

Phylogenetic relationships of the mustache toads inferred from mtDNA sequences

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Abstract

Species of the genus *Vibrissaphora* are unique among all anurans in that males exhibit external cornified spines in the maxillary region during the breeding season. They were separated from species of the genus *Leptobrachium* based on this unique character. We construct a phylogeny using the 16S, ND4, and cytochrome *b* mitochondrial genes of 42 individuals from eight species of *Vibrissaphora* and five species of *Leptobrachium* from mainland China, Southeast Asia, and Hainan Island. Species of both *Oreolalax* and *Scutigera* were used as outgroups. The results indicate that: *L. huashen* and *L. chapaense* form a clade that is nested within *Vibrissaphora*, and *L. hainanense* is the sister taxon to the clade comprising all *Vibrissaphora* plus *L. chapaense* and *L. huashen*; *V. boringiae* is grouped with a clade consisting of *V. leishanensis*, *V. liui*, and *V. yaoshanensis*; and *V. yaoshanensis* is a species separate from *V. liui*. We propose taxonomic changes that reflect these findings. Also based on the resulting phylogenetic trees, we propose that the mustache toads originated in the trans-Himalayan region of southwest China, and that the evolution of maxillary spines, large body size, and reverse sexual size dimorphism in these frogs was influenced by intrasexual selection due to adopting a resource-defense polygyny mating system.

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1. Introduction

The mustache toads of the genus *Vibrissaphora* (Anura: Megophryidae) are restricted to southern China and Vietnam. They are defined by having external cornified spines in the maxillary region of males during the breeding season and reverse sexual size dimorphism in which the males are generally larger than the females. Seven species of *Vibrissaphora* have been described from China; *V. ailaonica* Yang et al. (1983), *V. boringiae* Liu (1945), *V. jiulongshanensis* Wei and Zhao (1981), *V. leishanensis* Liu and Hu (1973), *V. liui* Pope (1947), *V. promustache* Rao et al. (2006), and *V. yaoshanensis* Ye et al. (1978), and two species from Viet-

nam, *V. echinata* (Dubios and Ohler, 1998) and *V. ngo-clinhensis* Orlov (2005). *Vibrissaphora jiulongshanensis* was later considered a synonym of *V. liui* (Huang et al., 1984; Wu and Liu, 1986; Fei et al., 1995), and *V. yaoshanensis* was demoted to a subspecies of *V. liui* (Wu and Zhao, 1987; Fei et al., 1995). *Vibrissaphora echinata* was considered a synonym of *V. ailaonica* by Ho et al. (1999), but its species rank was defended by Ohler et al. (2000) and Grosjean (2001) based on differences in eye color, number of maxillary spines, and larval characters.

Species within *Vibrissaphora* were divided into two species groups (a *V. boringiae* species group of *V. ailaonica*, *V. boringiae*, and *V. echinata*, and a *V. liui* species group of *V. leishanensis* and *V. liui*) based on the number and arrangement of external spines in the maxillary region but without examining the phylogeny of this genus (Fei et al., 1995; Dubios and Ohler, 1998; Fei et al., 2005). *V. ngo-clinhensis*

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and *V. promustache* were not placed within a species group. *V. promustache* in particular was considered a distant relative of other species of *Vibrissaphora* based on its possession of a mixture of morphological characters from *Vibrissaphora* and *Leptobrachium*. *Leptobrachium*-like characters included a small body size, typical anuran sexual size dimorphism with the females larger than males, and presence of vocal sacs, while *Vibrissaphora*-like characters were presence of maxillary spines, absence of linea masculina, indistinct tympanum, enlarged arms in the male, and rough skin (Rao et al., 2006).

Species of *Vibrissaphora* are morphologically very similar to species of the genus *Leptobrachium* to the point that *Vibrissaphora* was considered a junior synonym (Dubios, 1980) or subgenus of the latter (Tian and Hu, 1985; Dubios and Ohler, 1998). Dubios (1980) argued that the presence of spines was not sufficient to separate *Vibrissaphora* from *Leptobrachium*. However, Ho et al. (1999) argued that this character distinguishes the two genera and suggested that *Vibrissaphora* be retained pending a phylogenetic analysis. Recent molecular phylogenetic studies on megophryid frogs have even placed the two genera as sister taxa, with the genera *Oreolalax*, *Scutigera*, and *Leptolalax* occurring outside a *Leptobrachium/Vibrissaphora* clade (Zheng et al., 2004a,b). However, only two species of *Vibrissaphora* and one to two species of *Leptobrachium* were sampled in these studies; therefore, monophyly of these two genera could not be rigorously tested.

The purpose of the maxillary spines is not known but assumed to be for aggressive behavior (Duelman and Trueb, 1986). This assumption is probably based on the fact that: (1) maxillary spines are unique to this genus, and weapons (spines or tusks) and/or reverse sexual size dimorphism are uncommon, known in only 10% of frog species (Shine, 1979); and (2) in the few species whose males possess weapons and grow larger than females, male–male combat has been observed (Shine, 1979; Duelman and Trueb, 1986). Direct male–male interaction, such as fighting, has not been observed in mustache toads, as behavioral studies have not been conducted.

From limited observations, it is known that the maxillary spines develop on males only during the breeding season and drop off afterwards (Wu and Yang, 1981; Chen et al., 1984; Ho et al., 1999). During the breeding season, males move to streams from terrestrial habitat, construct submerged nest sites under stones by excavating sand creating a depression, and then call from within the underwater sites to attract females. Upon entering a nest site, the females are amplexed by the males and oviposit the eggs as an egg mass, which is then attached to the undersurface of the stone (Wu and Yang, 1981; Chen et al., 1984; Fei and Ye, 1984; Gu et al., 1986, 1988; Tang, 1990; Dubios and Ohler, 1998; Ho et al., 1999; Zheng and Fu, 2007). In *V. boringiae*, a female has been observed entering a nest site of a calling male, where the male engages her in amplexus. If the female leaves the nest site with the male in amplexus he immediately releases her and returns to

the nest site (Zheng and Fu, 2007) suggesting that he is defending the nest site and not just using it as a calling site.

Male nest building and defense are uncommon, and are usually associated with a resource-defense mating system (Sullivan et al., 1995). Halliday and Tejedo (1995) also noted that in frogs, a relatively larger male body size in relation to the female seems to be associated with resource-defense mating systems, whereas males that defend only calling sites or fight for possession of females are usually considerably smaller than females, even when large males are more likely to mate. The combination of large adult body size, reverse sexual size dimorphism, and weapons has been observed only in species that utilize resource-defense polygyny, such as *Adelotus brevis* (Katsikaros and Shine, 1997), *Hyla rosenborgi* (Kluge, 1981), *Pyxicephalus adspersus* (Cook et al., 2001), *P. edulis* (Brack and Maguire, 2005), and some species of *Limnonectes* (Emerson and Inger, 1992; Emerson and Voris, 1992; Orlov, 1997; Tsuji and Matsui, 2002; Tsuji, 2004).

