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Molecular phylogeny of Rhacophoridae (Anura): A framework of taxonomic reassignment of species within the genera *Aquixalus*, *Chiromantis*, *Rhacophorus*, and *Philautus*

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ABSTRACT

Phylogenetic relationships among representative species of the family Rhacophoridae were investigated based on 2904 bp of sequences from both mitochondrial (12S rRNA, 16S rRNA, the complete t-RNA for valine), and nuclear (tyrosinase, rhodopsin) genes. Maximum parsimony, maximum likelihood, and Bayesian analyses were employed to reconstruct the phylogenetic trees. This analysis, combined with previous phylogenetic studies, serves as a framework for future work in rhacophorid systematics. The monophyly of *Rhacophorus* is strongly confirmed except for the species *R. hainanus*, which is the sister taxon to *A. odontotarsus*. The non-monophyly of the newly designated genus *Aquixalus* by Delorme et al. [Delorme, M., Dubois, A., Grosjean, S., Ohler, A., 2005. Une nouvelle classification générique et subgénérique de la tribu des Philautini (Amphibia, Anura, Ranidae, Rhacophorinae). Bull. Mens. Soc. Linn. Lyon 74, 165–171] is further confirmed. *Aquixalus* (*Aquixalus*) forms a well-supported monophyletic group within *Kurixalus*, whereas, *Aquixalus* (*Gracixalus*) is more closely related to species of *Rhacophorus*, *Polypedates*, and *Chiromantis*. *Philautus* as currently understood, does not form a monophyletic group. *Philautus* (*Kirtixalus*) is the sister group to the clade comprising *Kurixalus* and *Aquixalus* (*Aquixalus*), and more remotely related to *Philautus* (*Philautus*). *Chiromantis romeri* does not cluster with species of *Chiromantis*, and forms a basal clade to all rhacophorids save *Buergeria*. We propose some taxonomic changes that reflect these findings, but further revision should await more detailed studies, which include combined morphological and molecular analyses, with greater species sampling.

1. Introduction

The frog family Rhacophoridae consists of 286 species in 10 genera, across a wide range: Tropical Africa; South Asia, including India and Sri Lanka; the Himalayas; South China east to Japan, and south across the Indochinese Peninsula to the Greater Sunda, and Philippine Islands (Frost, 2007). Although the genus *Chiromantis* (Peters, 1854) is found in Africa and Asia, the remaining nine genera (*Buergeria* Tschudi, 1838, *Aquixalus* Delorme, Dubois, Grosjean, and Ohler, 2005, *Kurixalus* Ye, Fei, and Dubois, 1999, *Nyctixalus* Boulenger, 1882, *Theloderma* Tschudi, 1838, *Philautus* Gistel, 1848, *Feihyla* Frost, Grant, Faivovich, Bain, Haas, Haddad, de Sá, Channing, Wilkinson, Donnellan, Raxworthy, Campbell, Blotto, Moler, Drewes, Nussbaum, Lynch, Green, and Wheeler, 2006, *Polypedates* Tschudi, 1838, and *Rhacophorus* Kuhl and Van Hssalt, 1822), are strictly Asian (Frost, 2007). Rhacophorids are mostly tree frogs, united morphologically in possessing intercalary elements between the two distal phalanges (a character shared with mantellids), and expanded digit discs (a character shared with some ranid lineages) (Frost et al., 2006; Liem, 1970). Several rhacophorid genera form aerial nests of foam (*Chiromantis*, *Rhacophorus*, *Polyedates*) or gelatin (*Aquixalus*, *Kurixalus*, *Nyctixalus*, *Theloderma*) that overhang water, into which developing tadpoles drop once they reach a certain size (Bain and Nguyen, 2004; Boulenger, 1903; Inger, 1966; Duellman and Trueb, 1986; Liem, 1970; Orlov et al., 2004). Finger webbing is variable, but extensive in many species (Liem, 1970).

Dubois (1992) proposed a major taxonomic revision of Rhacophoridae, considering it to be a subfamily of Ranidae, composed of three tribes: Buergerini, Philautini, and Rhacophorini. This revision has not been subsequently supported (e.g., Delorme et al., 2005; Frost et al., 2006; Richards and Moore, 1998; Wilkinson

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and Drewes, 2000; Wilkinson et al., 2002). Frost et al. (2006) used anatomical and molecular data to support a designation by Channing (1989) that Rhacophoridae includes two subfamilies, Buergeriinae and Rhacophorinae. However, a finely resolved family phylogeny has not yet been estimated due to the relatively limited rhacophorid sampling in Frost et al. (2006), as well as other studies (e.g., Channing, 1989; Delorme et al., 2005; Jiang et al., 1987; Liem, 1970; Richards and Moore, 1998; Wilkinson and Drewes, 2000; Wilkinson et al., 2002).

One problematic group is the genus *Rhacophorus* which contains 74 species, and is widely distributed across India, China, Japan, mainland Southeast Asia, the Greater Sunda Islands, and the Philippines (Frost, 2007). *Rhacophorus* is defined by a collection of morphological characters: Vertebrae procoelus; M. extensor radialis accessorius lateralis originates near crista ventralis; M. cutaneous pectoris thin, with a few layers; anal folds usually present; extensive dermal forearm and tarsal folds usually present; bright green or brown coloration usually present (after Jiang et al., 1987; Liem, 1970; Wilkinson and Drewes, 2000). A morphologically similar genus, *Polypedates*, is defined by a similar suite of characters: Vertebrae diplasiocoelus; M. extensor radialis accessorius lateralis originates on lateral side of humerus; M. cutaneous pectoris thick, muscular; anal folds usually absent; dermal forearm and tarsal folds only present as a slight ridge, if at all; dull gray coloration (after Jiang et al., 1987; Liem, 1970; Wilkinson and Drewes, 2000). Based on previous studies (e.g., Liem, 1970; Wilkinson and Drewes, 2000; Rao et al., 2006), Y-shaped terminal phalanges are synapomorphic for *Polypedates* and *Rhacophorus*, whereas webbing between the fingers in *Rhacophorus* separates it from *Polypedates*.

Regardless, there has been some disagreement over the monophyly of *Polypedates*, and the placement of species within *Rhacophorus* or *Polypedates*. Jiang et al. (1987) placed *Polypedates dugritei*, *P. hungfuensis*, *P. omeimontis*, *P. chenfui*, *P. nigropunctatus*, and *P. dennysi* in *Polypedates* without explanation. They considered two slips originating on the cristae ventralis as the ancestral character state. In a morphological reanalysis of their data, Rao et al. (2006) showed that the presence of only one slip on the cristae ventralis in *P. chenfui*, *P. dennysi*, *P. dugritei*, *P. hungfuensis*, and *P. nigropunctatus*, and one slip present on the tuberositas deltoidea in *R. reinwardtii* and *R. rhodopus* grouped these species at the exclusion of *P. leucomystax* and *P. mutus*. Rao et al. (2006) therefore suggested that these 'one slip species' (all green, and all known from China) be recognized as *Rhacophorus*, and the generic assignment of the remaining green rhacophorids from China (*P. gongshanensis*, *P. pingbianensis*, *P. puerensis*, *P. zhaojuensis*, and *P. yaoshanensis*) should be reconsidered. Rao et al. (2006) recognized that their evidence was not strong and that a molecular-based study would be needed to complement the previous morphological assays.

