of the genera *Chaparana* and *Nanorana*. The genus *Paa* contains more than 29 species (Frost, 2004) of which two were included in our study plus one undescribed species. The genus is paraphyletic. The genera *Nanorana* and *Chaparana* fall out as sister taxa within the genus *Paa*. Among the available generic names, *Nanorana* Günther, 1896 (type species *N. pleskei* by original designation) is the oldest available name having priority over *Altirana* Stejneger, 1927 (type species *N. parkeri* by original designation), *Chaparana* Bourret, 1939 (type species *Rana (Chaparana) fansipani* by original designation), and *Paa* Dubois, 1975 (type species *Rana liebigii* Günther, 1860, [named originally as a subgenus of *Rana*] by original designation). Paraphyletic relationships preclude retention of the subgenera within *Nanorana*. In addition to species already included in the genus *Nanorana*, we add those species previously recognized as *Paa*, as well as *Nanorana fansipani*, *Nanorana aenea*, *N. delacouri*, *N. quadranus*, *N. sikimensis*, *N. unculuanus*, *N. parkeri*, *N. pleskei* and *N. ventripunctata*.

Genus *Limnonectes*:

The type species of *Limnonectes* Fitzinger, 1843 is *L. kuhlii* by original designation. We recognize *Limnonectes* for the following species included in our study, *L. acanthi*, *L. blythii*, *Limnonectes* sp. ("duboisi"), *L. grunniens*, *L. ibanorum*, *L. ingeri*, *L. kuhlii*, *L. macrocephala*, *L. macrodon*, *L. magna*, *L. paramacrodon*, and *L. toumanoffi*, and exclusive of *Fejervarya limnocharis* and *F. cancrivora*. The tree of Roelants et al. (2004) does not conflict with this new taxonomy.

Although our analysis contains a small number of ranid frogs, major Asian groups are represented herein. No doubt the genus *Rana* remains a "megataxon" in that it is a paraphyletic assemblage of species. Our evaluation revealed that most assemblages of species contained paraphyletic grades of species, and not monophyletic assemblages. Consequently, in the interest of nomenclatorial stability we believe that further divisions of ranid frogs in the absence of a phylogenetic hypothesis will only result in additional confusion in an already incredibly complex history of names and species. We have initiated further biochemical studies on some genera, particularly *Amolops*, *Odorrana* and *Paa*, but also including Vietnamese species in the genus *Hylarana*. Future investigations using gene sequences from 12S and 16S rRNA of smaller subsets of species should prove equally fruitful for resolving relationships among the genera of ranid frogs.

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Table 1. Primers used for amplifying and sequencing fragments of RNA genes in the Subfamily Raninae. Sequence position indicates the starting position of the primer in the *Xenopus laevis* genome and is preceded by the amplification direction as indicated by (H) heavy or (L) light strand.

Name	Sequence 5' to 3'	<i>Sequence</i>	
		<i>position</i>	Reference
12S1L	CAAACCTGGGATTAGATAACCCACTAT	L2484	Kocher et al. (1989)
12S2H	AGGGTGACGGGCGGTGTGT	H2897	Kocher et al. (1989)
12S2L	ACACACCGCCCGTCACCCCTC	L2917	Fu (1999)
16S3H	GTA GCTCACTTGATTTCGGG	H3341	Fu (pers. comm..)
16S3L	CCCGAAATCAAGTGAGCTAC	L3362	Fu (pers. comm..)
16S1H	GGCTATGTTTGGTAAACAG	H3958	Modified from Palumbi (1996)
16S5H	CTACCTTGCACGGTTAGGATACCGCGGC	H4040	Fu (2000)
16S1M	CCGACTGTTACCAAAAACAT	L3955	Fu (1998)

16S2H CCGGATCCCCGGCCGGTCTGAACTCAGATCAC H4552 Palumbi (1996)

G

Table 2. Summary of genes sequenced from the ingroup and outgroup taxa. NT = Total number of taxa analyzed; TS = total number of homologous sites resolved; AS = number of ambiguous sites removed; NSR = number of homologous sites retained; NVS = number of variable sites; NPPIS = number of potentially phylogenetically informative sites; NMPTs = number of most parsimonious trees resolved; LMPTs = Length of most parsimonious solution; CI = consistency index; RI = retention index. Trees for the tRNA^{Val} gene were not calculated (n/a) owing to the limited number of characters (37) available to resolve nodes among the 52 taxa in the analysis.