Anurans that utilize this type of mating system are typically prolonged breeders as opposed to explosive breeders (Sullivan et al., 1995). Mustache toads breed over a period of 2 weeks to 2 months depending on the species (Fei, 1999). The skin of the breeding male becomes loose (Wu and Yang, 1981; Chen et al., 1984; Gu et al., 1986; Tang, 1990; Ho et al., 1999), which is thought to increase surface area facilitating cutaneous respiration (Ho et al., 1999). Cutaneous respiration would be needed for continual sub-aerial calling and defense of a nest site during the breeding season. Multiple egg masses have also been observed attached to the bottom of the stone within a nest that was defended by a single male of *V. ailaonica* (Ye et al., 1993) suggesting that this male mated with the several females that oviposited in the nest. Wu and Yang (1981) also stated that males of several species engage in multiple matings during a breeding season, though they did not state that they actually observed this behavior.

Based on these observations and comparisons with other anuran species, we hypothesize that the males of mustache toads engage in a resource-defense polygyny mating system by constructing and then defending nest sites in order to increase their attractiveness to females (Emlen and Oring, 1977). The nest site constructed under an in-stream flat bottom stone with the correct water parameters for embryonic development is likely the limiting resource that the male defends. We also hypothesize that adopting this type of mating system sets the stage for sexual selection, specifically intrasexual selection, to act on the evolution of maxillary spines concomitant with larger body sizes in males, producing reverse sexual size dimorphism in these species (Halliday, 1978; Bradbury and Davies, 1987; Halliday and Tejedo, 1995).

The purpose of this study is to reconstruct the phylogenetic relationships among most of the species of *Vibrissaphora* and between *Vibrissaphora* and *Leptobrachium* in order to examine the present taxonomy within *Vibrissaphora*. In particular, we test hypotheses of monophyly for *Leptob-*

rachium, *Vibrissaphora*, and the species groups, species, and subspecies of *Vibrissaphora*.

We also investigate the evolution of spine development, reverse sexual size dimorphism, and other characters within this group of frogs by determining where these historical transformations map onto the constructed phylogenetic tree(s). We then use these evolutionary patterns and the known breeding biology of each species to test the hypothesis that the mustache toads adopted a resource-defense polygyny mating system, and by doing so, the characters that define this group of frogs, mainly presence of maxillary spines, large adult body size, and reverse sexual size dimorphism, evolved as a result of intrasexual selection. We would expect a greater degree of sexual dimorphism to be exhibited in the derived taxa of the mustache toad clade due to intensive intrasexual selection on the species displaying these characters (West-Eberhard, 1983; Emerson, 1994).

Finally, we present an hypothesis of the biogeography of the mustache toads based on a relationship of the present distribution and phylogeny.

2. Materials and methods

2.1. Taxon sampling

A total of 42 samples corresponding to 13 ingroup species and five outgroup species were used in this study. Most of the individual specimens from China were collected in the field from 1993 to 2004 by the first author. The remaining specimens were loaned from colleagues in China and institutions outside China (Table 1). Tissues (liver or muscle) were kept in 95% ethanol and stored at -4°C until use.

Eight of the nine named taxa of *Vibrissaphora* were sampled; *V. ngoclinensis* was not sampled due to its unavailability. All specimens except *V. echinata* were collected from the type localities or near the type localities of the named taxa (see Table 1 for localities). Specimens of *V. echinata* were collected from Fenshuiling Mountain, Jinping County, and Dawei Mountain on the opposite side of the Hong He (Red River), Pinbian County, Yunnan Province, both near the Vietnamese border. These localities are approximately 65 km (Pinbian) to 80 km (Jinping) north of the type locality for this species. These specimens match the description by Dubios and Ohler (1998) by possessing a light green upper iris. This character was defended by Ohler et al. (2000) as a strong character to distinguish *V. echinata* from *V. ailaonica*. The maximum number of maxillary spines from specimens collected at these localities was one less than one of the two male specimens of *V. echinata* from Vietnam at 51, but consistently more than specimens of *V. ailaonica* from Ailao Mountain to the northwest (see, Fei et al., 1995; Dubios and Ohler, 1998). Additionally, specimens of *V. echinata* from Pinbian and Jinping Counties were found breeding in the fall, similar to those from Vietnam (Dubios and Ohler, 1998), whereas specimens of *V. ailaonica* from further north in Ailao and Wuliang

Mountains were found breeding in the spring, typical of this species (Chen et al., 1984). Based on the light green eye color, the higher number of maxillary spines, close proximity and same breeding season of these specimens to those from the type locality of *V. echinata*, we conclude that these specimens are *V. echinata* sensu Dubios and Ohler.

2.2. DNA amplification and sequencing

Most of the template DNA was extracted by a standard SDS/proteinase K digestion, phenol:chloroform extraction method (Sambrook et al., 1989; Hillis et al., 1996). The remaining DNA samples were extracted using the Qiagen DNeasy tissue kit (Qiagen Inc.) following manufacturer's instructions.

Polymerase chain reactions (PCRs) were run in a total volume of 100 or 50 μL in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems). Amplification of genomic DNA was conducted by first denaturing for 7 min at 94°C , then 40 cycles of denaturing at 94°C for 30 s, annealing at 46, 48, or 50°C (depending on sample) for 30 s, and elongation at 72°C for 1 min, with Life Technologies (GIBCO-BRL) Taq Polymerase, with a final extension at 72°C for 7 min. Negative controls were run on all amplifications to check for contamination. The primers used for PCR are listed in Table 2. Segments from the mitochondrial 16S rRNA gene (16S) and cytochrome *b* gene (cyt *b*) were respectively amplified by a pair of primers each, but the mitochondrial ND4 gene was amplified by one forward primer and two reverse primers (Leu and PII).

Some amplified products were purified using Promega Wizardprep[®] PCR Preps DNA Purification System (Promega) and others with Qiagen Qiaquick Purification Kit (Qiagen) according to manufacturers' instructions. Cycle-sequencing reactions were performed on purified PCR products using an ABI Prism Big Dye[®] Terminator version 3.1 DNA Sequencing Kit (Perkin-Elmer) with denaturation at 96°C for 10 s, annealing at 45°C for 5 s, and extension at 60°C for 4 min, for 50 cycles in 10 μL volumes in a Perkin-Elmer 9600 PCR Machine. The nucleotide sequences were determined on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Both forward and reverse chains of each individual template sample were sequenced. Sequences were aligned using the program CLUSTAL X 1.81 (Jeanmougin et al., 1998) and inspected by eye to maximize positional homology. All sequences were deposited in GenBank (Table 1).

2.3. Genetic diversity and phylogenetic analyses

Base compositional information for the three genes was estimated from aligned sequences using MEGA 3.0 (Kumar et al., 2004). A 1097 base pair (bp) sequence from the cyt *b* gene, together with a 572-bp sequence from the 16S gene, and an 897-bp sequence from the ND4 gene were combined. A maximum parsimony (MP) analysis using