Another problematic rhacophorid group is the genus *Aquixalus*; eight species that range from the Himalayan front ranges of eastern India through China and mainland Southeast Asia (Delorme et al., 2005). This genus was divided into two nominal subgenera, *Aquixalus* and *Gracixalus* (Delorme et al., 2005), however *Aquixalus* as defined by its describing authors is paraphyletic or polyphyletic (Delorme et al., 2005). This was later addressed by removing *A. idiootocus* and *A. verrucosus* to the genus *Kurixalus*, which resulted in there being no morphological syanpomorphies for the genus (Frost et al., 2006). Frost et al. (2006) did not include the type species of *Aquixalus* (*A. odontotarsus*) in their analysis and the validity of the subgenera remains largely untested.

Another genus of interest is *Philautus*, which has been characterized by the aerial direct development of eggs into froglets (Dring, 1979; Bossuyt and Dubois, 2001). *Philautus*, which includes 146 species, is the largest rhacophorid genus, with a wide distribution from India and Sri Lanka through China, mainland Southeast Asia, the Greater Sunda Islands, and the Philippines (Frost, 2007). Due to their small size and high intraspecific variability, systematic study of this genus is still preliminary (Bossuyt and Dubois, 2001). Dubois (1987) divided the genus *Philautus* into three subgenera: *Philautus*, *Gorhixalus*, and *Kirtixalus*. Later, Bossuyt and Dubois (2001) revised this taxonomy, transferring several Indian species from the subgenus *Kirtixalus* to *Philautus*. Frost et al. (2006) erected *Feihyla*, owing to the unique phylogenetic position of *Philautus palpebralis*. The phylogenetic positions of most species of *Philautus*, however, remain unknown.

To test these proposed relationships among rhacophorid tree frogs, we reconstructed a molecular phylogeny for the family using nuclear DNA sequence data (nuDNA; rhodopsin and tyrosinase genes), and mitochondrial DNA (mtDNA; t-RNA for valine, and parts of 12S and 16S ribosomal genes). Sequences from GenBank were also incorporated in our analysis. In the process, we reassessed the monophyly of several genera of rhacophorids, and provide a phylogenetic background for a revised classification.

2. Materials and methods

2.1. Taxon sampling and data collection

Forty-six species from the family Rhacophoridae were selected as ingroup taxa (Table 1), representing nine genera (*Aquixalus*, *Chiromantis*, *Feihyla*, *Kurixalus*, *Nyctixalus*, *Philautus*, *Polypedates*, *Rhacophorus*, *Theloderma*) from Rhacophorinae and one (*Buergeria*) from Buegeriinae. Three species from the family Mantellidae and one from Ranidae were chosen as outgroups (after Frost et al., 2006). We follow the taxonomy proposed by Frost (2007) and Yu et al. (2007), mainly for the purposes of discussion. All novel sequences produced in this study were deposited in GenBank (Accession Nos. are shown in Table 1), and we also obtained some sequences from GenBank (Table 1).

2.2. DNA extraction, amplification, and sequencing

Genomic DNA was extracted from toeclips, muscle, or liver tissues in 95% or 100% ethanol. Tissue samples were digested using proteinase K, and then followed a standard three-step phenol/chloroform extraction procedure (Sambrook et al., 1989; Hillis et al., 1996). The nuDNA fragments were a 532 bp fragment of exon 1 of tyrosinase and a 316 bp fragment of exon 1 of rhodopsin. The mtDNA fragment included 2056 bp from the 12S and 16S together with the complete t-RNA for valine. The primers used in this study are after Bossuyt and Milinkovitch (2000), and Wilkinson et al. (2002). Double stranded polymerase chain reaction (PCR) amplification for the mitochondrial genes was carried out using the following parameters: 95 °C initial hot start (5 min), 35 cycles of 94 °C denaturation (1 min), 55 °C annealing (1 min), and 72 °C extension (1 min). Final extension at 72 °C was conducted for 10 min. For rhodopsin and tyrosinase, the same procedure was used, but with annealing at 52 °C and 54 °C, respectively. Purified PCR products were directly sequenced with an ABI 3730 automated DNA sequencer and sequences were then determined in both directions for each species and submitted for BLAST searching (Altschul et al., 1997) in GenBank to ensure that the required sequences had been amplified.

2.3. Sequence alignment

Alignments were first conducted using the program Clustalx 1.81 (Thompson et al., 1997) with default parameters, and subsequently adjusted by eye. For the mtDNA gene sequences, five hyper-variable regions with 134 bp in total (168–180, 451–486,

