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A phylogeny of the high-elevation Tibetan megophryid frogs and evidence for the multiple origins of reversed sexual size dimorphism

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Abstract

A molecular phylogeny of the high-elevation Tibetan megophryid frogs was reconstructed using mitochondrial and nuclear gene sequences. Both parsimony and Bayesian analysis produced similar tree topologies, which identified the genus *Leptobrachium* as a sister group to *Vibrissaphora*, and the genera *Oreolalax* and *Scutiger* as monophyletic groups. Within the latter two genera, several species groups were also clearly recognized. At least 13 megophryids display reversed sexual size dimorphism (RSSD), where males are equal to or larger than females. According to our phylogenetic hypothesis, RSSD may have independently evolved at least five times or as many as seven times in this group. The multiple origins of RSSD within this group provide an excellent opportunity for studying the relationship between body size sexual dimorphism and other life-history traits and for determining the costs and benefits of RSSD.

Introduction

Females are larger than males in most animal species, with avian and mammalian species being the main exceptions. The widely accepted explanation for large female size is fecundity advantage, as egg production increases with body size (Andersson, 1994). Most anurans follow this rule, but in *c*. 10% of species, males tend to be of an equal or of a larger size than females (Shine, 1979). For instance, male *Vibrissaphora boringiae* may attain a body length of 69.8–89.0 mm, whereas females fall within the range of 58.6–76.0 mm (Liu & Hu, 1961). This is known as reversed sexual size dimorphism (RSSD). Several mechanisms have been proposed to explain the large male size in anurans: male-to-male physical combat (Shine, 1979), different selection gradients for size between males and females (Arak, 1988), female choice (Morris & Yoon, 1989) and several others (see Andersson, 1994 for a review). Monnet & Cherry (2002) pointed out that the previous studies suffered from either methodological problems (e.g. non-independent data points) or unrealistic assumptions, and rejected the sexual selection-based explanations. Instead, they suggested that the age difference between the sexes in breeding populations could explain most of the dimorphism. As such, the evolutionary factors operating on body size in anurans remain largely controversial, and a study of the origin of RSSD will undoubtedly improve our understanding of how natural and sexual selection operate in this large and important group. RSSD is particularly concentrated among the high-elevation Tibetan megophryid frog species of the genera *Oreolalax*, *Scutiger* and *Vibrissaphora*; in at least 13 of them, males are of an equal or a larger size than females (Liu & Hu, 1961; Yang, 1991; Ye, Fei & Hu, 1993; Fei & Ye, 2000, Supplementary Material Appendix S1). The repeated presence of RSSD in this group offers us an excellent opportunity to examine the adaptive values and tradeoffs that have shaped the degree of sexual size dimorphism over time, from both the male's and female's perspective.

Hypotheses on the evolutionary origin(s) of RSSD can only be tested within a solid phylogenetic framework. For instance, a shared single origin of RSSD would suggest that the multiple occurrences of RSSD simply reflect phylogenetic inertia, and are unrelated to variation in present-day selection pressures. Conversely, independent origins of

RSSD might reflect convergence due to similar selection pressures, a possibility that could be investigated by considering how RSSD and environmental conditions are distributed across the phylogeny. Phylogenetic hypotheses provide the foundation necessary for making informed evolutionary comparisons that account for shared history in addition to contemporary selective pressures (Harvey & Pagel, 1991).

Several phylogenetic works on the high-elevation Tibetan megophryids have been published. For example, Fu, Lathrop & Murphy (1997) and Fu & Murphy (1997) analysed some morphological data of the genera *Scutiger* and *Oreolalax*, respectively. A few recent studies have focused on inter-generic relationships within the Megophryidae; Delorme & Dubois (2001) presented a preliminary phylogeny of 15 megophryid frogs based on 54 external morphological characters, and Zheng (2004) studied the relationships among eight genera of the family Megophryidae using partial 16S and cytochrome *b* gene sequence data. Frost *et al*. (2006) also included eight megophryids in their amphibian tree of life. Unfortunately, these previous studies had either limited number of taxa or limited number of informative characters, as well as some analytical shortcomings, as Frost *et al*. (2006) pointed out. As such, the phylogenetic relationships of megophryid frogs in general and among species with RSSD in particular remain largely unknown. The lack of a solid phylogeny for this group has constrained the pursuit of several research avenues.

We recently initiated a project aiming at understanding the evolution of sexual dimorphism and sexual selection in these high-elevation megophryid frogs. As the first step of this long-term study, we used a combination of nuclear and mitochondrial DNA (mtDNA) sequence data to reconstruct a molecular phylogeny of the genera *Oreolalax*, *Scutiger* and *Vibrissaphora*. Furthermore, by mapping size dimorphism on to the reconstructed tree, we tested the single versus multiple origins hypotheses of RSSD among these megophryids.

Material and methods

Species sampling

A total of 34 specimens representing 23 species of the genera *Oreolalax*, *Scutiger* and *Vibrissaphora* were collected during 1999–2006. Zheng (2004) suggested that the three genera might form a monophyletic group. All eight species with reported RSSD in the genera *Oreolalax* and *Scutiger* (*O. jingdongensis*, *O. nanjiangensis*, *O. omeimontis*, *O. popei*, *O. rhodostigmatus*, *S. glandulatus*, *S. mammatus* and *S. muliensis*) were included in our analysis. Five of the six species of the genus *Vibrissaphora* (*V. ailaonica*, *V. boringiae*, *V. leishanensis*, *V. liui* and *V. ngoclinhensis*) have larger males, but we were only able to obtain two species in this analysis. One species from the genus *Leptobrachium*, which was often considered to be closely related to *Vibrissaphora* (but see Zheng, 2004), was also included in the analysis. None of the *Leptobrachium* species are known to display RSSD. Three species from two other genera, *Leptolalax* and *Megophrys*, were selected as outgroups for phylogenetic analysis. The genus *Leptolalax* has been considered to be closely related to the ingroup genera by many taxonomists (e.g. Dubois, 1980; Fei *et al*., 2005), while the genus *Megophrys* is relatively distantly related to the ingroup. All voucher specimens are deposited in the herpetological collections of the Chengdu Institute of Biology (Chengdu), the Institute of Zoology (Beijing) and the Royal Ontario Museum at Toronto (Table 1).