Table 1
Sequences used in this study

Species	Specimen No.	Source ^a	Source locality ^b	GenBank Accession No.		
				ND4	16S	Cyt <i>b</i>
<i>Leptobranchium chapaense</i>	RDQ193	DQR-Banna-Leptob001	Border area of Xishuangbanna Co. and Simao City, Yunnan Province	EU180939	EU180855	EU180897
<i>L. chapaense</i>	RDQ205	DQR-DDG Tadpole01	Border area of Xishuangbanna Co. and Simao City, Yunnan Province	EU180940	EU180856	EU180898
<i>L. chapaense</i>	RDQ088	DQR-Luchun Tadpole01	Huanglianshan, Luchun Co., Yunnan Province	EU180941	EU180857	EU180899
<i>L. hainanense</i>	RDQ120	SQL-Hainan-Leptob01	Diaoluoshan Mt., Lingshui Co., Hainan Province	EU180969	EU180885	EU180927
<i>L. huashen</i>	RDQ156	KIZ-L'95022	Kungang, Jingdong Co., Yunnan Province	EU180933	EU180849	EU180891
<i>L. huashen</i>	RDQ158	KIZ-LC96002	Huangcaoling, Jingdong Co., Yunnan Province	EU180934	EU180850	EU180892
<i>L. huashen</i>	RDQ157	KIZ-L'95024	Kungang, Jingdong Co., Yunnan Province	EU180935	EU180851	EU180893
<i>L. huashen</i>	RDQ183	KIZ-LC96003	Huangcaoling, Jingdong Co., Yunnan Province	EU180936	EU180852	EU180894
<i>L. huashen</i>	RDQ155	KIZ-L9504	Dahaoping, Tengchong Co., Yunnan Province	EU180937	EU180853	EU180895
<i>L. huashen</i>	RDQ258	KIZ-GLGS0798	Xiaoheishan, Longling Co., Yunnan Province	EU180938	EU180854	EU180896
<i>L. montanum</i>	RDQ221	FMNH236661	Tenom District, Sabah, Malaysia	EU180964	EU180880	EU180922
<i>L. montanum</i>	RDQ222	FMNH236663	Tenom District, Sabah, Malaysia	EU180965	EU180881	EU180923
<i>L. montanum</i>	403A80PII	FMNH236699	Sipitang District, Sabah, Malaysia	EU180966	EU180882	EU180924
<i>L. montanum</i>	RDQ346	FMNH236700	Sipitang District, Sabah, Malaysia	EU180967	EU180883	EU180925
<i>L. smithi</i>	329B29	CAS222215	Mon State, Myanmar	EU180968	EU180884	EU180926
<i>Oreolalax chuanbeiensis</i>	RDQ106	DQR-Pingwu-001J	Wangbachu, Pingwu Co., Sichuan Province	EU180971	EU180887	EU180929
<i>O. omeimontis</i>	RDQ109	KIZ-O.096002	Emei Mt., Emei City, Sichuan Province	EU180970	EU180886	EU180928
<i>O. jingdongensis</i>	RDQ162	KIZ-O.J'96011	Ailao Mt., Jingdong Co., Yunnan Province	EU180972	EU180888	EU180930
<i>Scutigera sp.</i>	RDQ356	CAS228188	Shibali, Fugong Co., Yunnan Province	EU180973	EU180889	EU180931
<i>S. cf. mammatum</i>	RDQ127	DQR-Scut-GS Yako01	Dulong Valley pass, Gongshan Co., Yunnan Province	EU180974	EU180890	EU180932
<i>Vibrissaphora ailaonica</i>	RDQ174	DQR-Jingdong 2001VA03	Mid-Wuliang Mt., Jingdong Co., Yunnan Province	EU180947	EU180863	EU180905
<i>V. ailaonica</i>	RDQ064	DQR-Nanjian VA01	Northern Wuliang Mt., Nanjian Co., Yunnan Province	EU180949	EU180865	EU180907
<i>V. ailaonica</i>	RDQ089	DQR-Nanjian VA02	Northern Wuliang Mt., Nanjian Co., Yunnan Province	EU180948	EU180864	EU180906
<i>V. ailaonica</i>	RDQ152	KIZ-VA96004	Mid-Ailao Mt., Jingdong Co., Yunnan Province	EU180946	EU180862	EU180904
<i>V. ailaonica</i>	RDQ153	KIZ-VA96007	Mid-Ailao Mt., Jingdong Co., Yunnan Province	EU180950	EU180866	EU180908
<i>V. boringiae</i>	RDQ118	DQR-Daguan Tadpole01	Huanglianhe, Daguan Co., Yunnan Province	EU180942	EU180858	EU180900
<i>V. boringiae</i>	RDQ134	DQR-Daguan VB01	Huanglianhe, Daguan Co., Yunnan Province	EU180943	EU180859	EU180901
<i>V. boringiae</i>	RDQ133	DQR-Daguan VB02	Huanglianhe, Daguan Co., Yunnan Province	EU180944	EU180860	EU180902
<i>V. boringiae</i>	RDQ150	KIZ-VB96011	Emei Mt., Emei City, Sichuan Province	EU180945	EU180861	EU180903
<i>V. echinata</i>	RDQ196	KIZ-JP2001A001	Fenshuiling Mt., Jinping Co., Yunnan Province	EU180951	EU180867	EU180909
<i>V. echinata</i>	RDQ197	KIZ-JP2001A002	Dawei Mt., Pinbian Co., Yunnan Province	EU180952	EU180868	EU180910
<i>V. jiulongshanensis</i>	RDQ148	DQR-Zhejiang VJ07	Jiulong Mt., Xuchang Co., Zhejiang Province	EU180958	EU180874	EU180916
<i>V. jiulongshanensis</i>	RDQ149	DQR-Zhejiang VJ08	Jiulong Mt., Xuchang Co., Zhejiang Province	EU180959	EU180875	EU180917
<i>V. leishanensis</i>	RDQ107	DQR-Leishan VL01	Leishan Mt., Leishan Co., Guizhou Province	EU180956	EU180872	EU180914
<i>V. leishanensis</i>	RDQ108	DQR-Leishan VL02	Leishan Mt., Leishan Co. Guizhou Province	EU180957	EU180873	EU180915
<i>V. liui liui</i>	RDQ146	DQR-Fujian VL03	Wuyi Mt., Chongan Co. (Wuyi City), Fujian Province	EU180960	EU180876	EU180918
<i>V. l. liui</i>	RDQ147	DQR-Fujian VL04	Wuyi Mt., Chongan Co. (Wuyi City), Fujian Province	EU180961	EU180877	EU180919
<i>V. l. yaoshanensis</i>	RDQ143	KIZ-VY95003	Yaoshan Mt., Jinxiu Co., Guangxi Province	EU180962	EU180878	EU180920
<i>V. l. yaoshanensis</i>	RDQ144	KIZ-VY95005	Yaoshan Mt., Jinxiu Co., Guangxi Province	EU180963	EU180879	EU180921
<i>V. promustache</i>	RDQ119	KIZ-PB-Vsp03004	Dawei Mt., Pinbian Co., Yunnan Province	EU180953	EU180869	EU180911
<i>V. promustache</i>	RDQ139	KIZ-PB-Vsp03009	Dawei Mt., Pinbian Co., Yunnan Province	EU180954	EU180870	EU180912
<i>V. promustache</i>	RDQ124	KIZ-PB-Vsp03001	Dawei Mt., Pinbian Co. Yunnan Province	EU180955	EU180871	EU180913

^a Institutional abbreviations are as listed in Leviton et al. (1985). DQR and SQL are personal collection numbers of Ding-Qi Rao and Shun-Qing Lu.

^b County and Province names in China, and equivalent administrative units from other countries.

Table 2
Primers used for PCR and cycle-sequencing

Primer	Sequence	Source
16S		
Sar	5' CGCCTGTTTATCAAAAACAT 3'	Palumbi et al. (1991)
Sbr	5' CCGGTCTGAACTCAGATCACGT 3'	Palumbi et al. (1991)
ND4		
ND4	5' TGACTACCAAAAAGCTCATGTAGAAGC 3'	Arévalo et al. (1994)
Leu	5' CATTACTTTTACTTGGATTGACCA 3'	Arévalo et al. (1994)
PII	5' TACTTTTACTTGGAGTTGCA 3'	Arévalo et al. (1994)
Cyt b		
FrogGlu-f	5' TGATCTGAAAAACCACCGTTG 3'	Hillis et al. (1996)
FrogThr-r	5' CTCATTCTTCGRCTTACAAG 3'	Hillis et al. (1996)

PAUP* 4.0b10 (Swofford, 2002) and a Bayesian analysis using MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) were performed on this 2566-bp composite sequence. Each nucleotide was treated as an unordered character with four alternative states, and gaps were considered missing data in all analyses.