Gene	NT	TS	AS	NSR	NVS	NPPIS	NMPTs	LMPTs	CI	RI
12S rRNA	53	546	29	517	335	236	6	1664	0.276	0.524
tRNA ^{Val}	52	72	2	70	48	37	n/a	n/a	n/a	n/a
16S rRNA	69	1509	25	1484	910	728	16	6279	0.237	0.530
All RNAs	70	2127	56	2071	1301	1012	3	8512	0.239	0.527

Table 3. A representative summary of the history of names applied to some of the Asian species of ranid frogs investigated in this study.

Specific epithet	Frost 1985	Dubois 1987 “1986”	Fei et al. 1990	Dubois 1992	Ye et al. 1993	Fei 1999
<i>hongkongensis</i>	<i>Amolops</i>	—	<i>Amolops</i>	<i>A. (Amolops)</i>	<i>Amolops</i>	<i>Amolops</i>
<i>loloensis</i>	<i>Amolops</i>			<i>A. (Amolops)</i>		
<i>ricketti</i>	<i>Amolops</i>	—	<i>Amolops</i>	<i>A. (Amolops)</i>	<i>Amolops</i>	<i>Amolops</i>
<i>erythraea</i>	<i>R. (Hylarana)</i>	—	—	<i>R. (Hylarana)</i>	—	—
<i>guentheri</i>	<i>R. (Hylarana)</i>	—	<i>H. (Hylarana)</i>	<i>R. (Sylvirana)</i>	<i>H. (Hylarana)</i>	<i>Hylarana</i>
<i>nasica</i>	<i>Amolops</i>	—	<i>Amolops</i>	<i>A. (Huia)</i>		<i>Amolops</i>
<i>johnsi</i> (as <i>sauteri</i>)	<i>R. (Hylarana)</i>	—	<i>Pseudorana</i>	<i>R. (Pseudorana)</i>	<i>Pseudorana</i>	<i>Pseudorana</i>
<i>livida</i>	<i>R. (Hylarana)</i>	—	<i>Odorrana</i>	<i>R. (Eburana)</i>	<i>Odorrana</i>	<i>Odorrana</i>
<i>macrodactyla</i>	<i>R. (Hylarana)</i>	—	<i>Hylarana</i> <i>(Tenuirana)</i>	<i>R. (Hylarana)</i>	<i>H.</i> <i>(Tenuirana)</i>	<i>H.</i> <i>(Tenuirana)</i>