Table 1
Samples and sequences used in this study

Specific epithet	Frost (2007)	Present genus	Specimen voucher No.	Locality	GenBank No. (12S and 16S)	GenBank No. (rhodopsin, tyrosinase)
	Rhacophoridae Buergeriinae	Rhacophoridae Buergeriinae				
buergeri	*Buergeria*	*Buergeria*	TTU-R-11759	—	AF458122	AY880623
oxycephala	*Buergeria*	*Buergeria*	SCUM 050267YJ	China: Hainan	EU215524[a]	EU215556[a], EU215585[a]
	Rhacophorinae	Rhacophorinae				
eiffingeri	*Kurixalus*	*Kurixalus*	UMFS 5969	China: Nantou, Taiwan	DQ283122	DQ283880, DQ282931
idiootocus	*Kurixalus*	*Kurixalus*	SCUM 061107L	China: Lianhuachi, Taiwan	EU215547[a]	EU215577[a], EU215607[a]
idiootocus	*Kurixalus*	*Kurixalus*	UMFS 5702	China: Nantou, Taiwan	DQ283054	DQ283783, DQ282905
odontotarsus	*Aquixalus*	*Kurixalus*	SCUM 060688L	China: Mengyang, Jinghong	EU215549[a]	EU215579[a], EU215609[a]
hainanus	*Rhacophorus*	*Kurixalus*	HNNU A1180	China: Mt. Diaoluo, Hainan	EU215548[a]	EU215578[a], EU215608[a]
gracilipes	*Aquixalus*	*Gracixalus*	AMNH A163897	Vietnam	DQ283051	DQ283780
jinxiuensis	*Philautus*	*Philautus*	KIZ 061210YP	China: Mt. Dayao, Guangxi	EU215525[a]	EU215557[a], EU215587[a]
romeri	*Chiromantis*	*Liuixalus*	KIZ 061205YP	China: Mt. Shiwan, Guangxi	EU215528[a]	EU215559[a], EU215589[a]
Unidentified	—	*Liuixalus*	KIZ 061209YP	China: Mt. Dayao, Guangxi	EU215526[a]	EU215558[a], EU215588[a]
acutirostris	*Philautus*	*Philautus*	—	—	AF458137	
surdus	*Philautus*	*Philautus*	CAS 219932	Philippine	AF458138	
microtympanum	*Philautus*	*Philautus*	GenBank	Sri Lanka	DQ346974	AF249126, AF249189
wynaadensis	*Philautus*	*Philautus*	GenBank	India	DQ346966	AF249127, AF249190
charius	*Philautus*	*Philautus*	GenBank	India	DQ346967	AF249128, AF249191
rhododiscus	*Philautus*	*Theloderma*	SCUM 061102L	China: Mt. Dayao, Guangxi	EU215530[a]	EU215555[a], EU215586[a]
rhododiscus	*Philautus*	*Theloderma*	AMNH A163892	Vietnam	DQ283392 DQ283393	DQ284007, DQ282998
corticale	*Theloderma*	*Theloderma*	AMNH A161499	Vietnam	DQ283050	DQ283779, DQ282904
spinosus	*Nyctixalus*	*Nyctixalus*	ACD 1043	Philippine: Mindanao	DQ283114	DQ283827
pictus	*Nyctixalus*	*Nyctixalus*	FMNH 231095	Malaysia	DQ283133	DQ283834
leucomystax	*Polypedates*	*Polypedates*	CAS 219931	Philippine	AF458140	DQ283777
megacephalus	*Polypedates*	*Polypedates*	—	—	AF458141	AY880650
megacephalus	*Polypedates*	*Polypedates*	SCUM 0607116L	China: Huidong, Guangdong	EU215550[a]	EU215580[a], EU215610[a]
megacephalus	*Polypedates*	*Polypedates*	SCUM 060602L	China: Tengchong, Yunnan	EU215553[a]	EU215583[a], EU215613[a]
megacephalus	*Polypedates*	*Polypedates*	SCUM 050508C	China: Mt. Daiyun, Fujian	EU215552[a]	EU215582[a], EU215612[a]
mutus	*Polypedates*	*Polypedates*	SCUM 37940C	China: Xishuangbanna, Yunnan	EU215551[a]	EU215581[a], EU215611[a]
kio	*Rhacophorus*	*Rhacophorus*	SCUM 37941C	China: Xishuangbanna, Yunnan	EU215532[a]	EU215562[a], EU215592[a]
rhodopus	*Rhacophorus*	*Rhacophorus*	SCUM 060692L	China: Mengyang, Jinghong	EU215531[a]	EU215561[a], EU215591[a]
bipunctatus	*Rhacophorus*	*Rhacophorus*	SN 030035	China: Hainan	EU215529[a]	EU215560[a], EU215590[a]
bipunctatus	*Rhacophorus*	*Rhacophorus*	ROM 99944	—	AF458144	AY844737
annamensis	*Rhacophorus*	*Rhacophorus*	AMNH A161414	Vietnam	DQ283047	DQ283776
orlovi	*Rhacophorus*	*Rhacophorus*	AMNH A161405	Vietnam	DQ283049	DQ283778
calcaneus	*Rhacophorus*	*Rhacophorus*	AMNH A163749	Vietnam	DQ283380	DQ283999, DQ282991
malabaricus	*Rhacophorus*	*Rhacophorus*	—	India	DQ346957	AF249125, AF249188
dugritei	*Rhacophorus*	*Rhacophorus*	SCUM 051001L	China: Baoxing, Sichuan	EU215541[a]	EU215571[a], EU215601[a]
dugritei	*Rhacophorus*	*Rhacophorus*	SCUM 051017L	China: Hongya, Sichuan	EU215540[a]	EU215570[a], EU215600[a]
hungfuensis	*Rhacophorus*	*Rhacophorus*	SCUM 060425L	China: Wenchuan, Sichuan	EU215538[a]	EU215568[a], EU215598[a]
minimus	*Rhacophorus*	*Rhacophorus*	KIZ 061214YP	China: Mt. Dayao, Guangxi	EU215539[a]	EU215569[a], EU215599[a]
puerensis	*Rhacophorus*	*Rhacophorus*	SCUM 060649L	China: Puer, Yunnan	EU215542[a]	EU215572[a], EU215602[a]
pingbianensis	*Rhacophorus*	*Rhacophorus*	SCUM 061104L	China: Mt. Dawei, Yunnan	EU215536[a]	EU215566[a], EU215596[a]
omeimontis	*Rhacophorus*	*Rhacophorus*	SCUM 0606137L	China: Pengxian, Sichuan	EU215535[a]	EU215565[a], EU215595[a]
taronensis	*Rhacophorus*	*Rhacophorus*	SCUM 060614L	China: Mt. Gaoligong, Yunnan	EU215537[a]	EU215567[a], EU215597[a]
arboreus	*Rhacophorus*	*Rhacophorus*	TTU-R-11748	—	AF458142	AY880653
moltrechti	*Rhacophorus*	*Rhacophorus*	SCUM 061106L	China: Lianhuachi, Taiwan	EU215543[a]	EU215573[a], EU215603[a]

nigropunctatus	*Rhacophorus*	*Rhacophorus*	SCUM 070657L	China: Weining, Guizhou	EU215533[a]	EU215563[a], EU215593[a]
feae	*Rhacophorus*	*Rhacophorus*	SCUM 050642W	China: Hekou, Yunnnan	EU215544[a]	EU215574[a], EU215604[a]
dennysi	*Rhacophorus*	*Rhacophorus*	SCUM 060401L	China: Shaoguan, Guangdong	EU215545[a]	EU215575[a], EU215605[a]
chenfui	*Rhacophorus*	*Rhacophorus*	SCUM 060404L	China: Mt. Omei, Sichuan	EU215534[a]	EU215564[a], EU215594[a]
palpebralis	*Feihyla*	*Feihyla*	SCUM 0606132L	China: Mt. Dawei, Yunnan	EU215546[a]	EU215576[a], EU215606[a]
doriae	*Chiromantis*	*Chiromantis*	SN 030051	China: Hainan	EU215527[a]	EU215554[a], EU215584[a]
rufescens	*Chiromantis*	*Chiromantis*	CAS 207601	Equatorial Guinea	AF458126	DQ347356, DQ347139
xerampelina	*Chiromantis*	*Chiromantis*	–	–	AF458132	DQ284012
Outgroup						
	Mantellidae	Mantellidae				
tephraeomystax	*Boophis*	*Boophis*	AMNH A168144	Madagascar	DQ283032	DQ283761, AF249168
madagascariensis	*Aglyptodactylus*	*Aglyptodactylus*	UMMZ 198472	Madagascar	DQ283056	DQ283785, AF249166
aurantiaca	*Mantella*	*Mantella*	UMMZ 201411	Madagascar	DQ283035	DQ283766, DQ282901
	Ranidae	Ranidae				
poilani	*Limnonectes*	*Limnonectes*	AMNH A163717	Vietnam	DQ283378	DQ283997, DQ282989

[a] Sequences new to this study. HNNU, Hainan Normal University; KIZ, Kunming Institute of Zoology, the Chinese Academy of Sciences; SCUM, Sichuan University Museum. Additionally SN is from the field numbers of Shunqing Lu. "–" represents the unknown information.

633–679, 1033–1063, and 1417–1423) were excluded from further analysis due to the ambiguous alignments. Such exclusion increases the reliability of the phylogenetic analysis (after Swofford et al., 1996). The aligned sequences have been submitted to TreeBASE at http://www.treebase.org under accession number (SN3819). Gaps resulting from the alignment were treated as missing data. Considering that all mtDNA gene sequences are effectively inherited as one locus, the 12S and 16S ribosomal gene fragments and the complete t-RNA for valine were concatenated into a single fragment for the analyses.