Laboratory protocols

DNA sequence data were used for phylogenetic reconstruction. Two fragments were targeted for sequencing. One is from the mitochondrial genome, and includes the second half of the 12S gene, most of the 16S gene, plus the $tRNA^{-val}$ gene between them. This fragment is one of the most conservative parts of the mitochondrial genome and has been used extensively in the reconstruction of species-level phylogenies in various vertebrate groups, especially in amphibians (e.g. Vences *et al*., 2004). The other fragment is part of the recombination activating gene 1 (RAG-1), a single-copy protein-coding nuclear gene that has also been widely used in amphibian phylogenetics (e.g. Mauro *et al*., 2004).

DNA was extracted from muscle tissues preserved in 95% ethanol or liquid nitrogen using the Promega Wizard genomic DNA extraction protocols. A standard polymerase chain reaction (PCR) was used to amplify the mitochondrial 12S–16S gene fragment. PCR products were purified using Qiaquick protocols (Qiagen, Germantown, MA, USA), and were directly sequenced with BigDye-labelled terminator sequencing kits on an ABI 377 automatic sequencer (Applied Biosystems, Foster City, CA, USA). The primers, 12SA-5′, 16Sbr-3′ (Palumbi, 1996), 1602L (5′-GTA TAC CGG AAG GTG TAC TTG GAA CAG-3′, this study) and 2571H (5′-TAC CTT CGC ACG GTC AGA ATA CCG C-3′, this study), were used to amplify and sequence the 12S–16S fragment. Most sequencing was conducted in both directions with a 70–80% overlap. In a few cases, strings of eight or more consecutive guanines or cytosines disrupted sequencing and, as a result, sequences from only one direction were obtained. For the RAG-1 gene, five of the samples were amplified, purified and directly sequenced using the same protocols as the mitochondrial genes. The primers, Amp-RAG1 F (5′-AGC TGC AGY CAR TAC CAY AAR ATG TA-3′, Mauro *et al*., 2004) and RAG1-R (5′-GCA AAG TTT CCG TTC ATT CTC AT-3′, this study), were used for both amplification and sequencing. For the rest of the samples, the targeted fragments were first amplified and then cloned into the pCR2.1/TOPO vector (Invitrogen, Carlsbad, CA, USA). The standard M13 forward and reverse primers were then used to sequence the inserted fragment.

Analysis

Sequence alignment was conducted with the computer programs ClustalX (version 1.8; Thompson *et al*., 1997)

Table 1 Specimen information and GenBank accession numbers

Species	Specimen number	Collection location	Coordinates	12S-16S GenBank accession numbers	RAG-1 Genbank accession numbers
Oreolalax chuanbeiensis	CIB-ZYC074	Mao Xian Co., Sichuan Province, China	31°45.615′N 104°07.184′E	EF397266	N/A
O. jingdongensis	IOZCAS2691	Ailoushan, Jingdong Co., Yunnan Province, China	24°32.721′N 101°01.701′E	EF397255	EF397287
O. liangbeiensis	IOZCAS3796	Puxiong, Yuexi Co., Sichuan Province, China	28°32.002′N 102°45.659′E	EF397253	EF397286
O. lichuanensis	CIB-ZYC787	Jin Fu Shan, Chongqing Municipality, China	29° 02.390′N 107°12.302′E	EF397260	N/A
O. major	ROM40452	Bai Sha He, Hongya Co., Sichuan Province, China	29°26.533′N, 102°53.779′E	EF397252	N/A
O. multipunctatus	ROM40463	Omei Mt., Omei Co., Sichuan Province, China	29°33.5′N 103°25.0′E	EF397268	EF397291
O. nanjiangensis	CIB-XM804	Da Jiang Kou, Nan Jiang Co., Sichuan Province, China	32°34.858′N 106°42.775′E	EF397265	N/A
O. omeimontis	CIB-XM297	Omei Mt., Omei Co., Sichuan Province, China	29°33.131′N 103°21.061′E	EF397263	N/A
	CIB-XM439	Omei Mt., Omei Co., Sichuan Province, China	29°33.023′N 103°22.224′E	EF397264	N/A
	ROM40454	Wa Wu Shan, Hongya Co., Sichuan Province, China	29°39.111′N, 102°57.065′E	EF397261	N/A
	CIB-XM379	Da Yi Co., Sichuan Province, China	30°37.915′N 103°21.061′E	EF397262	EF397289
O. pingii	CIB-XM980	Xi Chang Co., Sichuan Province, China	27°52.177′N 102°30.947′E	EF397259	EF397293
O. popei	CIB-XM0107	Pengxian Co., Sichuan Province, China	31°14′N 103°45′E	EF397267	EF397290
O. rhodostigmatus	CIB-ZYC724	Siu Yang Co., Gui Zhou Province, China	28°14.256′N 107°12.325′E	EF397249	N/A
	CIB-ZYCA746	Da Fang Co., Guizhou Province, China	27°05.936′N 105°40.247′E	EF397248	EF397294
O. rugosus	CIB-XM340	Shi Mian Co., Sichuan Province, China	29°03.007′N 102°31.058′E	EF397254	N/A
O. schmidti	ROM40457	Wa Wu Shan, Hongya Co., Sichuan Province	29°39.111′N 102°57.065′E	EF397257	EF397292
	CIB-XM417	Da Yi Co., Sichuan Province, China	30°40.409′N 103°09.798′E	EF397258	N/A
O. sp.	CIB-XM092	Peng Xian Co., Sichuan Province, China	31°14′N 103°45′E	EF397256	EF397288
O. xiangchengensis	CIB-3LW008	Li Jiang Co. Yunnan Province, China	26°54.289′N 99°42.343′E	EF397250	N/A
	CIB-3LW0032	Zhongdian Co., Yunnan Province, China	28°01.400′N 99°29.776′E	EF397251	EF397285
Scutiger chintingensis	ROM39065	Wa Wu Shan, Hongya Co., Sichuan Province, China	29°39.111′N 102°57.065′E	EF397269	EF397303
	ROM40460	Wa Wu Shan, Hongya Co., Sichuan Province, China	29°39.111′N 102°57.065′E	EF397270	EF397301
S. boulengeri	ROM40423	Dao Fu Co., Sichuan Province, China	30°23.094′N 101°34.021′E	EF397272	EF397296
	ROM40405	Linxia Co., Gansu Province, China	35°28.215′N 102°54.373′E	EF397271	EF397295
	ROM 40445	Xiaojin Co., Sichuan Province, China	30°53.933′N 102°54.662′E	EF397273	N/A
S. glandulatus	ROM40448	Dao Fu Co., Sichuan Province, China	30°23.094′N 101°34.021′E	EF397276	EF397297
	CIB-XM958	Kang Ding Co., Sichuan Province, China		EF397275	N/A