<i>maosonensis</i>	<i>R. (Hylarana)</i>	—	—	<i>R. (Sylvirana)</i>	—	—
<i>milleti</i>	<i>R. (Hylarana)</i>	—	—	<i>R. (Sylvirana)</i>	—	—
<i>taipehensis</i>	<i>R. (Hylarana)</i>	—	<i>Hylarana</i> <i>(Tenuirana)</i>	<i>R. (Hylarana)</i>	<i>H.</i> <i>(Tenuirana)</i>	<i>H.</i> <i>(Tenuirana)</i>
<i>lateralis</i>	<i>Rana</i>	<i>Rana (Rana)</i>	—	<i>R. (Pelophylax)</i>	—	—
<i>pipiens</i>	<i>R. (Rana)</i>	<i>R. (Rana)</i>	—	<i>R. (Pantherana)</i>	—	—
<i>catesbeiana</i>	<i>R. (Rana)</i>	<i>R. (Rana)</i>	—	<i>R. (Aquarana)</i>	<i>R.</i> <i>(Aquarana)</i>	<i>R.</i> <i>(Aquarana)</i>
<i>fansipani</i>	<i>Rana (Paa?)</i>	—	—	<i>C. (Chaparana)</i>	—	—
<i>kuhlii</i>	<i>R. (Limnonectes)</i>	<i>L. (Limnonectes)</i>	<i>Limnonectes</i>	<i>L. (Limnonectes)</i>	<i>Limnonectes</i>	<i>Limnonectes</i>
<i>toumanoffi</i>	<i>R. (Euphlyctis)</i>	<i>L. (Bourretia)</i>	—	<i>L. (Bourretia)</i>	—	—
<i>blythii</i>	<i>R. (Euphlyctis)</i>	<i>L. (Limnonectes)</i>	—	<i>L. (Limnonectes)</i>	—	—
<i>rugulosus</i>	<i>R. (Euphlyctis)</i>	<i>L.</i> <i>(Hoplobatrachus)</i>	<i>Tigrina</i>	<i>Hoplobatrachus</i>	<i>Hoplobatrach</i> <i>us</i>	<i>Hoplobatra</i> <i>chus</i>
<i>cancrivorus</i>	<i>R. (Euphlyctis)</i>	<i>L.</i>	<i>Euphlyctis</i>	<i>L. (Fejervarya)</i>	<i>Euphlyctis</i>	<i>Fejervarya</i>

		(<i>Hoplobatrachus</i>)				
<i>limnocharis</i>	<i>R. (Euphlyctis)</i>	<i>L. (Fejervarya)</i>	<i>Euphlyctis</i>	<i>L. (Fejervarya)</i>	<i>Euphlyctis</i>	<i>Fejervarya</i>
<i>labrosa</i>	<i>Tomopterna</i>	<i>T. (Sphaeroteca)</i>	—	<i>T. (Sphaeroteca)</i>	—	—
<i>yunnanensis</i>	<i>R. (Paa)</i> <i>phrynoïdes</i>	<i>R. (Paa)</i>	<i>Paa (Paa)</i> <i>phrynoïdes</i>	<i>Paa</i> <i>(Gyandropaa)</i>	<i>Paa (Paa)</i>	<i>Paa (Paa)</i>
<i>parkeri</i>	<i>Altirana</i>	—	<i>Altirana</i>	<i>N. (Altirana)</i>	<i>Altirana</i>	<i>Nanorana</i>
<i>pleskei</i>	<i>Nanorana</i>	—	<i>Nanorana</i>	<i>N. (Nanorana)</i>	<i>Nanorana</i>	<i>Nanorana</i>
<i>chapaensis</i>	<i>Rana (Hylarana)</i>	—	—	<i>R. (Nidirana)</i>	—	—
<i>spinosa</i>	<i>R. (Paa)</i>	<i>R. (Paa)</i>	<i>Paa</i>	<i>Paa (Quasipaa)</i>	<i>R. (Paa)</i>	<i>Paa (Paa)</i>
<i>laevis</i>	<i>Occidozyga</i>	<i>Occidozyga</i>				
<i>lima</i>	<i>Occidozyga</i>	<i>Phrynobatrachus</i>				