In the MP analysis, data were treated with equal weight (Kjer, 1995; Allard and Carpenter, 1996; Cibois et al., 1999). We performed 100 heuristic searches with the starting trees obtained by random stepwise addition, followed by tree-bisection-reconnection (TBR) branch-swapping. We also performed a nonparametric bootstrap analysis (Felsenstein, 1985) with 1000 replicates, each executed as a heuristic search as above, to evaluate support for relationships, as implemented in PAUP* 4.0b10.

In the Bayesian analysis, the dataset was partitioned into three sets according to the three genes for separate evaluations in the program Modeltest version 3.7 (Posada and Crandall, 1998). The general-time-reversal model of base substitution (Nei and Kumar, 2000) was selected for all three partitions using the Akaike Information Criterion in Modeltest. For the tree-partition analysis, the Monte Carlo Markov Chains were simulated for 10,000,000 generations and sampled every 100 generations. Four chains were run and 2500 initial trees were discarded (burn-in).

For both phylogenetic analyses, three species of *Oreolalax* and two species of *Scutiger*, the two closest relatives of *Vibrissaphora* and *Leptobranchium* (Zheng et al., 2004a,b), were designated as outgroup taxa to root the trees. We conducted a parsimony based Wilcoxon signed-ranks' test (Templeton, 1983) to determine if the molecular data were significantly inconsistent with a *Vibrissaphora* monophyly. We consider bootstrap support of 70% and higher and Bayesian posterior probability of 95% and higher to be strong support for a clade.

2.4. Ancestral state reconstruction

We examined the historical patterns of change in sexual dimorphism and reproductive behavior by mapping morphological and behavioral characters onto the resulting

phylogenetic trees using MacClade version 4.03 (Maddison and Maddison, 2001). Table 3 is a matrix of the following characters and their states expressed in the respective species: (1) linea masculina (0 = present, 1 = absent), (2) tadpole size (0 = small, 1 = large), (3) upper iris (0 = dark, 1 = light), (4) maxillary spines (0 = absent, 1 = present), (5) V-shaped mark at base of tail of tadpole (0 = absent, 1 = present), (6) nest construction by male (0 = no, 1 = yes), (7) mean body size in male (0 = less than 60 mm, 1 = between 60 and 80 mm, 2 = over 80 mm), (8) vocal sacs (0 = present, 1 = absent), (9) skin in breeding males (0 = not loose, 1 = loose), (10) number of maxillary spines (0 = under 20, 1 = between 10 and 20, 2 = over 20). We performed a parsimony based ancestral character state reconstruction analysis on these characters as implemented in the program Mesquite version 1.2 (Maddison and Maddison, 2006) to assess the evolution of each character on the trees.

Table 3

A character matrix of morphological and behavioral characters to be used in an ancestral state reconstruction analysis

Species/characters	1	2	3	4	5	6	7	8	9	10
<i>Oreolalax</i> spp.	0	0	0	0	0	0	0	0	0	N/A
<i>Scutiger</i> spp.	0	0	0	0	0	0	0	0	0	N/A
<i>L. montanum</i>	0	0	0	0	0	0	0	0	0	N/A
<i>L. smithi</i>	0	0	0	0	0	0	0	0	0	N/A
<i>L. hainanense</i>	0	1	1	0	0	0	0	0	0	N/A
<i>V. promustache</i>	1	1	1	1	1	1	0	0	0	2
<i>V. ailaonica</i>	1	1	1	1	1	1	1	1	1	2
<i>V. echinata</i>	1	1	1	1	1	1	1	1	1	2
<i>V. boringiae</i>	1	1	1	1	1	1	1	1	1	1
<i>V. yaoshanensis</i>	1	1	1	1	1	1	2	0	1	0
<i>V. leishanensis</i>	1	1	1	1	1	1	2	1	1	0
<i>V. liui</i>	1	1	1	1	1	1	2	1	1	0
<i>L. chapaense</i>	0	1	1	0	1	0	0	0	0	N/A
<i>L. huashen</i>	0	1	1	0	1	0	0	0	0	N/A

(1) Linea masculina (0 = present, 1 = absent), (2) tadpole size (0 = small, 1 = large), (3) upper iris (0 = dark, 1 = light), (4) maxillary spines (0 = absent, 1 = present), (5) V-shaped mark at base of tail of tadpole (0 = absent, 1 = present), (6) nest construction by male (0 = no, 1 = yes), (7) mean body size in male (0 = less than 60 mm, 1 = between 60 and 80 mm, 2 = over 80 mm), (8) vocal sacs (0 = present, 1 = absent), (9) skin in breeding males (0 = not loose, 1 = loose), (10) number of maxillary spines (0 = under 20, 1 = between 10 and 20, 2 = over 20).

3. Results

The MP analysis produced eight most parsimonious trees, a strict consensus of which is presented in Fig. 1. The Bayesian analysis produced a very similar tree (Fig. 2), differing only where bootstrap values (MP analysis) and posterior probability estimates (Bayesian analysis) were low.

In both trees, specimens of *L. huashen* form a clade separate from *L. chapaense* with high bootstrap (100%) and posterior probability (1.00) support. However, these two species form a strongly supported (100% bootstrap and

1.00 posterior probability) clade that is nested within *Vibrissaphora*. *Vibrissaphora promustache* is outside the clade comprising *L. chapaense*, *L. huashen* and all other *Vibrissaphora* species. This *Vibrissaphora* (including *L. chapaense* and *L. huashen*) clade has high bootstrap (100%) and posterior probability (1.00) support. *Leptobrachium hainanense* is the sister taxon to this clade, which in turn, is the sister taxon to a clade containing *L. montanum* and *L. smithi* (Figs. 1 and 2). When the MP tree was constrained so that *L. chapaense* and *L. huashen* were outside a *Vibrissaphora* clade, the tree (3633 steps) was significantly longer ($p = 0.0008$) than the unconstrained MP tree (3602 steps).