Table 1. Continued.

Species	Specimen number	Collection location	Coordinates	12S-16S GenBank accession numbers	RAG-1 Genbank accession numbers
			29°54.242′N 101°35.005′E		
	CIB-XM1188	Dao Cheng Co., Sichuan Province, China	29°11.34′N 100°06.61′E	EF397274	EF397298
S. mammatus	CIB-XM972	Xin Du Qiao, Kangding Co., Sichuan Province, China	30°01.950′N, 101°28.496′E	EF397279	EF397300
S. muliensis	IOZCAS3638	Yanyuan Co., Sichuan Province, China	27°20.513′N 101°32.423′E	EF397277	EF397302
S. tuberculatus	CIB-XM988	La Ji, Yuexi Co., Sichuan Province, China	28°31.682′N 102°30.684′E	EF397278	EF397299
Vibrissaphora boringiae	CIB-XM454	Omei Mt., Omei Co., Sichuan Province, China	29°34.589′N 103°23.476′E	EF397247	EF397284
V. leishanensis	CIB-XM594	Leishan, Guizhou Province, China	26°24′N 108°11′E	EF397246	EF397283
Leptobrachium xanthospilum	ROM32184	Tram Lap, Gia Lai Province, Vietnam	14°26.40′N 108°32.97′E	EF397245	EF397282
Leptolalax pelodytoides	ROM18282	Tam Dao, Vinh Phu, Vietnam	21°27′N 105°39′E	EF397244	N/A
Megophrys nankiangensis	CIB-XM835	Dajiangkou, Nanjiang Co., Sichuan province, China	32°34.858′N 106°42.775′E	EF397243	EF397281
M. omeimontis	ROM40462	Omei Mt., Omei Co., Sichuan Province, China	29°34.589′N 103°23.476′E	EF397242	EF397280

Coordinates with decimal numbers are GPS reading and coordinates without decimal numbers are estimates from maps.
CIB, Chengdu Institute of Biology, Chengdu; IOZCAS, Institute of Zoology, Beijing; ROM, Royal Ontario Museum, Toronto.

with the default multiple alignment parameters. Minor modifications were made by eye in MacClade (version 4.06, Maddison & Maddison, 2003) to remove obvious misalignments. Both maximum parsimony and Bayesian inference were used for reconstructing the phylogenetic hypothesis, and the mtDNA data and RAG-1 gene data were analysed both separately and as a combined dataset.

For maximum parsimony analysis, each unique haplotype was treated as a taxon and each nucleotide site was treated as a character. All characters were unordered and equally weighted. Gaps were treated as missing data. Phylogenetically uninformative characters were excluded from the analysis. A heuristic search was conducted in PAUP (version 4.0b10; Swofford, 2002) using TBR branch-swapping with 1000 random step-wise addition replicates. Bootstrap proportions (BSP) (Felsenstein, 1985) with 1000 pseudoreplicates were used to estimate the support for the recovered nodes.

All nucleotide sites were included for the Bayesian analysis. A likelihood ratio test was first conducted to select an evolutionary model that best fit the observed data, using the computer program MrModeltest (version 2.2; Nylander, 2004). The Bayesian analysis was conducted using MrBayes (version 3.02; Ronquist & Huelsenbeck, 2003). Four Markov chains were used and the dataset was run for six million generations to allow for adequate time of convergence. A flat 'prior' was used and all states were equally probable. Trees were sampled every 100 generations and we designated the first 50 000 sample trees as 'burn in' and used the last 10 000 sample trees to estimate the consensus tree and the Bayesian posterior probabilities (BPP). Two separate runs, which include a total of four independent tree searches, were conducted and the resulting trees were compared and pooled. For the combined data analysis, the data were separated into two partitions (mtDNA and RAG-1) and each partition followed its own evolutionary model.