2.4. Phylogenetic analyses

For each mtDNA and nuDNA fragment, possible saturation of substitution types was checked by plotting the number of transitions (Ti) and transversions (Tv) versus TN93 distance using DAMBE (Xia, 2000). To examine possible incongruence between genes and gene combinations (tyrosinase + rhodopsin; mtDNA + tyrosinase + rhodopsin), we used an incongruence length difference (ILD test) (Farris et al., 1994) referred to as a partition homogeneity test in PAUP 4.0b 10a (Swofford, 2003). One hundred replicates of the ILD test with 10 random addition sequences were implemented. Sequence data of mtDNA and nuDNA genes were analyzed both separately and combined. All characters were weighted equally and unordered. Maximum parsimony (MP) and maximum likelihood (ML) analysis were calculated using PAUP 4.0b 10a (Swofford, 2003). The best fitting models of sequence evolution to all three partitions (mtDNA; tyrosinase + rhodopsin; mtDNA + tyrosinase + rhodopsin) were obtained by Modeltest 3.7 (Posada and Crandall, 1998) for ML and Bayesian inference (BI). Maximum parsimony analyses were performed using a heuristic search with 1000 random stepwise additions followed by TBR branch swapping. Bootstrap branch support (BBP) values were calculated with 1000 replicates. For model-based ML analysis, we selected the GTR + I + G model under the Akaike Information Criterion (AIC) in Modeltest. Heuristic searches were executed in 10 replicates using TBR branch swapping and bootstrap branch support (BBP) were calculated with 10 mL replicates. Due to time limitations, ML analysis was confined to the final combined data (mtDNA + rhodopsin + tyrosinase). MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) was used for BI. For the Bayesian posterior probabilities (BPP), the following settings were applied: Number of Markov chain Monte Carlo (MCMC) generations = 3,000,000 and sampling frequency = 100. For combined nuclear (rhodopsin + tyrosinase) and mtDNA Bayesian analyses, each partition was followed as its own DNA evolution model under the Akaike Information Criterion (AIC) in Modeltest. The best-fit models for mtDNA, rhodopsin and tyrosinase genes are GTR + I + G, HKY + I + G and SYM + I + G, respectively. The first 7500 were discarded as a conservative burn-in. The remaining samples were used to generate a majority rule consensus tree. All MCMC runs were repeated twice to confirm consistent approximation of the posterior parameter distributions.

3. Results

3.1. Sequence variation and data partitions

Alignment of the mitochondrial gene fragments resulted in 2056 sites, corresponding to sites 712 through 2666 of *P. megacephalus* mitochondrial genome (AY458598). At the exclusion of hyper-variable regions, the remaining mtDNA data had a total of 1922 characters, with 799 constant characters and 912 parsimony-informative characters. The alignment of the combined data (rhodopsin + tyrosinase) produced 848 bp of sequences, of which 533 sites were constant and 224 sites were parsimony-informa-

tive. The combined and aligned data matrix of mtDNA + rhodopsin + tyrosinase presented a total of 2770 characters, of which 1317 were constant and 1151 were parsimony-informative. Transitions and transversions in the case of the three genes were accumulating linearly and gave no indication of saturation effect (data not shown), thus all substitutions in these genes were used for phylogenetic reconstructions.

3.2. Phylogenies of mtDNA gene data

Analysis of mtDNA data under unweighted parsimony resulted in two trees with $L = 6240$ steps, consistency index (CI) = 0.302, retention index (RI) = 0.553, and rescaled consistency index (RC) = 0.167. For BI, the likelihood values of the 50% majority consensus tree was $\ln L = -33215.6367$. Since the 50% majority consensus tree from BI was consistent with the MP strict consensus tree, we show only the BI tree, but bootstrap values from MP and Bayesian posterior probabilities are indicated for all nodes to show the degree of congruence among results (Fig. 1).

3.3. Phylogenies of nuclear gene data

For tyrosinase and rhodopsin nuclear genes, partition homogeneity test presented no evidence of phylogenetic conflict ($P = 0.06$), so a combined dataset was constructed for phylogenetic inferences. We only used the complete nuclear data for analysis. The combined nuclear gene data resulted in a total of 384 most parsimonious trees of 766 steps. These trees exhibited the following descriptive statistics: CI = 0.544, RI = 0.681, $RC = 0.371$. For the BI analyses, the likelihood values of the 50% majority consensus tree was $\ln L = -5234.5884$. In BI, the 50% majority consensus trees based on combined nuclear genes were almost the same as the MP strict consensus tree. The combined nuclear data showed little resolution. However, tree topologies supported by the combined nuclear data were largely congruent with the results from mtDNA. Fig. 2 showed the Bayesian tree.

3.4. Phylogenies of combined mtDNA and nuclear gene data

Parsimony analysis of the combined data resulted in four equally parsimonious trees ($L = 7169$, CI = 0.324, RI = 0.560, $RC = 0.182$). For BI analyses, the likelihood values of the 50% majority consensus tree was $\ln L = -34841.9531$. All analyses based on the combined data yielded almost the same topology. Only the 50% majority consensus tree from BI is shown in Fig. 3. In MP, ML, and BI, all the nodes for recently divergent taxa had higher bootstrap values, and some basal nodes of the topology were well resolved only in BI (Fig. 3). The results are reported as follows:

(1) Species in the family Rhacophoridae form a strongly supported clade with respect to the outgroups (1.00, 100, and 99 support in Bayesian posterior probability, ML, and MP bootstrap values, respectively).
(2) *Buergeria buergeri* and *B. oxycephalus* form a strongly supported clade (1.00, 100, and 100) that is basal to all remaining rhacophorids.
(3) *Chiromantis romeri* and one unidentified species form a strongly supported clade (1.00, 100, and 100), that is a sister clade to the remaining rhacophorids (1.00, 100, and 99), excluding *Buergeria*.
(4) *Nyctixalus pictus* and *N. spinosus* form a strongly supported clade (1.00, 100, and 100), that is sister to a *Theloderma corticale*, *T. rhododiscus* clade (1.00, 100, and 100). *Theloderma rhododiscus* from Guangxi Province, China, is sister to a population from Ha Giang Province, Vietnam. This strongly supported (1.00, 100, and 100) *Nyctixalus*, *Theloderma* clade is basal to the remaining rhacophorids, excluding the abovementioned species, with relatively strong posterior probability (0.99, –, and –).
(5) *Philautus acutirostris* and *P. surdus* form a strongly supported clade (1.00, 100, and 100) that is part of a polytomy with other rhacophorid clades. *Philautus charius*, *P. wynaadensis*, and *P. microtympanum* constitute a strongly supported clade (1.00, 100, and 100) that is sister to a strongly supported clade (1.00, 100, and –) consisting of *Kurixalus eiffingeri*, *K. idiootocus*, *Aquixalus odontotarsus*, and *Rhacophorus hainanus*.
(6) *Kurixalus eiffingeri* and *K. idiootocus* form a strongly supported clade (1.00, 100, and 100) that is sister to a strongly supported clade (1.00, 100, and 100) consisting of *R. hainanus* and *A. odontotarsus*.
(7) *Aquixalus gracilipes* and *P. jinxiuensis* constitute one strongly supported clade (1.00, 90, and 84) that is basal to a clade consisting of *Feihyla palpebralis* and species of *Chiromantis*, *Polypedates*, and *Rhacophorus*.
(8) *Chiromantis rufescens* and *C. xerampelina* are sister species (1.00, 100, and 100), and together form a sister clade to *C. doriae* (1.00, 100, and 100). This *Chiromantis* clade forms a sister group relationship with moderate Bayesian support (0.93, –, and –) with a clade consisting of a polytomy between *Feihyla palpebralis* and clades consisting of species of *Rhacophorus* and *Polypedates*.
(9) *Rhacophorus kio*, *R. rhodopus*, *R. bipunctatus*, *R. annamensis*, *R. orlovi*, *R. calcaneus*, and *R. malabaricus* constitute a well-supported clade (1.00, 100, and 100) that forms a strongly supported (1.00, 100, and 95) sister relationship to another strongly supported clade (1.00, 100, and 100) consisting of *Rhacophorus dugritei*, *R. hungfuensis*, *R. minimus*, *R. puerensis*, *R. pingbianensis*, *R. omeimontis*, *R. taronensis*, *R. arboreus*, *R. moltrechti*, *R. chenfui*, *R. nigropunctatus*, *R. feae*, and *R. dennysi*. A Hainan population and population of unknown provenance of *R. bipunctatus* are sister species (1.00, 100, and 100), and together form a sister group relationship with *R. rhodopus* (1.00, 100, and 94). A maculated individual of *R. dugritei* is sister to an un-maculated individual.
(10) Species of *Polypedates* form a monophyletic clade (1.00, 100, and 100) within which Chinese populations previously assigned to *P. megacephalus* split into two clades. One clade consists of Yunnan and Fujian populations, and an unknown population of *Polypedates megacephalus* with strong support (1.00, 100, and 100). A Guangdong population of *P. megacephalus* and a Philippine population of *Polypedates leucomystax* show sisters group relationships with strong support (1.00, 100, and 90), and together form a sister group to *P. mutus* (1.00, 100, and 100).