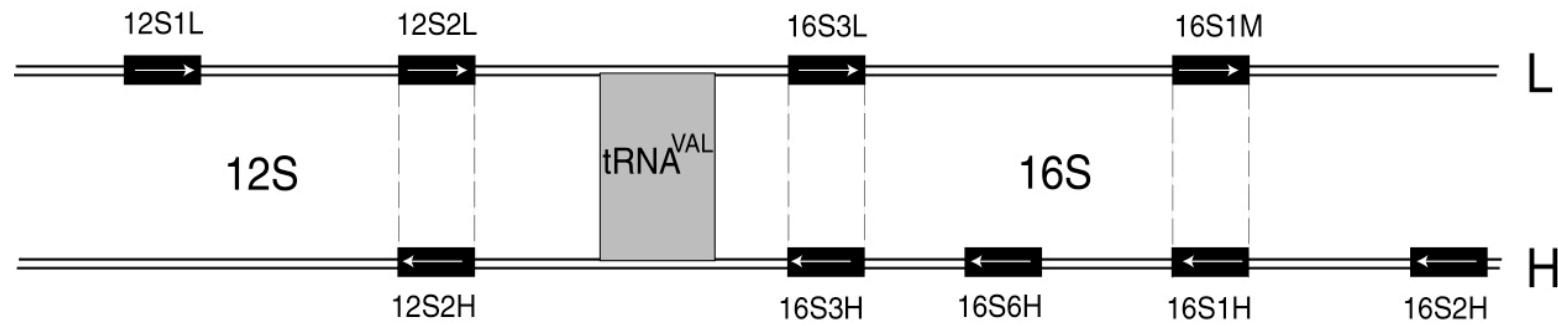


Figure 1. Schematic drawing of the 12S, tRNA^{Val}, and 16S mitochondrial gene and the relative positions of the primers used in this study. Hatched lines refer to primers that are complements to each other; L and H denote light and heavy strands, respectively. Specific primers are listed in Table 1.