Finally, the species with RSSD were mapped on the resulting phylogeny using MacClade. Evolutionary scenarios of RSSD were reconstructed using both ACCTRAN and DELTRAN algorithms under the parsimony principle. Alternative tree topologies and evolutionary scenarios were examined using the SH test (Shimodaira & Hasegawa, 1999) and Templeton's test (Templeton, 1983). Both statistical tests provided the same conclusion in all cases, and therefore, only the results of Templeton's test were reported for brevity.

Results

Sequence data

For the mitochondrial fragment, a total of 38 sequences were obtained, and all but three were between 1910 and 1959 base pairs in length. After alignment, a total of 2015 sites were found, including 492 from 12S, 69 from $tRNA^{-val}$ and 1454 from 16S. The intra-specific uncorrected pairwise sequence distance (uncorrected *p*-distance) ranged from 0 (*O. xiangchengensis*) to 3.7% (*O. rhodostigmatus*). The inter-

specific variation ranged from 0.7% (*O. schmidti* vs. *O. pingii*) to 10.8% (*O. lichuanensis* vs. *O. popei*) in the genus *Oreolalax*, 1.9% (*S. muliensis* vs. *S. tuberculatus*) to 5.4% (*S. tuberculatus* vs. *S. chintingensis*) in the genus *Scutiger* and 5.0% in the genus *Vibrissaphora*. The lowest inter-generic *p*-distance was 13.5% between *Scutiger* and *Oreolalax* and the highest was 22.6% between *Leptolalax* and *Leptobrachium*, excluding the *Megophrys* outgroups.

For the RAG-1 gene, a total of 24 sequences with 1133 base pairs each were resolved. Our inability to obtain sequence data for the rest of the samples was likely due to poor matching between the primers and templates. Several attempts to design new primers also failed. All sequences can be properly translated into amino acids, and the alignment did not produce any gaps. The intra-specific uncorrected *p*-distance ranged from 0.4% (*S. boulengeri*) to 0.7% (*S. chintingensis*). The inter-specific variation ranged from 0.2% (*O. schmidti* vs. *O. pingii*) to 1.7% (*O. xiangchengensis* vs. *O. multipunctatus*) in the genus *Oreolalax*, 0.1% (*S. boulengeri* vs. *S. glandulatus*) to 1.9% (*S. chintingensis* vs. *S. tuberculatus*) in the genus *Scutiger*, and 2.7% in the genus *Vibrissaphora*. Inter-generic variation ranged from 2.3% (*Scutiger* vs. *Oreolalax*) to 4.6% (*Leptobrachium* vs. *Scutiger*) excluding the outgroup *Megophrys*. All sequences are deposited in GenBank and accession numbers are listed in Table 1.

Phylogenetic hypothesis

The mtDNA sequences produced a dataset with 38 taxa and 2015 characters. Among the characters, 890 were variable and 663 were parsimoniously informative. The parsimony analysis resulted in three equally most parsimonious trees (MPTs) with 2137 steps, a consistency index of 0.5063, and a retention index of 0.7903. Most nodes received higher than 70% BSP support. Figure 1 presents one of the MPTs. The two other MPTs differed from the tree in Fig. 1 in that *O. nanjiangensis* was sister group to *O. popei* and clade B was sister group to clade D (Fig. 1). The likelihood ratio test suggested that the GTR + I + G model best fit the observed data. The Bayesian analysis produced a very similar tree to the parsimony analysis, and most nodes received higher than 90% BPP support. Only two nodes differed from the MPT presented in Fig. 1; the *Leptobrachium*/*Vibrissaphora* clade was sister group to the genus *Scutiger* and clade G was sister group to clade H. Neither association was well supported (BPP < 90). Both the MP and Bayesian analyses supported the monophyly of the genera *Oreolalax*, *Scutiger* and *Vibrissaphora*, as well as the close relationship between *Vibrissaphora* and *Leptobrachium*. The clade including *Leptobrachium* and *Vibrissaphora* was most closely related to the genus *Oreolalax* in the MP analysis and was most closely related to the genus *Scutiger* in the Bayesian analysis. Several species groups (clades A–H) were also clearly identified (Fig. 1).

The RAG-1 gene sequences produced a dataset with 24 taxa and 1133 characters. Among these characters, 239 were variable and 150 were parsimoniously informative. The parsimony analysis resulted in 99 equally MPTs with 214 steps and a consistency index of 0.7664 and a retention index of 0.8724. The strict consensus presents a topology similar to the mtDNA data, but with a much lower bootstrap support (Fig. 2). Most of the differences were in the designation of species group members. The likelihood ratio test suggested that the TrN + G model best fit the observed data for the Bayesian analysis. The Bayesian 50% majority rule consensus tree was identical to the strict consensus tree from the parsimony analysis (Fig. 2).

Overall, the support of the recovered nodes from RAG-1 gene was low relative to the mitochondrial data. For example, only five nodes received bootstrap support higher than 70. This was probably due to there being far fewer variable nucleotide sites within the RAG-1 gene. A striking difference between the nuclear and mitochondrial gene trees was regarding the monophyly of the genus *Vibrissaphora*. While the mitochondrial data resolved the genus *Vibrissaphora* as monophyletic, the nuclear gene suggested that *V. boringiae* was more closely related to *Leptobrachium xanthospilum* than to congeneric *V. leishanensis*. Both solutions were strongly supported by their respective data. Constraining the monophyly of the genus *Vibrissaphora* resulted in a significantly longer tree for the RAG-1 data ($P = 0.0075$–0.0118, Templeton's test); therefore, the RAG-1 data rejected the monophyly of the genus.