4. Discussion

Nuclear rhodopsin and tyrosinase have been widely used to infer amphibian phylogenies (Bossuyt and Milinkovitch, 2000; Che et al., 2007; Frost et al., 2006). In our analyses, the nuclear tree was less resolved than mtDNA gene tree. We favor the hypothesis from combined data to guide our discussion and taxonomic conclusions for two reasons. First, there were no strongly supported nodes that were in conflict with the trees from the partitioned data. Second, the combined data increased our phylogenetic resolution (in the combined data, 42 nodes with BBP > 70 from MP,

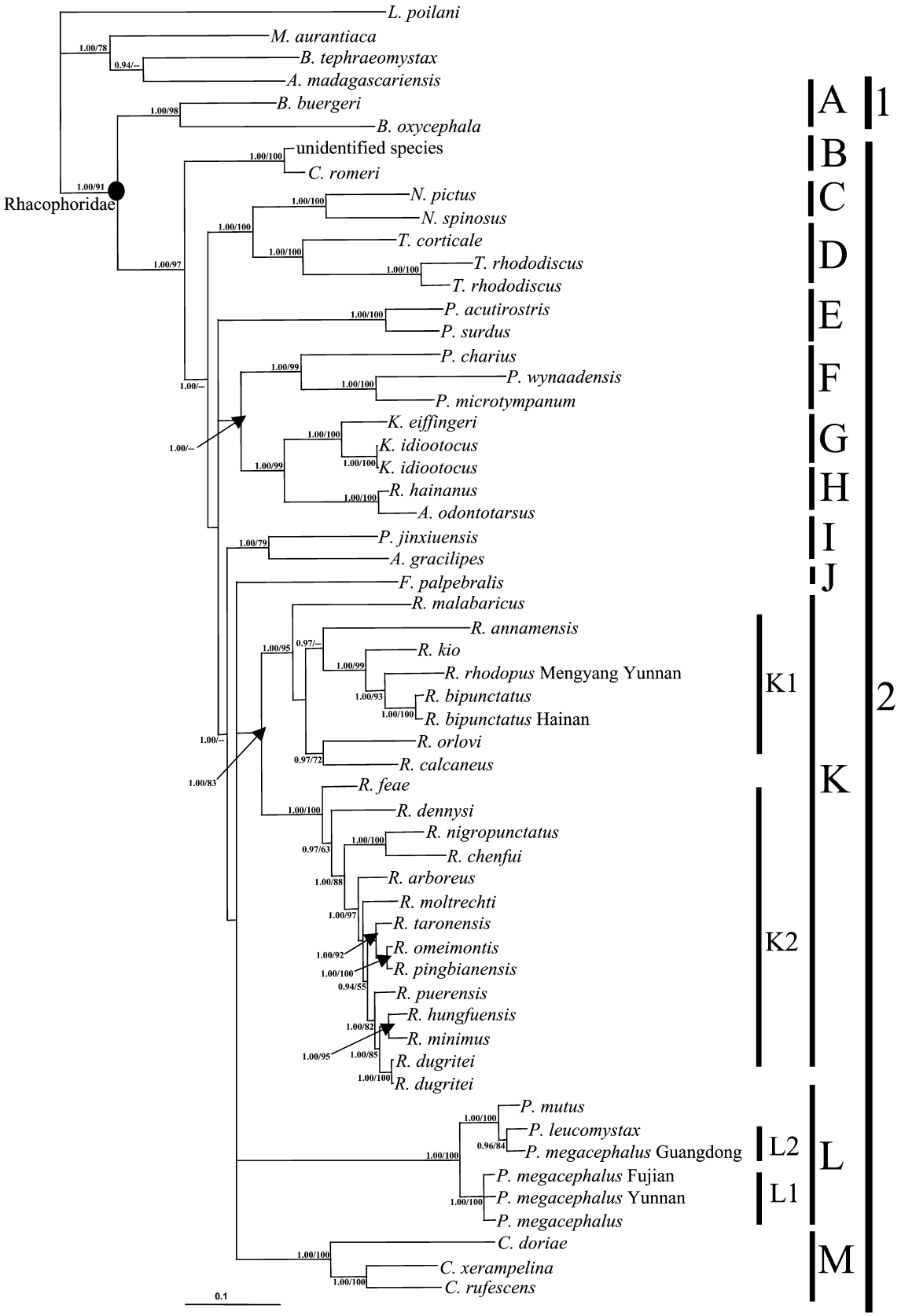

Fig. 1. Bayesian inference tree derived from the part of 12S and 16S ribosomal genes together the complete t-RNA for valine. Numbers above the lines or besides the nodes are Bayesian posterior probabilities (⩾90% retained)/bootstrap support for maximum parsimony analyses (1000 replicates, ⩾50 retained). "--" represents Bayesian posterior probabilities and bootstrap value lower than 90% and 50%, respectively.