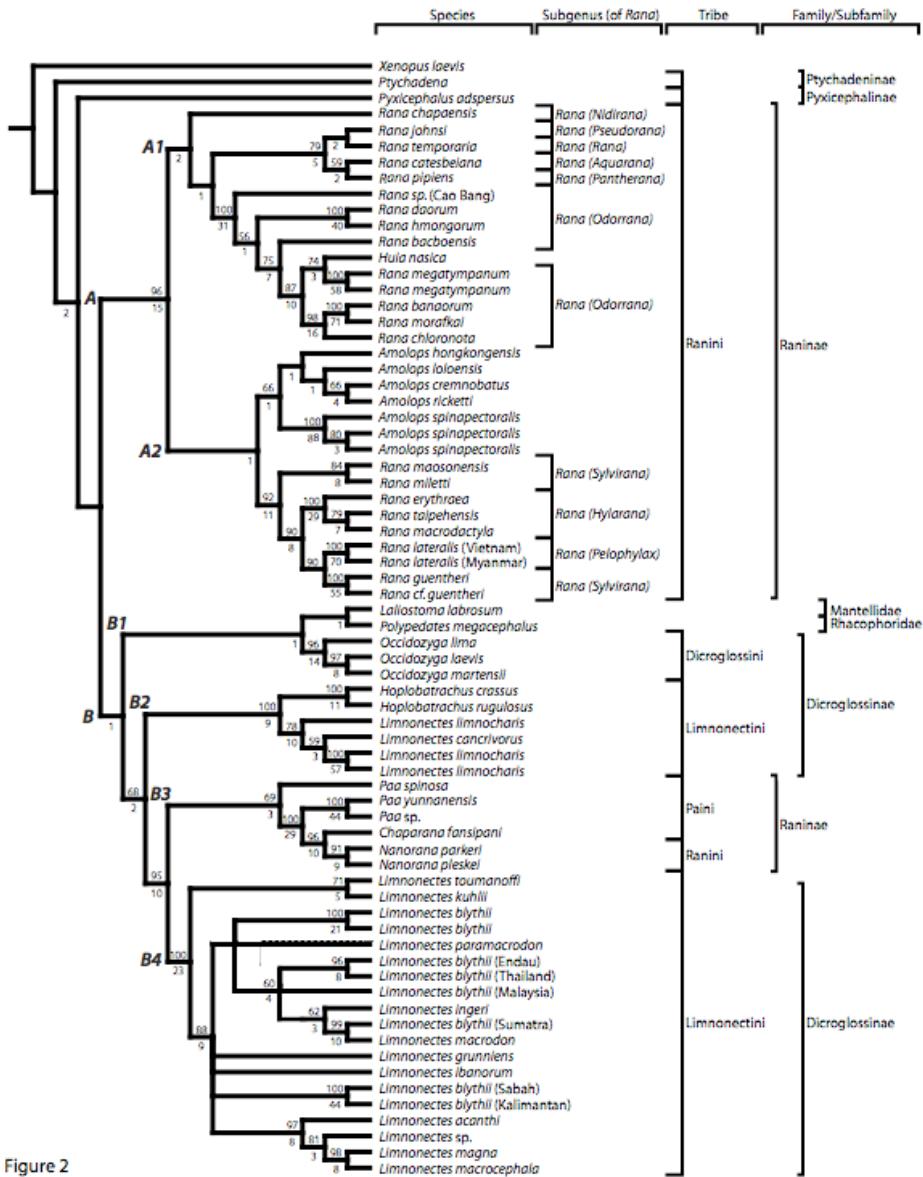


Figure 2

Figure 2. The strict consensus tree of the two most parsimonious explanations of mtDNA sequence data for Southeast Asian ranids. *Xenopus laevis* was used to root the tree. Taxonomy reflects current usage. Taxonomic groupings proposed by Dubois, 1992 appear to the right of the tree. Numbers above the line are bootstrap proportions (≥ 50) and those below are Bremer decay indices examined up to six steps longer than the most parsimonious trees.