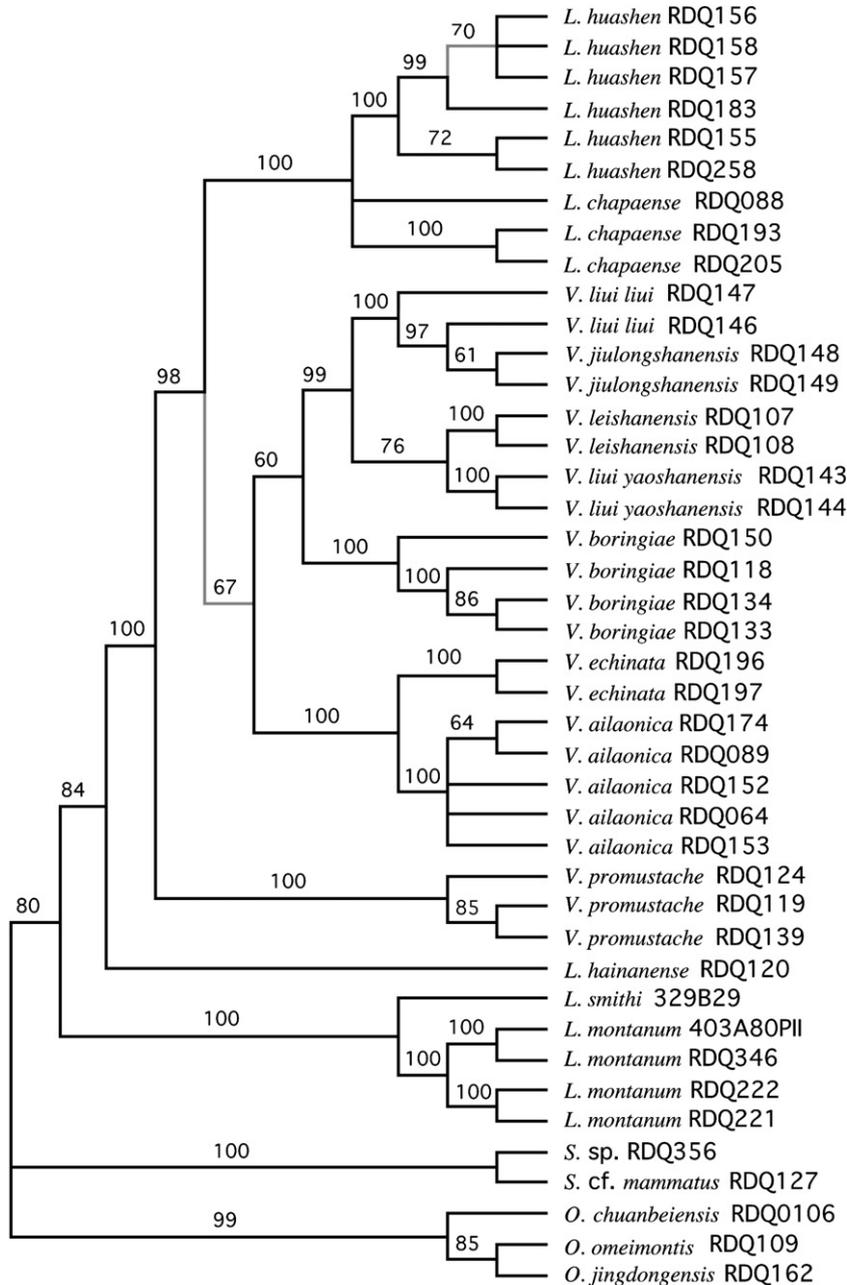


Fig. 1. Maximum parsimony tree (strict consensus) obtained from a combined analysis of 16S, cyt b, and ND4 genes. Numbers at branches indicate bootstrap support for those branches. Lightly shaded branches indicate discrepancies between this tree and the Bayesian tree in Fig. 2. *L.*, *Leptobrachium*; *O.*, *Oreolalax*; *S.*, *Scutigera*; *V.*, *Vibrissaphora*.

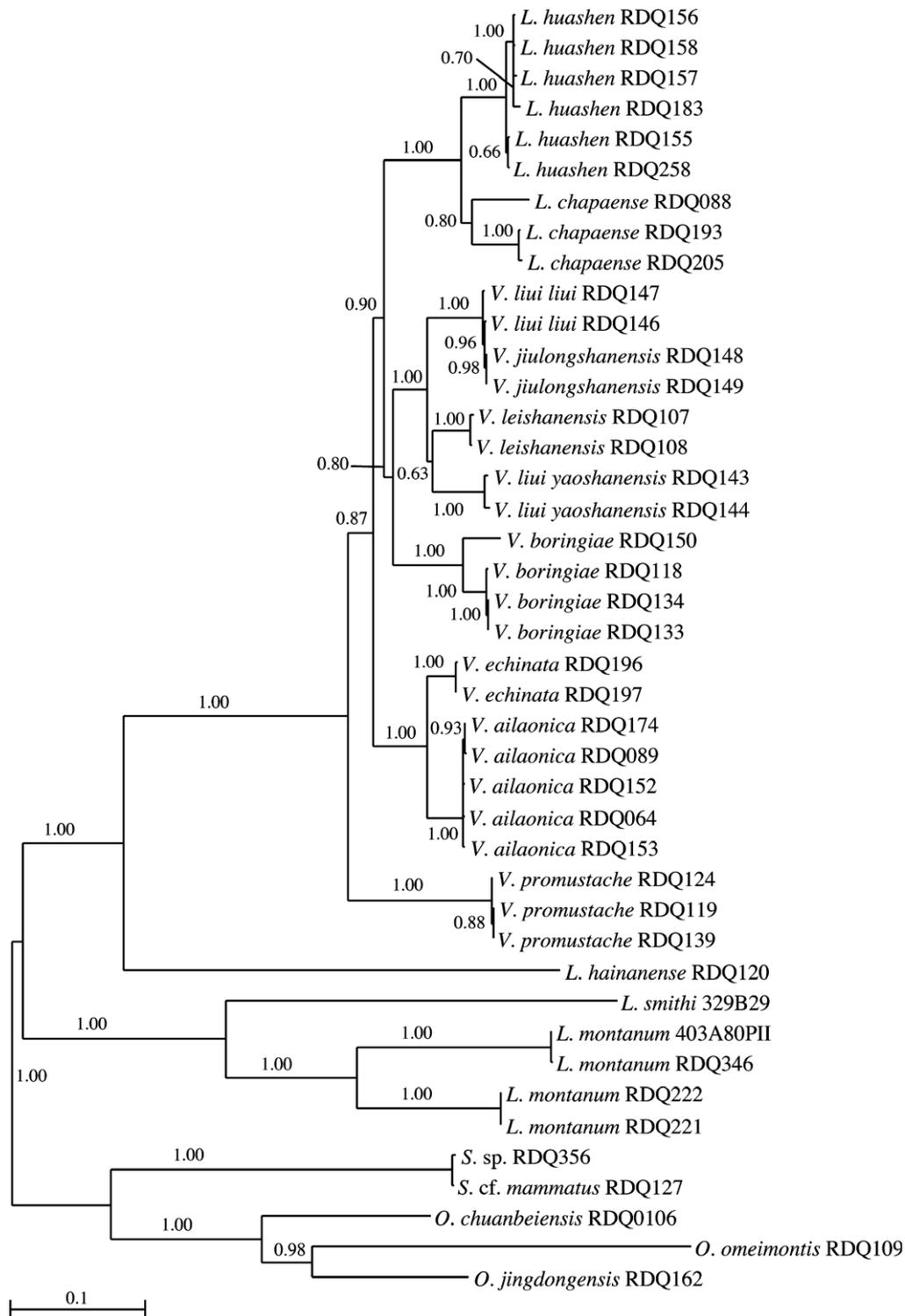


Fig. 2. Bayesian phylogram (50% majority rule) obtained from a combined analysis of 16S, *cyt b*, and ND4 genes. Numbers at branches indicate posterior probabilities for those branches. *L.*, *Leptobranchium*; *O.*, *Oreolalax*; *S.*, *Scutigera*; *V.*, *Vibrissaphora*.

The two specimens of *V. echinata* are separated from *V. ailaonica* with high bootstrap (100%) and posterior probability (1.00) support, but these clades are most closely related to each other with equally strong support.