The combined data, which included only taxa that have both mitochondrial and nuclear sequences, yielded a total of 24 taxa and 3148 characters. Among them, 1044 are variable and 771 are parsimoniously informative. The parsimony analysis resulted in one MPT, with 1964 steps, a consistency index of 0.5764 and a retention index of 0.7609 (Fig. 3). The tree was most similar to the mitochondrial gene tree. This was most likely due to the fact that a large portion of the informative characters was from the mitochondrial sequences (621 out of 771 informative characters). All four genera were monophyletic, and the genus *Oreolalax* is most closely related to the clade including *Leptobrachium* and *Vibrissaphora*. Species were grouped into species groups in the same way as the mtDNA data, although the relationships among them were slightly different. The Bayesian analysis revealed a very similar tree to the MP analysis, although the relationships among several species groups were different (Fig. 3). The conventional taxonomy considered the genera *Oreolalax* and *Scutiger* as sister groups (e.g. Fei *et al.*, 2005). Constraining the two genera to be sister groups did not result in a significantly longer tree ($P = 0.5059$, Templeton's test). Therefore, the molecular data could not reject the conventional taxonomic view.

Multiple origin of RSSD

Considering all evidence including the previous phylogenetic studies, we have chosen the tree derived from the parsimony analysis of the combined data as our preferred phylogenetic hypothesis for the high-elevation Tibetan megophryid group (Fig. 3). This topology shared more compatible elements with previous morphology hypotheses than

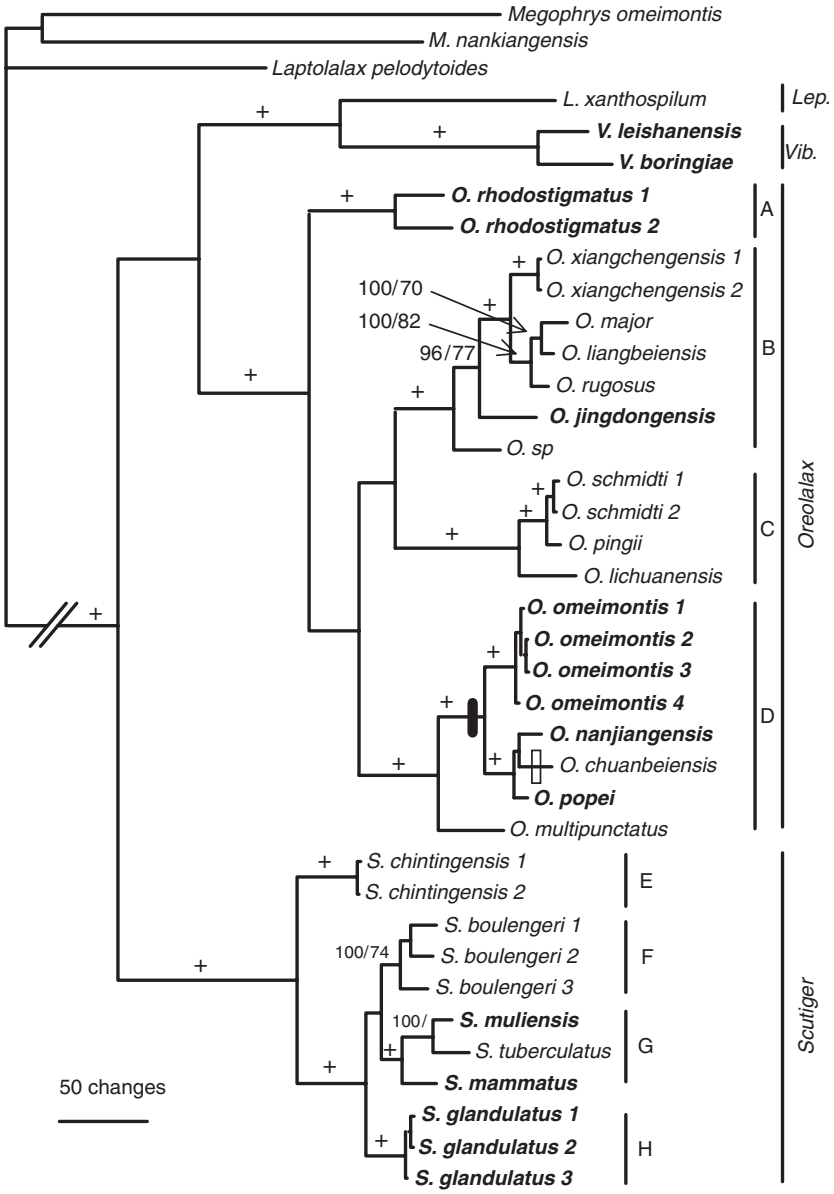

Figure 1 A most parsimonious tree derived from the mitochondrial 12S and 16S partial sequences. Bayesian posterior probability (BPP; in front of the '/') >90% and bootstrap proportions (BSP; after the '/') >70% are mapped on the tree. A '+' indicates 100% BPP and >95% BSP. Bold species names are species with reversed sexual size dimorphism. When multiple individuals were sequenced for a species, a number, 1–4, was added to the species name to distinguish the specimens. '*Lep*' = *Leptobrachium*; '*Vib*' = *Vibrissaphora*. Letters 'A'–'H' denote major clades. The vertical solid bar in clade D represents a hypothetical origin of RSSD and the open bar represents a hypothetical loss of RSSD. Refer to Fig. 3 for more hypothetical origins of RSSD among other clades.

any other alternative topologies (see the 'Discussion' below). Mapping the sexual size dimorphism trait on the phylogeny suggested that the evolution of RSSD can be most parsimoniously explained by at least five or as many as seven independent origins (Figs 1 and 3).