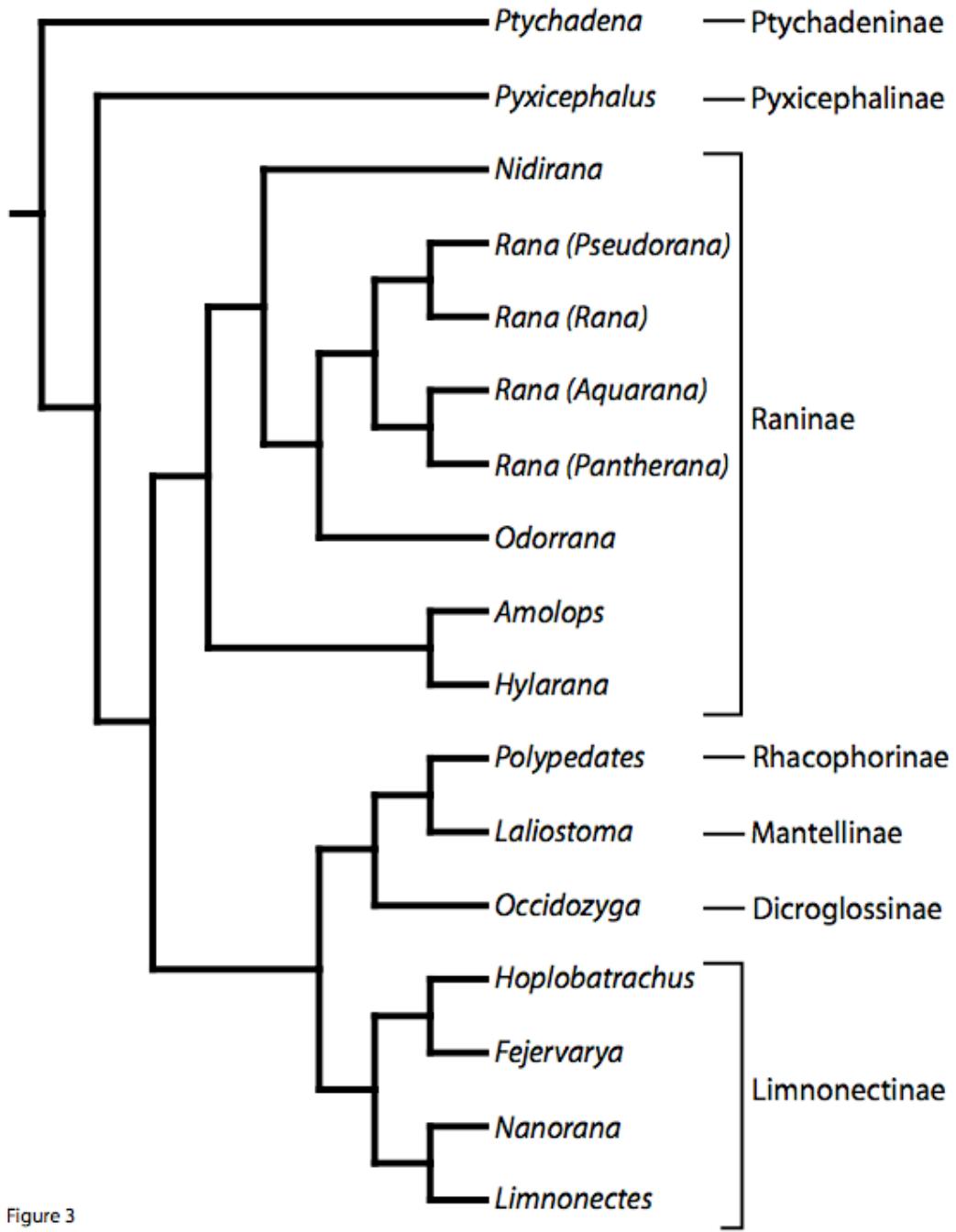


Figure 3

Figure 3. A reduced cladogram with genera as terminal taxa depicting nomenclatorial adjustments. To the right of the tree are the subfamilial taxonomic groupings supported by this study. Taxonomy reflects the recommendations of this manuscript.

Appendix 1. Species sequenced, voucher specimen catalog numbers, and collecting locality. The taxonomy generally follows Frost (2004).

GenBank				
Taxon number		Species	Voucher No.	Locality
#	12S/tRNA			
		/16S		
		AF206077		
1	206122	<i>Amolops cremnobates</i>	ROM 14534	Khe Moi R., Nghe An Prov., Vietnam
	206458			
		AF206072		
2	206117	<i>Amolops hongkongensis</i>	ROM 16300	Hong Kong, China
	206453			
		AF206112		
3	206157	<i>Amolops lolensis</i>	JF101	Sichuan, China
	206493			
		AF206073		
4	206118	<i>Amolops ricketti</i>	ROM 16818	Tam Dao, Vinh Phu Prov., Vietnam
	206454			
		AF206074		
5	206119	<i>Amolops spinapectoralis</i>	ROM 7548	Tram Lap, Gia Lai Prov., Vietnam
	206455			

		AF206075			
6	206120	<i>Amolops spinapectoralis</i>	ROM 7555	Tram Lap, Gia Lia Prov.,	Vietnam
	206456				
		AF206076		Ngoc Linh, Kon Tum	
7	206121	<i>Amolops spinapectoralis</i>	ROM 27456	Prov., Vietnam	
	206457				
		AF206093		Sa Pa, Lao Cai Prov.,	
8	206138	<i>Chaparana fansipani</i>	ROM 28286	Vietnam	
	206474				
		AF206080		Tam Dao, Vinh Phu Prov.,	
9	206125	<i>Huia nasica</i>	ROM 16640	Vietnam	
	206461				
		AF206084		Ba Be, Cao Bang Prov.,	
10	206129	<i>Hoplobatrachus rugulosus</i>	ROM 19405	Vietnam	
	206465				
		AF206082		Tram Lap, Gia Lia Prov.,	
11	206127	<i>Limnonectes blythii</i>	ROM 7144	Vietnam	
	206463				
		AF206092		Dumaguete City, Negros	
12	206137	<i>Limnonectes cancrivorus</i>	ROM 1059	Island, Philippines	
	206473				
13	AF206083	<i>Limnonectes kuhlii</i>	ROM 19384	Khe Moi R., Nghe An	

	206128			Prov., Vietnam
	206464			
14	AF206081	<i>Limnonectes</i>		Hanoi, Ha Tay Prov.,
	206126	<i>limnocharis</i>	ROM 12574	Vietnam
	206462			
15	AF206085	<i>Limnonectes</i>	USNM	
	206130	<i>limnocharis</i>	520407	Myanmar
	206466			
16	AF206115			
	206160	<i>Limnonectes toumanoffi</i>	ROM 22081	Yok Don, Dac Lac Prov.,
	206496			Vietnam
17	AF206110			
	206155	<i>Nanorana parkeri</i>	JF037	Tibet, China
	206491			
18	AF206111			
	206156	<i>Nanorana pleskei</i>	JF118	Sichuan, China
	206492			
19	AF206116			
	206161	<i>Occidozyga lima</i>	ROM 25003	Chi Linh, Hai Duong,
	206497			Vietnam
20	AF206086			
	206131	<i>Occidozyga martensii</i>	ROM 22222	Yok Don, Dac Lac Prov.,
	206467			Vietnam