A clade with the nominal species *V. jiulongshanensis*, *V. liui*, and *V. leishanensis* and one subspecies *V. l. yaoshanen-*

sis has high bootstrap (99%) and posterior probability (1.00) support. *Vibrissaphora boringiae* forms a clade with this *V. liui* clade instead of the *V. ailaonica/V. echinata* clade, with a bootstrap value of 60% and a posterior probability value of 0.80. *Vibrissaphora jiulongshanensis* groups with *V. liui liui*, whereas *V. liui yaoshanensis* groups with *V.*

leishanensis instead of *V. l. liui* with 76% bootstrap and 0.63 posterior probability support.

The main difference between the results of the MP and Bayesian analyses is the phylogenetic position of the *L. chapaense*/*L. huashen* clade. In the Bayesian analysis this clade is more closely related to a clade consisting of *V. boringiae*, *V. leishanensis*, and *V. liui*, with a 0.90 posterior probability (Fig. 2), whereas, in the MP analysis the *L. chapaense*/*L. huashen* clade is the sister taxon to all *Vibrissaphora* species except *V. promustache* with a 67% bootstrap support (Fig. 1).

Fig. 3 illustrates the MP tree with the morphological and behavioral characters mapped onto it. Tables 4 and 5 are ancestral state matrices of these characters for both

the MP and Bayesian trees. The MP tree at 16 steps was illustrated because it contained a more parsimonious ancestral state reconstruction than the Bayesian tree (19 steps). The MP tree was more parsimonious for characters 7 (mean body size in male), 8 (presence or absence of vocal sacs), and 9 (skin in breeding males).

4. Discussion

4.1. Taxonomic considerations

Based on the resulting phylogenies, in which *Vibrissaphora liui yaoshanensis* forms a clade with *V. leishanensis* instead of *V. liui*, we elevate *V. l. yaoshanensis* to full spe-

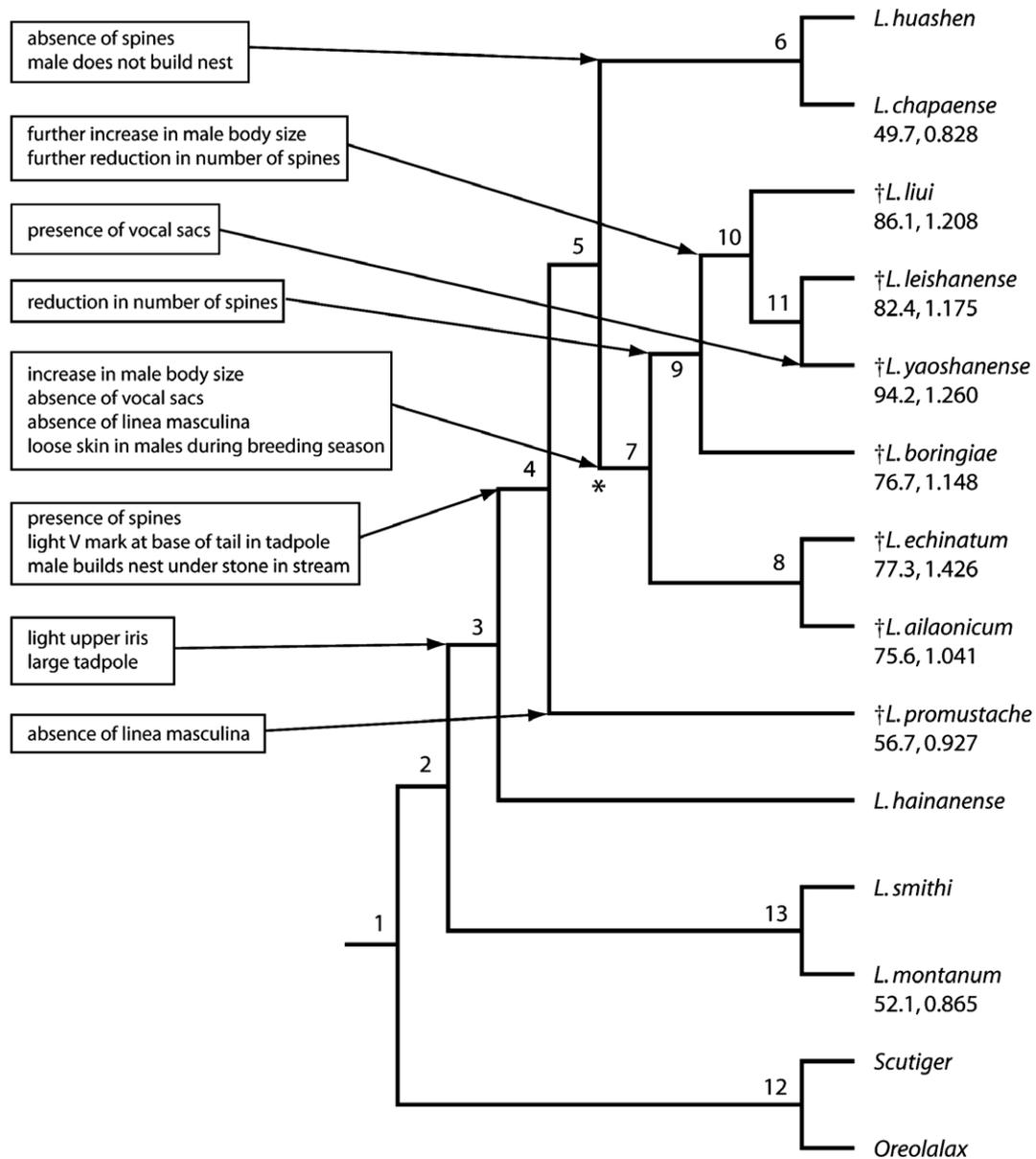


Fig. 3. Morphological and behavioral characters mapped onto the MP tree from Fig. 1. Numbers under species are average male snout–vent length (SVL) in millimeters and ratio of male to female SVL according to Dubios and Ohler (1998) and Rao et al. (2006). Numbers at nodes correspond to numbers for nodes in Tables 4 and 5. The asterisk indicates where we hypothesize the adoption of a resource-defense polygyny mating system. A dagger (†) indicates a mustache toad, formerly *Vibrissaphora* as shown in Figs. 1 and 2. *L. Leptobranchium*.

Table 4

Resulting ancestral character state matrix of the morphological and behavioral characters in Table 3 on the MP tree

Character/ node	1	2	3	4	5	6	7	8	9	10	11	12	13
1	1	1	1	0,1	0,1	1	0	0	0	0	0	1	1
2	0	0	1	1	1	1	1	1	1	1	1	0	0
3	0	0	1	1	1	1	1	1	1	1	1	0	0
4	0	0	0	0,1	0,1	0	1	1	1	1	1	0	0
5	0	0	0	1	1	1	1	1	1	1	1	0	0
6	0	0	0	0,1	0,1	0	1	1	1	1	1	0	0
7	0	0	0	0	0	0	1	1	1	2	2	0	0
8	0	0	0	0	0	0	1	1	1	1	1	0	0
9	0	0	0	0	0	0	1	1	1	1	1	0	0
10	0	0	0	0	0	0	0	0	0,1,2	2	2	0	0

The numbers at the top correspond to the nodes in Fig. 3.