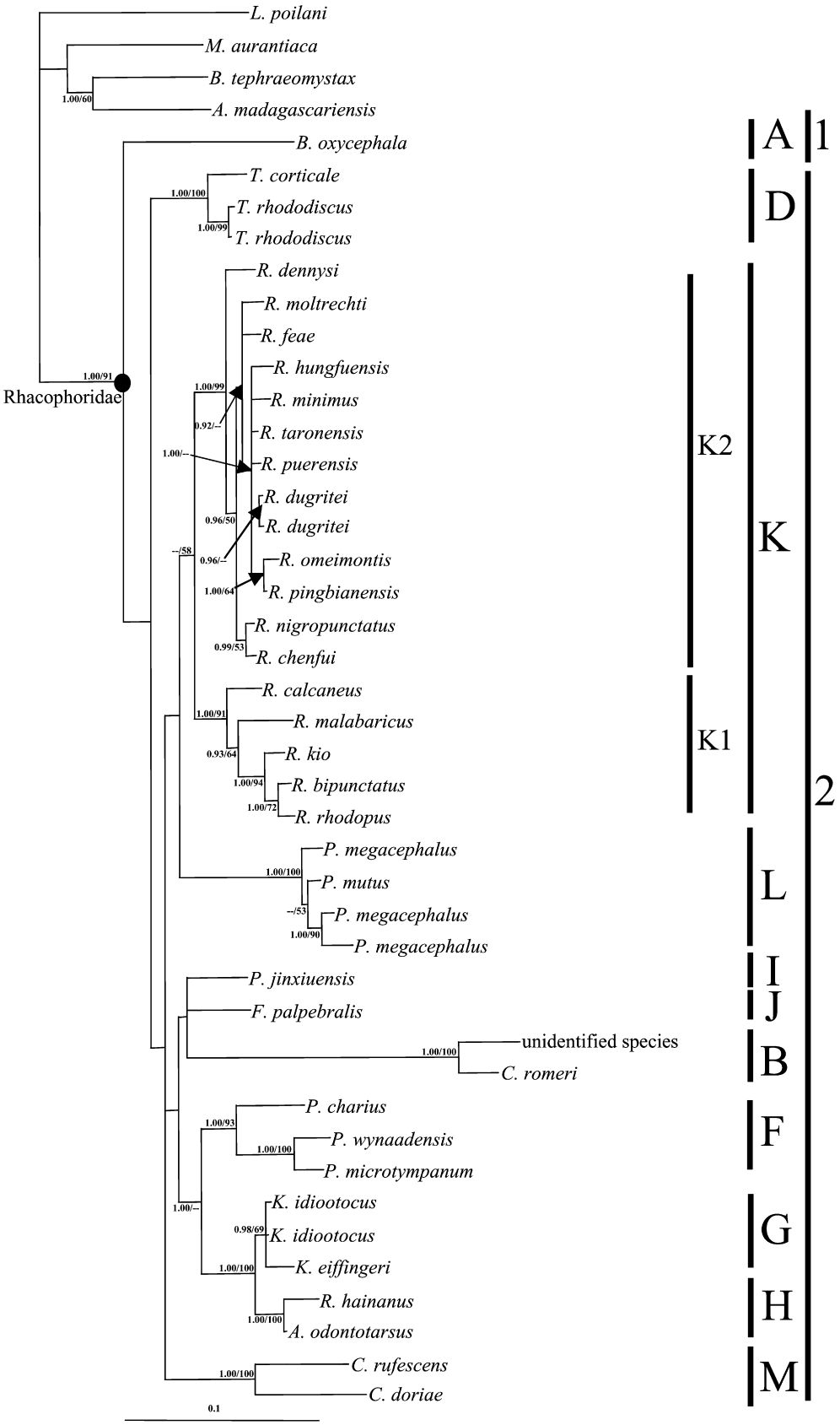

Fig. 2. Bayesian inference tree derived from the partial DNA sequence of the nuclear genes rhodopsin and tyrosinase. Numbers above the lines or besides the nodes are Bayesian posterior probabilities (⩾90% retained)/bootstrap support for maximum parsimony analyses (1000 replicates, ⩾50 retained). "--" represents Bayesian posterior probabilities and bootstrap value lower than 90% and 50%, respectively.

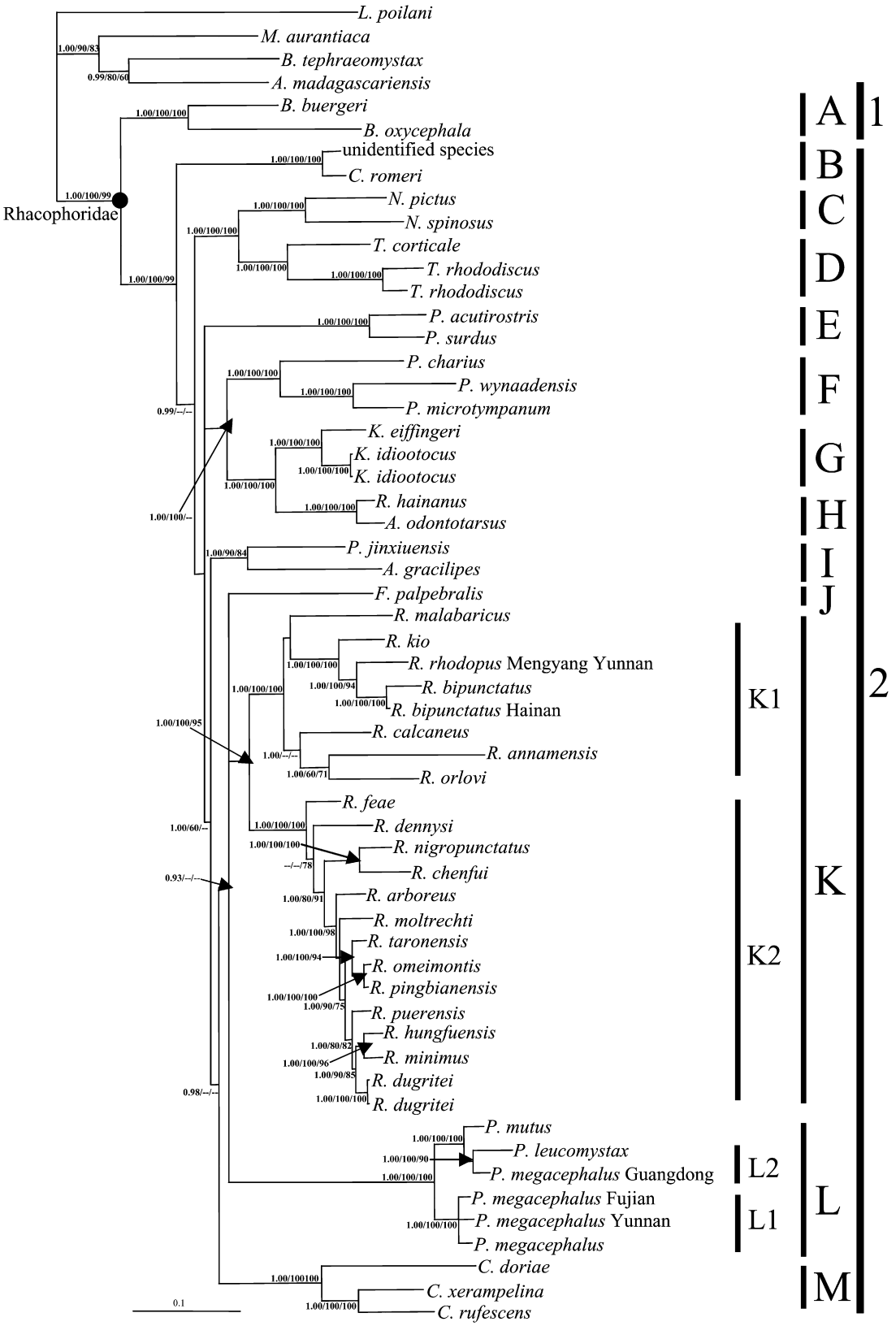

Fig. 3. Bayesian inference tree derived from the combined gene fragment (mtDNA + rhodopsin + tyrosinase). Bayesian posterior probabilities (⩾90% retained), bootstrap support from maximum likelihood (10 replicates), and maximum parsimony (1000 replicates) (⩾50 retained) are showed at the nodes, respectively. "--" represents Bayesian posterior probabilities and bootstrap value from MP and ML lower than 90% and 50%, respectively.

and 47 nodes with BPP > 90 from BI; in mtDNA data, 39 and 46; and in nuDNA, even lower at 15 and 25). We do acknowledge, however, that mtDNA gene data contributes greatly to the combined data topology.

4.1. Phylogeny of Rhacophorus (sensu Frost, 2007)

In this study, *Rhacophorus* is more closely related to *Polypedates* than it is to *Chiromantis*, although this result has poor support. This is not consistent with the molecular analyses of Wilkinson et al. (2002) and Frost et al. (2006), but it does support the morphological study of Wilkinson and Drewes (2000). However, these relationships are far from being conclusive and await improved taxonomic sampling and more gene data collection.