			AF206087		
21	206132	<i>Paa</i> sp.		AMNH 13199	Sa Pa, Lao Cai Prov., Vietnam
	206468				
			AF206088		
22	206133	<i>Paa spinosa</i>		ROM 13189	Khe Moi R., Nghe An Prov., Vietnam
	206469				
			AF206089		
23	206134	<i>Paa yunnanensis</i>		ROM 19128	Sa Pa, Lao Cai Prov., Vietnam
	206470				
			AF206090		
24	206135	<i>Ptychadena</i> sp.		JPB6253	Africa
	206471				
			AF206091		
25	206136	<i>Pyxicephalus adspersus</i>		JPB6584	Africa
	206472				
			AF206099		
26	206144	<i>Rana bacboensis</i>		ROM 13044	Khe Moi R., Nghe An Prov., Vietnam
	206480				
			AF206106		
27	206151	<i>Rana banaorum</i>		ROM 7472	Tram Lap, Gia Lai Prov., Vietnam
	206487				

			<i>Rana chapaensis</i>		
28	206124	AF206079		ROM 28070	Sa Pa, Lao Cai Prov., Vietnam
	206460				
		AF206104			
29	206149		<i>Rana chloronota</i>	ROM 14885	Hong Kong, China
	206485				
		AF206101			
30	206146		<i>Rana daorum</i>	ROM 19053	Sa Pa, Lao Cai Prov., Vietnam
	206482				
		AF206094			
31	206139		<i>Rana erythraea</i>	ROM 7296	Tram Lap, Gia Lai Prov., Vietnam
	206475				
		AF206095			
32	206140		<i>Rana guentheri</i>	ROM 12573	Hanoi, Ha Tay Prov., Vietnam
	206476				
		AF206102			
33	206147		<i>Rana hmongorum</i>	ROM 19112	Sa Pa, Lao Cai Prov., Vietnam
	206483				
		AF206096			
34	206141		<i>Rana johnsi</i>	ROM 24230	Chi Linh, Hai Duong, Vietnam
	206477				

		AF206097		
35	206142	<i>Rana lateralis</i>	ROM 22153	Yok Don, Dac Lac Prov., Vietnam
	206478			
		AF206098	USNM	
36	206143	<i>Rana lateralis</i>	520399	Myanmar
	206479			
		AF206108		
37	206153	<i>Rana macrodactyla</i>	ROM 25697	Chi Linh, Hai Duong, Vietnam
	206489			
		AF206107		
38	206152	<i>Rana cf. maesonensis</i>	ROM 24274	Chi Linh, Hai Duong, Vietnam
	206488			
		AF206100		
39	206145	<i>Rana megatymanum</i>	ROM 13046	Khe Moi R., Nghe An Prov., Vietnam
	206481			
		AF206105		
40	206150	<i>Rana megatymanum</i>	ROM 7038	Pac Ban, Tuyen Quang Prov., Vietnam
	206486			
		AF206109		
41	206154	<i>Rana milleti</i>	ROM 7240	Tram Lap, Gia Lai Prov., Vietnam
	206490			

		AF206103		
42	206148	<i>Rana morafkai</i>	ROM 7446	Tram Lap, Gia Lia Prov., Vietnam
	206484			
		AF206113		
43	206158	<i>Rana sp. cf. guntheri</i>	ROM 25908	Chi Linh, Hai Duong Prov., Vietnam
	206494			
		AF206078		
44	206123	<i>Rana sp.</i>	ROM 26476	Cao Bang, Cao Bang Prov., Vietnam
	206459			
		AF206114		
45	206159	<i>Rana taipehensis</i>	ROM 7193	Tram Lap, Gia Lia Prov., Vietnam
	206495			