Table 5

Resulting ancestral character state matrix of the morphological and behavioral characters in Table 3 on the Bayesian tree

Character/ node	1	2	3	4	5	6	7	8	9	10	11	12	13
1	1	1	1	0	0	0	0	1	0	0	0	1	1
2	0	0	1	1	1	1	1	1	1	1	1	0	0
3	0	0	1	1	1	1	1	1	1	1	1	0	0
4	0	0	0	1	1	1	1	0	1	1	1	0	0
5	0	0	0	1	1	1	1	1	1	1	1	0	0
6	0	0	0	1	1	1	1	0	1	1	1	0	0
7	0	0	0	0	0,1	1	0,1	0	0,1,2	2	2	0	0
8	0	0	0	0,1	1	0,1	0	1	1	1	1	0	0
9	0	0	0	0,1	1	0,1	0	1	1	1	1	0	0
10	0	0	0	0	0	0,1,2	0,1,2	0,1,2	2	2	2	0	0

The numbers at the top correspond to the nodes on Fig. 3. The tree structure in Fig. 3 is the same as the Bayesian tree except that the *L. chapaense*/*L. huashen* clade would be reversed with the *L. ailaonica*/*L. echinatum* clade.

cies, differentiating it from *V. liui* by the presence of four to six maxillary spines and *V. leishanensis* by the presence of vocal sacs. We keep *V. julongshanensis* in synonymy with *V. liui*, which typically contains two to four maxillary spines, one or two on each side of the head. While independent lines of morphological and molecular evidence both support the recognition of *V. yaoshanensis* as a species, we recognize that the low bootstrap and posterior probability support warrant concern. We therefore also recommend that additional work be performed to confirm this taxonomic change.

We also propose a *V. boringiae* species group of *V. boringiae*, *V. leishanensis*, *V. liui*, and *V. yaoshanensis* based on the consistent presence of this clade in both analyses. This species group can be distinguished from a *V. ailaonica* species group of *V. ailaonica*, *V. echinata*, and *V. ngoclinhensis* by containing along the maxillary arch a linear arrangement of spines that become progressively fewer in number from *V. boringiae* to *V. leishanensis*, *V. yaoshanensis*, and *V. liui*. Again, we concede that this arrangement does not have strong bootstrap or posterior probability support but is most consistent with the resulting trees.

We also accept *V. echinata* as a species separate but closely related to *V. ailaonica*, as stated by Dubios and Ohler (1998), Ohler et al. (2000), and Grosjean (2001). The range of spines from individuals of *V. echinata* collected from Jinping County is 31–45 with a mean of 34.8 ($n = 6$). Those collected from Pinbian County are 38–51 with a mean of 40.9 ($n = 10$). The range of spines of *V. ailaonica* counted from Jingdong County approximately 300 km to the northwest were 22–48, but a lower mean of 28.1 (Fei et al., 1995). The two specimens of *V. echinata* from Vietnam, 65–80 km to the southeast, possessed 52 and 61 spines, respectively (Dubios and Ohler, 1998). The single male specimen of *V. ngoclinhensis* from over 900 km southeast of the Vietnamese specimens of *V. echinata* contained 66 spines. This species was not included in our study, but if it also groups with *V. ailaonica* and *V. echinata*, this would suggest a trend in increasing number of spines as one travels south along these northwest to southeast transverse mountains. Further sampling of specimens in the intervening areas along this mountain range is needed to clarify the relationships of these species. Phylogenetic analyses including *V. ngoclinhensis* are needed to determine whether this species should be placed in the *V. ailaonica* species group as recognized here.

We also accept the assessment by Fei and Ye (2005) that *L. huashen* from mid-elevation areas of the Trans-Himalayas (Gaoligongshan Mountain in Tengchong and Longling Counties, and Wuliang and Ailao Mountains in Jingdong County) are a separate but closely related species with *L. chapaense* from southern Yunnan Province (border area of Simao City and Xishuangbanna County, and Huanglianshan Mountain in Luchun County, near northern Vietnam and Laos). We believe that the wide-ranging *L. chapaense* is a species complex that requires further systematic investigation due to known morphological variation between populations (Yang, 1991; Dubios and Ohler, 1998; Lathrop et al., 1998).

Finally, we place *Vibrissaphora* Liu (1945) in synonymy with *Leptobrachium* Tschudi (1838) to be consistent with these phylogenetic results but suggest that further taxon sampling of other Southeast Asian *Leptobrachium* be performed to confirm this taxonomic change.

The new taxonomic changes are as follows:

Leptobrachium ailaonicum (Yang et al., 1983)

Leptobrachium boringiae (Liu, 1945)

Leptobrachium echinatum Dubios and Ohler, 1998

Leptobrachium leishanense (Liu and Hu, 1973)

Leptobrachium liui (Pope, 1947)

Leptobrachium ngoclinhense (Orlov, 2005)

Leptobrachium promustache (Rao et al., 2006)

Leptobrachium yaoshanense (Liu and Hu, 1978)

4.2. Character evolution within *Leptobrachium*

Until recently, several characters have been used to differentiate species of mustache toads (*Vibrissaphora* sensu

Liu) from members of *Leptobrachium* sensu stricto, including presence of maxillary spines, absence of linea masculina, a larger body size, reverse sexual size dimorphism, indistinct tympani, rougher dorsal skin, absence of vocal sacs, enlarged forearms in males, loose skin in males during the breeding season, light colored upper iris, large tadpole size, and the presence of a “Y”-shaped mark at the dorsal base of the tail of the tadpole (Tian and Hu, 1985; Ye et al., 1993; Zhao and Adler, 1993; Dubios and Ohler, 1998; Ho et al., 1999; Fei et al., 2005). However, other than the presence of the maxillary spines, the other characters have not consistently differentiated all species of the two genera (Dubios and Ohler, 1998; Rao et al., 2006). In fact, some of these characters, such as large tadpoles and light blue to white dorsal eye color in *L. chapaense*, *L. hainanense*, and *L. huashen*, and a “Y”-shaped mark at the base of the tail of the tadpole of *L. chapaense* and *L. huashen* are more similar to species of mustache toads than they are to other species of *Leptobrachium*, suggesting relatedness (Dubios and Ohler, 1998). *Leptobrachium promustache*, which is the sister taxon to the other mustache toads, contains a mixture of characters between *Vibrissaphora* sensu Liu and *Leptobrachium* sensu stricto (Rao et al., 2006).

By mapping these characters onto the constructed phylogenies, we propose that a light-colored upper iris and a large tadpole may be synapomorphies for the clade of *L. chapaense*, *L. hainanense*, *L. huashen*, and the mustache toads, and a light “Y”-shaped mark in the tadpoles is a synapomorphy for a subclade of *L. chapaense*, *L. huashen*, and all mustache toads (Fig. 3). We also propose that the appearance of maxillary spines occurs after the subclade splits from *L. hainanense*. The spines are then secondarily lost in the *L. chapaense*/*L. huashen* clade (Fig. 3). An equally parsimonious hypothesis would be independent evolution of spines in *L. promustache* and the remaining mustache toads in the MP tree but would require independent occurrences of spines in *L. promustache*, the *L. ailaonicum*/*L. echinatum* clade, and the *L. boringiae* species-group clade in the Bayesian tree. In the MP tree, the possession of loose skin in males during the breeding season may be a synapomorphy for the subclade of mustache toads excluding *L. chapaense*, *L. huashen*, and *L. promustache*. This character corresponds to the subaerial defense of the nest site by these frogs. In the Bayesian tree the loose skin in males would either be gained separately in the *L. echinatum*/*L. ailaonicum* lineage and the *L. boringiae* group or secondarily lost in the *L. chapaense*/*L. huashen* lineage.