The RSSD in the genus *Oreolalax* has at least three origins. The RSSD in *O. rhodostigmatus* and *O. jingdongensis* each unambiguously has its own independent origin (Fig. 3). Considering the phylogenetic position of *O. chuanbeiensis* (Fig. 1), the RSSD in *O. popei*, *O. nanjiangensis* and *O. omeimontis* can be best explained by a single shared origin, followed by a loss event (Figs 1 and 3). Within the genus *Scutiger*, the presence of RSSD can be equally parsimoniously explained by three independent origins or one origin, followed by two subsequent losses. In the latter case, RSSD evolved in the common ancestor of *S. glandulatus*, *S. boulengeri*, *S. mammatus* and *S. tuberculatus*, and *S. tuberculatus* and *S. boulengeri* subsequently lost the feature. Five of the six species in the genus *Vibrissaphora* have RSSD; with limited sampling, they likely share a single origin.

Discussion

Preferred phylogeny and its taxonomic implications

All data unequivocally and strongly supported the close tie between genera *Vibrissaphora* and *Leptobrachium* and this is in agreement with the previous phylogenetic study (Zheng, 2004) and the conventional taxonomic view (Liu, 1950; Dubois & Ohler, 1998; Fei *et al*., 2005). However, it is less

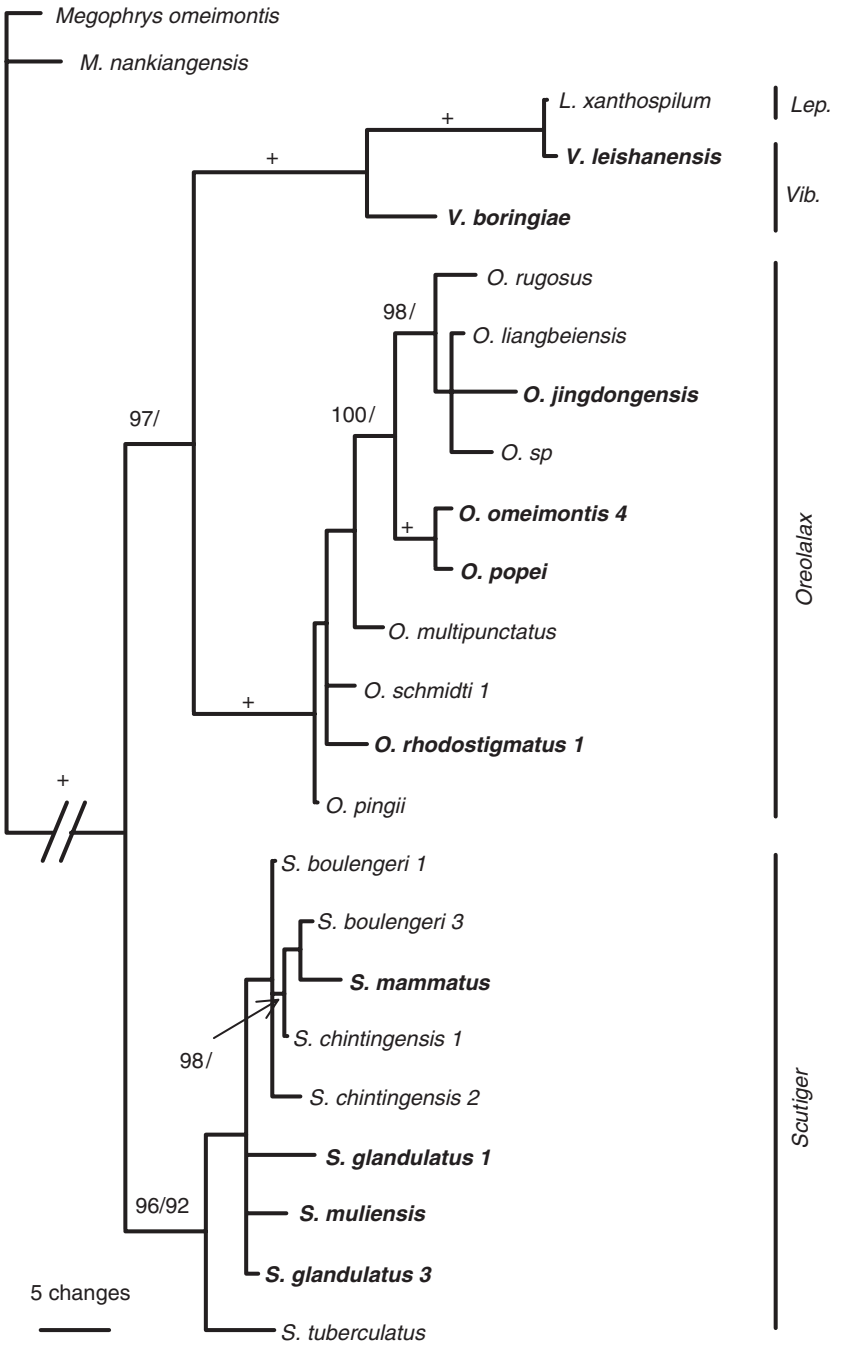

Figure 2 Strict consensus tree derived from parsimony analysis of the recombination activating gene (RAG-1) partial sequences. Bayesian posterior probability (BPP; in front of the '/') >90% and bootstrap proportions (BSP; after the '/') >70% are mapped on the tree. A '+' indicates 100% BPP and >95% BSP. Bold species names are species with reversed sexual size dimorphism. '*Lep*' = *Leptobrachium*; '*Vib*' = *Vibrissaphora*.