Rhacophorus is shown to be non-monophyletic and further divided into two monophyletic clades (H and K) in this study. In Clade H, *Rhacophorus hainanus* is the sister taxon to *Aquixalus odontotarsus*, and in this study *A. odontotarsus* is moved to genus *Kurixalus* (see below). In order to avoid paraphyly within *Rhacophorus*, we recommend transferring *R. hainanus* to *Kurixalus* as *K. hainanus*.

At the exclusion of *R. hainanus*, the monophyly of *Rhacophorus* (Clade K), as currently recognized, is well-supported by all analyses. We recognize Clade K as *Rhacophorus* sensu stricto because it contains *R. kio*, which has been shown to be closely related to the type species, *R. reinwardtii* (Ohler and Delorme, 2006). In our topology, Clade K is divided into two well-supported monophyletic subclades (K1 and K2). Members of K1 are nested in genus *Rhacophorus*, which was supported by numerous morphological characters (Jiang et al., 1987; Liem, 1970; Wilkinson and Drewes, 2000) and has been confirmed in previous molecular analyses (Frost et al., 2006; Wilkinson et al., 2002). Members of the strongly supported sister subclade to subclade K1 (K2; Fig. 3) have alternately been recognized as *Rhacophorus* or *Polypedates* (Jiang et al., 1987; Rao et al., 2006; Wilkinson and Drewes, 2000; Wilkinson et al., 2002), but our increased sample size affords us greater resolution and stronger support than previous assays. Our molecular phylogenetic analysis strongly suggests that members of subclade K2 should lie inside of *Rhacophorus* rather than *Polypedates*. This is consistent with the suggestion that rhacophorid species with green dorsal color and webbing between the fingers are members of *Rhacophorus* (Rao et al., 2006; Wilkinson et al., 2002). Our results further confirm that these 'one slip species' (all green, and all known from China), such as *Polypedates dugritei*, *P. taronensis*, *P. pingbianensis*, *P. puerensis*, *P. chenfui*, *P. dennysi*, *P. hungfuensis* and *P. nigropunctatus* should be recognized as *Rhacophorus*.

Inger et al. (1999) considered *R. rhodopus* to be a synonym of *R. bipunctatus*, which was adopted by Frost, (2007). Recently, Yu et al. (2007) showed that this is a polytypic species with a complicated genetic structure across a very wide range, and suggested that *R. rhodopus* be removed from the synonymy of *R. bipunctatus* as a valid species, which was further confirmed by the morphological study (Bordoloi et al., 2007). In our analysis, *R. rhodopus* from the type locality is shown to be an independent lineage remote from *R. bipunctatus*. However, it is difficult to know which of our taxa (if any) are *R. bipunctatus* sensu stricto, since there are at least a few species present in the region, and the type locality is in the "Khasi Hills" of India. We therefore treat our type locality specimens as *R. rhodopus* and the other specimens as *R. cf. bipunctatus*.

Our results also indicated that *R. pingbianensis* and *R. omeimontis*, as well as *R. hungfuensis* and *R. minimus* are very closely related. We suggest that these species be more closely studied (both morphologically and molecularly) to be certain that they are each valid species. Additionally, our results support Li et al. (2006), who considered that the un-maculated pattern of *R. dugritei* are independent of chromosomal data, and the dorsal color pattern of *R. dugritei* should not be considered to be diagnostic.

4.2. Phylogeny of Aquixalus (sensu Frost, 2007)

Our results suggest that the genus *Aquixalus* is paraphyletic across Clades G and H, which is largely consistent with Delorme et al. (2005). Frost et al. (2006) preserved the monophyly of *Aquixalus* by placing *A. verrucosus* within *Kurixalus*. Our results further suggest that Clade H, which included *A. odontotarsus*, *R. hainanus*, and the type species of *Kurixalus*, *K. eiffingeri*, are sister with strong support not only from mtDNA genes but also nuDNA genes. This is not surprising, given the great morphological similarity between the two genera (Delorme et al., 2005; Frost et al., 2006). Orlov et al. (2002) suggested that *A. odontotarsus*, the type species of *Aquixalus*, is conspecific with *K. verrucosus*. Zhao et al. (2004) placed *R. hainanus* in the genus *Rhacophorus*, due to its similarity to Chinese specimens of *R. cavirostris*, which is also thought to be synonymous with *K. verrucosus* (Inger et al., 1999). All of this suggests that the genus *Aquixalus* might lie inside of *Kurixalus*. We therefore move *A. odontotarsus*, the type species of *Aquixalus*, and *R. hainanus* to the genus *Kurixalus*, and consider the genus *Aquixalus* a junior synonym of genus *Kurixalus*.

A unique clade (Clade I) containing *A. gracilipes* (the type species of the genus *Aquixalus* subgenus *Gracixalus*), and *P. jinxiuensis* (see below) is found to be distantly related to *Kurixalus* (Clade H), *Aquixalus* (Clade H), and *Philautus* (Clade E; see below). The *A. gracilipes–P. jinxiuensis* clade is the most basal member in the well-supported monophyletic group of *A. gracilipes–P. jinxiuensis*, *Chiromantis*, *Feihyla*, *Rhacophorus*, and *Polypedates*. Frost et al. (2006) suggested that *A. gracilipes* is the most basal rhacophorid, except for *Buergeria* (with taxonomic sampling that differed from ours). This indicates that the phylogenetic position of the *A. gracilipes–P. jinxiuensis* clade is unique. Frost et al. (2006) suggested some provisional morphological diagnosis of *Gracixalus*, such as spines on the upper eyelid, rictal gland connected to the mouth, foot very thin and so on, which can separate it from the nominate subgenus *Aquixalus*. Following our exclusion of *A. odontotarsus* and *R. hainanus* from genus *Aquixalus* to *Kurixalus*, a putatively monophyletic *Gracixalus* should be recognized, which is consistent with the implication of Frost et al. (2006). By this definition, however, *Gracixalus* should be raised to the rank of genus, and it contains the type species *G. gracilipes*, as well as *G. supercornutus* (Orlov et al., 2004) (not studied by us).

4.3. Phylogeny of Philautus (sensu Frost, 2007)

Presently, *Philautus* contains approximately 150 species, widely distributed in South and Southeast Asia (Frost, 2007). Our data suggest *Philautus*, as currently understood, does not constitute a monophyletic group. Clearly, resolving the paraphyly/polyphyly of the whole genus is beyond the scope of this paper, but our findings warrant comment and taxonomic revisions.

We did not include the type species of *Philautus*, *P. aurifasciatus*, but we did include *P. acutirostris*, long considered a synonym of *P. aurifasciatus* (see Inger, 1966). Dring (1987) removed *P. acutirostris* from the synonymy of *P. aurifasciatus*, but placed it into the *P. aurifasciatus* group. These are indicative of the probable close relationship between *P. acutirostris* and *P. aurifasciatus*. Therefore, as with Wilkinson et al. (2002), we consider the clade that includes *P. acutirostris* and *P. surdus* (Clade E) as *Philautus* sensu stricto. In this study, *Philautus* forms a polytomy with a "*Kurixalus*" clade, an *A. gracilipes–P. jinxiuensis* clade, and a *Feihyla–Rhacophorus–Polypedates*, and a *Chiromantis* clade, which is incongruent with the hypothesis made by Wilkinson et al. (2002) of a sister group relationship between *Philautus* and *Kurixalus*. However, the relationship is far from being conclusive and is still in need of a rigorous phylogenetic analysis.