We also see two general trends in maxillary-spine evolution. The first trend is for the number of spines to decrease in the *L. boringiae* species group. The spines become more regular in size and arrangement as in *L. boringiae*, and finally fewer, with two to three spines on a side as in *L. leishanense* and *L. yaoshanense*, and one to two spines on a side as in *L. liui*. Concomitant with this is an increase in male body size and an increase in the difference between the sizes of the male and female, with the male becoming much larger (reverse sexual size dimorphism; Fig. 3). The

other trend in the *L. ailaonicum* species group is for the number of spines initially to decrease from that in *L. promustache* but not to the point of the *L. boringiae* species group, while also exhibiting an increase in body size and reverse sexual size dimorphism. The arrangement and size of the spines do not become regular, and the number may even increase as seen in *L. echinatum* and possibly *L. ngoclinhense* (if it is shown to be part of this species group).

4.3. Sexual selection and the evolution of sexually dimorphic characters

The transformation history of sexually dimorphic characters (maxillary spines and reverse sexual size dimorphism) in mustache toads appears to follow the predicted patterns of change as a result of intrasexual selection (Fig. 3). Spine development appears first as very small and numerous spines, as seen in *L. promustache*. Observations by the first author indicate that males of this species call from under stones at the shallow edges of streams. Egg masses have also been observed at these sites, attached to the undersides of stones. However, males have not been found under stones with egg masses. Males do not seem to develop the loose skin for seasonally cutaneous respiration, indicating that they do not defend a nest site from under water over a prolonged period.

The much smaller and more numerous spines in *L. promustache* may have developed to protect the snout when digging a nest site but not to defend it (see below for *L. echinatum*). The spines are secondarily lost in *L. chapaense* and *L. huashen* (Fig. 3), species in which the male calls from loose soil near a stream that is separated from the oviposition site, which is also under a stone in the stream (Rao and Wilkinson, personal observations). Amplectant pairs have been observed moving to the stream from a terrestrial site, indicating that the female chooses a site to oviposit (Rao, personal observations).

The remaining subclade of mustache toads has larger but fewer spines, a result, we suggest, of adopting a resource-defense polygyny mating system (at the internode with the asterisk in Fig. 3), in which the male calls from a constructed nest site under an in-stream flat-bottom stone (Chen et al., 1984). In conjunction with larger and fewer spines, the adult body size is larger and reverse sexual size dimorphism exists, as would be expected (West-Eberhard, 1983; Emerson, 1994). The males have also developed loose skin that is believed to aid in cutaneous respiration and allow for prolonged defense of the aquatic nest site. It has been proposed that *L. ailaonicum* males are guarding the egg masses from potential predators (Ho et al., 1999), but we hypothesize that the males are defending the nest site itself from conspecific males, and that any protective benefit to the egg masses is secondary. The first author observed individual males of *L. echinatum* forcing their heads under stones while digging with their forearms, indicating that the spines may help protect the snout during

this activity, which suggests a possible dual function for these spines.

Because the maxillary spines become fewer in the *L. boringiae* species group, we propose that the function of the spines is primarily for defending the nest site from conspecific males, not for protecting the snout during nest site construction. Natural history observations of *L. liui* constructing nest sites by digging with the hind legs support this hypothesis (Gu et al., 1986). The first author observed agonistic behavior in *L. ailaonicum*, in which males extended the limbs and butted the head forward, but has yet to observe this behavior between competing males. Actually observing males fighting in natural conditions would be difficult as this activity would occur within the nest site under a stone within a stream and would probably stop when an observer lifted the stone. However, based on a lack of observations of male-male agonistic behavior, lack of observed scars on the frogs from fighting, but observations of a female touching the snout of the male when entering a nest site (Zheng and Fu, 2007), an alternative hypothesis has been proposed that the role of the spines is for species recognition of the male by the female (Fu, personal communication). Intrasexual selection due to female choice could give similar patterns of evolution of these characters as seen here (Sullivan et al., 1995).

These hypotheses can be tested by conducting ecological and behavioral studies that would determine the presence of agonistic behaviors between males (Katsikaros and Shine, 1997; Tsuji and Matsui, 2002), the stability of nest sites during the breeding season, the factors that determine relative nest site quality, the relationship between nest-site quality and male characters, and mating success of individual males (Wells, 1977; Howard, 1978; Greer and Wells, 1980; Höbel, 2000; Tsuji, 2004; Pröhl, 2005).

However, intrasexual selection for larger and fewer spines, larger adult body size, and increased reverse sexual size dimorphism due to adopting a resource-defense polygyny mating system is consistent with the pattern of evolution of these characters on the phylogenetic tree in this study and the occurrence of similar characters in other species of frogs that engage in this type of mating system. We therefore consider character states of a small body size and normal sexual size dimorphism as expressed in *L. promustache*, *L. chapaense* and other species of *Leptobrachium* plesiomorphic for the mustache toad clade. An increase in body size and reverse sexual size dimorphism were derived after the remaining members of the clade split with *L. chapaense/L. huashen* as in the MP tree (Fig. 2) and adopted a resource-defense polygyny mating system; or *L. chapaense/L. huashen* secondarily lost these characters as in the Bayesian tree. The first scenario is more parsimonious if these characters are considered independently derived. However, the expression of this suite of sexually dimorphic characters may simply be influenced by androgen levels, thus not independent and potentially homoplastic (Emerson and Hastings, 1998).

4.4. Biogeography

All species of mustache toads have montane distributions in mid-elevation forests. These frogs require small streams with moderate currents to breed, and forested terrestrial uplands to occupy outside the breeding season. The four species with only one to three spines inhabit forests from 800 to 1600 m in the mountains of southeast China, and are more closely related to *L. boringiae*, which occurs from 700 to 1700 m in the mountains of southern Sichuan and northeastern Yunnan (Fei, 1999). *Leptobrachium ailaonicum*, *L. echinatum*, *L. promustache*, *L. chapaense*, and *L. huashen* are from southwest China and Vietnam, at higher elevations of 2000 to 2500 m associated with the southern trans-Himalayan region (Dubios and Ohler, 1998; Fei, 1999; Fei et al., 2005; Rao et al., 2006). The present distribution and phylogeny of the mustache toad group suggests a trans-Himalayan origin based on the presence in this region of species spanning deep phylogenetic divergences, as suggested by Inger (1999) and Zhao (1999) for other groups.

We propose that the basal lineage of the mustache toad clade within *Leptobrachium* originated in the southern trans-Himalayas of southwest China and dispersed along the north to south mountain ranges, probably as far south as central Vietnam (locality of *L. ngoclinense*), and east from Yunnan along the mountains bordering the southern Chengdu Basin, then via the Nanling and Wuyi ranges into the provinces of Fujian and Zhejiang. The Yunnan–Guizhou Plateau probably acted as a barrier to dispersal between these areas as it lacks the moderate currents of mountain streams needed by these species to breed (Inger, 1999). Subsequent isolating events, such as contraction and expansion of the montane zones in the trans-Himalayas, may have allowed for speciation of certain species now in sympatry (Inger, 1999), such as *L. echinatum* and *L. promustache* (Rao et al., 2006). Other isolating events, such as the creation of large river valleys between the older mountain ranges to the east (Wan and Zhu, 2002) probably allowed for allopatric speciation of *L. boringiae*, *L. leishanense*, *L. yaoshanense* and *L. liui*.

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