clear whether the genus *Vibrissaphora* is monophyletic. Analysis of the combined data, which supported the monophyly, reflected the dominance of the mtDNA data in the combined dataset, but the RAG-1 gene data suggested that *V. boringiae* was more closely related to *L. xanthospilum* than to the congeneric *V. leishanensis*. This latter association was well supported by both bootstrap analysis in the parsimony framework and the BPP (Fig. 2). Statistically, the RAG-1 data also rejected the monophyly of the genus (Templeton's test). Although the status of *Vibrissaphora* has long been controversial, and many have argued that *Vibrissaphora* species are specialized *Leptobrachium* with labial spines, the monophyly of *Vibrissaphora* has never been challenged (see Dubois &

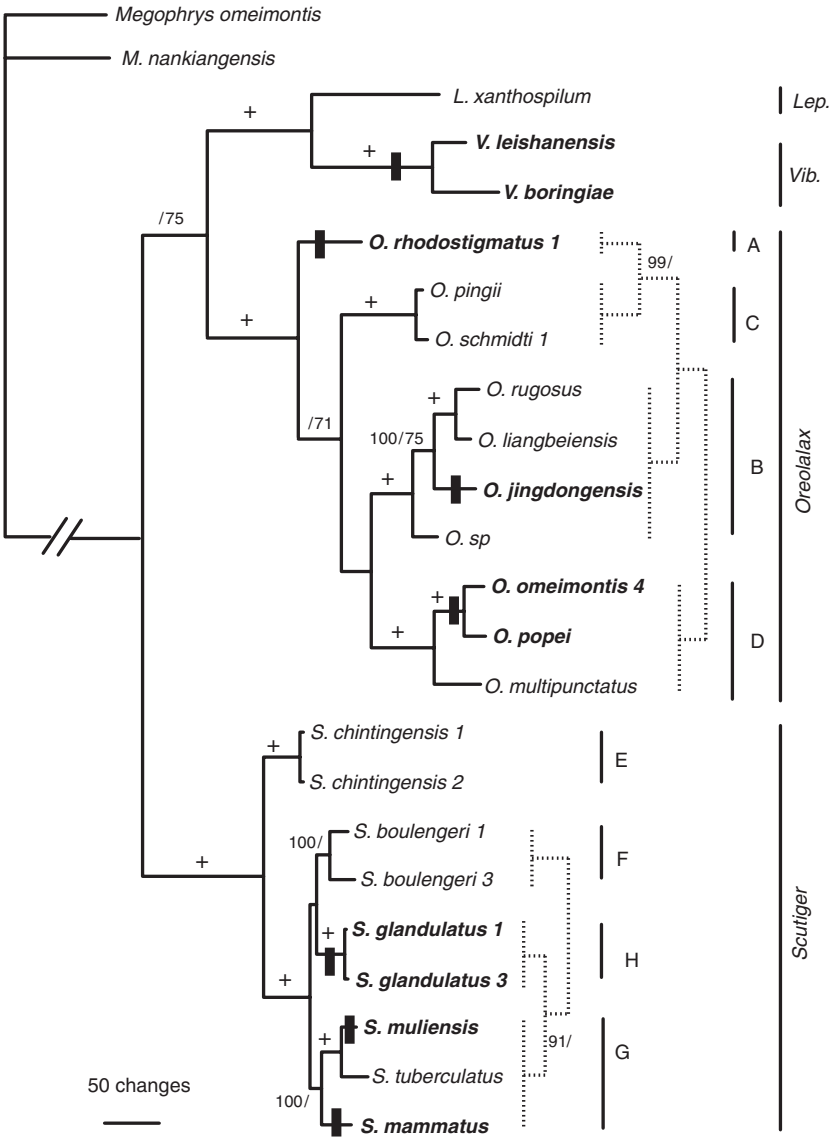

Figure 3 The most parsimonious tree derived from the combined mitochondrial and nuclear gene sequence data. The dashed lines depict the alternative topology from the Bayesian analysis. Bayesian posterior probability (BPP; in front of the '/') >90% and bootstrap proportions (BSP; after the '/') >70% are mapped on the tree. A '+' indicates 100% BPP and >95% BSP. Bold species names are species with reversed sexual size dimorphism (RSSD). Letters 'A'–'H' denote major clades. The vertical solid bars on the branches indicate the hypothetical origins of RSSD in this group, as inferred according to the parsimony principle. Alternative scenarios are also possible.

Ohler, 1998 for a review). Both morphologically and behaviourally, *Vibrissaphora* species share many derived characters, such as the male nuptial spines on the upper jaws (Liu, 1945). Here, we tentatively accept the genus as a monophyletic group as depicted on our preferred phylogeny. The phylogenetic studies of Zheng (2004), which were based on mtDNA sequences, also supported the monophyly of *Vibrissaphora*. A non-monophyletic *Vibrissaphora* would suggest an additional independent origin or reversal events of RSSD, and therefore has a large impact on the directions of our future evolutionary studies. A more thorough sampling of the species from both genera is needed to reach a more conclusive decision.

This study strongly supported the monophyly of both *Scutiger* and *Oreolalax*, and therefore provided additional evidence for the validity of the two genera. Although the two generic names have been widely accepted, as opposed to the suggestion that the two groups should be considered subgenera in the genus *Scutiger* (e.g. Dubois, 1980), the inclusion of species in the two genera has not always been consistent. For example, *S. chintingeneis* has been placed in both *Scutiger* (e.g. Zhao & Adler, 1993; Fei *et al*., 2005) and

Oreolalax (e.g. Dubois, 1980; Yang, 1991). This study unambiguously placed *chintingensis* in the genus *Scutiger*.