Dubois (1987) divided *Philautus* into three distinct subgenera: *Philautus*, *Gorhixalus*, and *Kirtixalus*. Only two species, *P. hosii* and *P. ingeri*, are included in *Philautus* (*Gorhixalus*). *Philautus* (*Philautus*) contains most of the species previously referred to the genus *Philautus*. *Philautus* (*Kirtixalus*), type species *P. microtympanum*, included species from Sri Lanka and India. However, Bossuyt and Dubois

(2001) proposed that the species from India should be provisionally placed in the subgenus *Philautus* (*Philautus*) pending more data. Our data strongly suggest that the type species of *Kirtixalus*, *P. microtympanum* from Sri Lanka, as well as *P.* (*Philautus*) *charius* and *P.* (*Philautus*) *wynaadensis* from India form a distinct Clade F, which was clearly divergent from other members of the *Philautus* (*Philautus*) (i.e., Clade E). Whereas members of Clade F form a monophyletic group with members of "*Kurixalus*" (Clade G and H) with strong support, and our nuclear tree also indicates the same relationship with high Bayesian support; a novel hypothesis. Some previous analyses, such as Frost et al. (2006), Wilkinson et al. (2002) do not include species of this clade, and it likely involves many more species, as shown in Meegaskumbura et al. (2002). Additionally, no morphological characters are given to suggest that these species separate from members of *Kurixalus*. However, to the large genus with approximately 150 species, to determine whether or not members of Clade F are recognized as *Kurixalus* or whether they represent a new genus will require further analysis, with more sufficient taxon sampling and morphological character coding.

Philautus jinxiuensis is clearly divergent from *Philautus* (Clade E and F) in this study. It is the sister taxon to *A. gracilipes* with high Bayesian support and moderate MP support from mtDNA data. Because of the incomplete nuclear data of *A. gracilipes*, the sister relationship is treated as provisional. We currently refrain from recognizing *P. jinxiuensis* as a member of *Gracixalus*.

4.4. Phylogeny of Chiromantis (sensu Frost, 2007)

Chiromantis was shown to be paraphyletic across Clades M and B. Clade M, which included the type species *C. xerampelina*, is recognized as *Chiromantis* sensu stricto.

The present study is the first to include *Chiromantis romeri* in a phylogenetic analysis, a taxon whose proper generic placement has not definitively been made (e.g., Frost et al., 2006). Our results suggest *C. romeri* is basal to all rhacophorids, save *Buergeria*, with robust Bayesian support. The phylogenetic position of *C. romeri* is novel, which is supported by mtDNA and combined data, but not nuDNA data. However, our nuclear topology indicates that *C. romeri* is remotely related to *Philautus* (Clade E), *Kirtixalus* (Clade F), *Kurixalus* (Clade G), and *Chiromantis* (Clade M). Since Smith's (1953) description of *Chiromantis romeri* (as *Philautus*), this species had been transferred among several genera (Bossuyt and Dubois, 2001; Frost, 2007; Smith, 1953; Wilkinson et al., 2003). Due to the presence of a tadpole stage in *Philautus romeri*, *P. romeri* was tentatively assigned to *Chirixalus* (Bossuyt and Dubois, 2001). In addition, Wilkinson et al. (2003) suggested that *P. romeri* may be a putative member of *Kurixalus*, pending further study of the type specimens and specimens in the field. Recently, Frost et al. (2006) moved this species to the genus *Chiromantis*, pending resolution of its true phylogenetic position. All of the evidence indicates that the morphological character of *C. romeri* is very unique. Furthermore, *C. romeri* shares 28 base characters with one unidentified species, which is distinct from any other genus. In order to recognize the unique position of this clade, we consider it to be a new genus *Liuixalus* gen. n. (type species: *Chiromantis romeri* Smith, 1953. Etymology: Cheng-chao Liu + *ixalus* [the genus *Ixalus* Duméril and Bibron, 1841, a traditional generic root for treefrogs] to commemorate the contribution to Chinese herpetology by Cheng-chao Liu). The molecular synapomorphies are presented in Appendix A. The new genus provisionally contains present *C. romeri* and one unidentified species.

4.5. The paraphyly of Rhacophorus megacephalus (sensu Fei et al., 2005)

Our data is congruent with Frost et al. (2006) and Wilkinson et al. (2002), in capturing *Polypedates* as a clade distinct from *Rhacophorus*. Matsui et al. (1986) resurrected *P. megacephalus* from the synonymy of *P. leucomystax*, based on chromosomal, morphological, and bioacoustic data. They also suggested that Chinese mainland populations of these frogs required further taxonomic investigation (Matsui et al., 1986), although Chinese taxonomists have consistently regarded mainland populations as *P. megacephalus* (Fei, 1999; Fei et al., 2005 [as *Rhacophorus megacephalus*]; Zhao and Adler, 1993). Our results suggest that mainland Chinese populations of *P. megacephalus* are likely a complex of at least two species. The taxonomic resolution of these populations is beyond the scope of this study, since we do not have topotypes of either *P. leucomystax*, or *P. megacephalus* with which to compare our specimens. Their taxonomic positions remain unresolved until a comprehensive taxonomic revision of this widespread complex can be undertaken.

The phylogeny of Rhacophoridae is incompletely resolved, and a much more thorough taxonomic and character sampling are needed for future study. However, several patterns are clearly demonstrated on our phylogenetic trees, warranting some taxonomic revisions.

4.6. Taxonomic implication

Our results strongly support the division of rhacophorids into two groups, which correspond with Buergeriinae and Rhacophorinae (Channing, 1989; Frost et al., 2006; Wilkinson et al., 2002). Comparing Rhacophorinae sensu Dubois (1992) and Rhacophoridae sensu Frost et al. (2006), we prefer to accept that of Frost et al. (2006).

Within Rhacophoridae, our results support some currently understood generic placements: *Buergeria* (corresponding to Clade A); *Nyctixalus* (corresponding to Clade C); *Theloderma* (corresponding to Clade D); *Feihyla* (corresponding to Lineage J) and *Polypedates* (corresponding to Clade L). *Aquixalus* is not monophyletic. Transferring *Aquixalus (Aquixalus)* to genus *Kurixalus*, we consider the monophyletic *Gracixalus* (corresponding to Clade I) to be a full genus. The genus *Philautus* as presently configured is not monophyletic. We retained species distributed in Java and Philippines as *Philautus* (corresponding to Clade E), and kept the subgenus *Kirtixalus* (corresponding to Clade F), which include Sri Lankan and Indian species, in their current taxonomic position. At the exclusion of *R. hainanus* from *Rhacophorus* to *Kurixalus*, the monophyly of *Rhacophorus* (corresponding to Clade K) is recognized. *Chiromantis* as currently understood is paraphyletic. To avoid this, we erected a new genus, *Liuixalus* gen. n. (corresponding to Clade B), which includes *C. romeri* and one unidentified species of the genus, to ensure the monophyly of *Chiromantis* (corresponding to Clade M).

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Appendix A

Molecular synapomorphies of the new genus *Liuixalus* gen. n. (They must be cross-referenced with the aligned matrix).

42/A, 622/C, 816/A, 1156/G, 1245/T, 1599/T, 1624/A, 1816/A, 1929/C, 1953/G, 1962/G, 1965/G, 1998/C, 2016/G, 2052/G, 2109/G, 2116/C, 2118/C, 2133/C, 2137/C, 2145/C, 2163/G, 2172/C, 2184/C, 2358/T, 2413/T, 2702/G, 2710/T.

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