The relationships among the genera are poorly resolved and poorly supported. Both separate analysis of the mitochondrial genes and the RAG-1 gene data affiliated the genus *Oreolalax* with the genus *Leptobrachium* (including *Vibrissaphora*), although the association was not well supported by either gene. Nevertheless, a sister group relationship between the genera *Oreolalax* and *Scutiger* has long been accepted among taxonomists, as the two genera had previously been considered as subgenera in the genus *Scutiger* until the work of Zhao & Adler (1993). Two previous phylogenetic studies had supported the relationship (Delorme & Dubois, 2001; Zheng, 2004). Templeton's test based on the combined data suggested that the current molecular data could not reject the previous hypothesis. We tentatively consider the relationships among the genera unresolved.

Within the genera *Scutiger* and *Oreolalax*, species were grouped into several well-supported major clades. In the genus *Oreolalax*, four major clades (A–D) were apparent, and there were four major clades in the genus *Scutiger* (E–H; Figs 1 and 3). For example, *O. rhodostigmatus* made up clade A and *O. xiangchengensis*, *O. major*, *O. liangbeiensis*, *O. rugosus*, *O. jingdongensis* and an unnamed species of *Oreolalax* constituted clade B. These major clades received most of their support from the mtDNA data. Considering the relatively short branches within these clades, and the relatively long branches among the clades, these clades likely represent closely related species groups. Many of these close associations were also supported by morphological data. For example, Fu & Murphy (1997) suggested close relationships of *O. popei* with *O. omeimontis*, *O. xiangchengensis*, *O. jingdongensis* with *O. rugosus* and the uniqueness of *O. rhodostigmatus*, and Fu *et al.* (1997) suggested close relationships of *S. mammatus*, *S. muliensis*, *S. tuberculatus* and *S. glandulatus* (*S. brevipes* as in Fu *et al.*, 1997).

Evolution of RSSD

The resulting phylogenetic hypothesis unambiguously suggested that the RSSD has evolved multiple times in high-elevation Tibetan megophryid frogs. As such, the group provides an excellent opportunity to study the causes and selection on the sexual dimorphism in anurans.

Several hypotheses may account for the observed RSSD in these megophryid frogs. First, a large male size in several frog species was found to be associated with male–male combat behaviour (e.g. Shine, 1979; Emerson & Ward, 1998). During the breeding season, male *Vibrissaphora* develop keratinized spines on the upper jaw, which resemble weaponry structures. Dubois & Ohler (1998) speculated that the spines are possibly used for direct combat between males. However, to our knowledge, combat between males has not been reported for any of the 13 megophryid species with RSSD. Observation suggests that male *Vibrissaphora* display no territory behaviour, and can coexist in close proximity and in high numbers while sharing the same breeding site. Ye *et al.* (1993) reported that multiple males, as many as 20, of *V. leishanensis* were commonly found under the same rock during the breeding season, while multiple breeding males of *V. boringiae* were also found under the same rock frequently. Furthermore, we have two seasons of detailed field study of *V. boringiae*, and not a single occasion of male–male combat has been observed; our examination of 105 breeding males did not reveal any wounds or scars near the heads (J. Fu, unpubl. data). So far, no evidence from these megophryid frogs supports the male–male combat hypothesis.

Alternatively, a large male size could be the consequence of female choice. Ho *et al.* (1999) suggested that an increase in male body size might be associated with parental care. A larger male with more energy reserve can provide better care for its offspring, a direct benefit for the female. During the breeding season, Ye *et al.* (1993) found male frogs of the genus *Vibrissaphora*, as well as *S. glandulatus* and *S. mammatus*, stationed under rocks and surrounded by one or more clutches of eggs, but females were seldom encountered. These males are often found to have loose skin, suggesting that they may remain underwater for prolonged periods of time to guard the eggs, while the folds of skin are used for cutaneous respiration (Hutchison, Haines & Engbretson, 1976). We also found that unguarded egg masses of *V. boringiae* suffer higher mortality than guarded ones, mostly due to fungus infection (J. Fu, unpubl. data). In this scenario, the spines of *Vibrissaphora* may be used for defending eggs, rather than for combat (Ho *et al.*, 1999). However, most *Vibrissaphora* species lives in fast-moving mountain streams, where no large-size predators are known to exist. The selective advantage of the spines as weaponry is questionable. Our preliminary observation suggested that the labial spine of male *Vibrissaphora* may be associated with stimulating females and nest construction/maintenance (J. Fu, unpubl. data). Finally, body size and the dominant calling frequency are often correlated, and the selection of male body size is possibly realized via female choice of the male mating call. We are currently analysing recorded mating calls of *V. boringiae*, and the data will shed more light on the female choice hypothesis.

The sexual dimorphism is likely correlated with other life-history traits, an area of research that remains largely unexplored (Gustafsson, Qvarnstrom & Sheldon, 1995). The limited availability of life-history data for these megophryids prevents us from presenting a more rigorous analysis. Nevertheless, the current phylogeny will help us to identify species groups where we will focus our effort on collecting life-history data, and eventually conduct a comprehensive phylogenetic comparative study. The phylogeny also provides guidance for selecting species for detailed field studies and experiments. Comparisons between closely related species with and without RSSD (e.g. *O. popei*/*nanjiangensis* vs. *O. chuanbeiensis*) but otherwise similar natural histories will better our understanding of the causes of sexual size dimorphism.

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Supplementary material

The following material is available for this article online:

Appendix S1. Sexual size dimorphism data for the species examined. Recent literature was preferred because of historical name changes. Measurement with stated sample size and large sample size was preferred. When available, the sample sizes are provided in parentheses.

This material is available as part of the online article from http://www.blackwell-synergy.com/doi/abs/10.1111/j.1469-7998.2007.00330.x

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