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Re-examination of the phylogeny of Rhacophoridae (Anura) based on mitochondrial and nuclear DNA

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ABSTRACT

The phylogenetic relationships among rhacophorid frogs are under dispute. We use partial sequences of three mitochondrial (12S rRNA, 16S rRNA, and cytochrome *b*) and three nuclear protein-coding (Rag-1, rhodopsin exon 1, and tyrosinase exon 1) genes from 57 ingroup taxa and eight outgroup taxa to propose a hypothesis for phylogenetic relationships within Rhacophoridae. Our results support recognition of the genus *Feihyla*, and *Chiromantis* is the sister taxon to the clade formed by *Feihyla*, *Polypedates* and *Rhacophorus*. We place *Aquixalus odontotarsus* within *Kurixalus*, and the remaining species of *Aquixalus* and *Philautus jinxiuensis* into the genus *Gracixalus*. We give *Philautus* (*Kirtixalus*) the rank of genus and place *Philautus menglaensis* within it. The division of species groups among Chinese *Rhacophorus* needs revision, and a cryptic species is revealed within *Rhacophorus nigropunctatus*. *Rhacophorus pingbianensis* is considered a synonym of *Rhacophorus omeimontis*. The validity of *Rhacophorus hui* is confirmed by present molecular evidence.

1. Introduction

The Rhacophoridae treefrogs originated before the Madagascan and India-Seychelles land masses separated (Bossuyt and Milinkovitch, 2000). They dispersed out of India after its collision with Eurasia (Bossuyt and Milinkovitch, 2001) and radiated almost exclusively in the Oriental Realm into about 290 species covering two subfamilies and 11 genera (Frost, 2008). Although many studies of phylogeny based on morphological or molecular datasets have been reported for this rich and diverse group, and previous molecular studies (Richards and Moore, 1998; Wilkinson et al., 2002; Frost et al., 2006; Grosjean et al., 2008; Li et al., 2008; Yu et al., 2008) have provided compelling evidence in support of the Buergeriinae–Rhacophorinae dichotomy among Rhacophoridae, the phylogenetic placement and taxonomy of some genera and species of Rhacophoridae are still under debate.

Feihyla was erected by Frost et al. (2006) based on the results of Wilkinson et al. (2002), in which *Feihyla palpebralis*, the type species of *Feihyla*, was recovered as the sister taxon to all other rhacophorid frogs except for *Buergeria* by analyzing approximately 2000 bp of genes encoding 12S rRNA, 16S rRNA, and t-RNA for valine. Using 1676 bp of genes encoding 12S rRNA, 16S rRNA, and rhodopsin exon 1, Grosjean et al. (2008) found that *F. palpebralis* was the sister taxon to *Chiromantis*, and they suggested that *F. palpebralis* does not deserve a new generic status. However, using 2904 bp of mitochondrial (12S rRNA, 16S rRNA, and t-RNA for valine) and nuclear (tyrosinase exon 1and rhodopsin exon 1) genes, Li et al. (2008) reconstructed a clade formed by *Feihyla*, *Rhacophorus*, and *Polypedates*, although the relationships among taxa within this clade are not clear. Additionally, these differences in the phylogenetic placement and validity of *Feihyla* make the phylogenetic placement of *Chiromantis* ambiguous. Based on the results of Grosjean et al. (2008), in which *Feihyla* was placed into the synonymy of *Chiromantis*, *Chiromantis*, *Rhacophorus*, and *Polypedates* form a clade. However, according to Li et al. (2008), *Chiromantis* is the sister taxon to the clade consisting of *Feihyla*, *Rhacophorus*, and *Polypedates*. Richards and Moore (1998) and Frost et al. (2006), using, respectively, 1081 bp of 12S rRNA, 16S rRNA, and t-RNA for valine genes and approximately 4700 bp of 12S rRNA, 16S rRNA, t-RNA for valine, rhodopsin exon 1, tyrosinase exon 1, histone H3, 28S, and seventh in absentia genes, also obtained the clade of *Chiromantis*, *Rhacophorus*, and *Polypedates*; however, *F. palpebralis* was not sampled in these two studies. The phylogenetic placement of *F. palpebralis* and the validity of *Feihyla* need further examination.

Controversies on the phylogenetic placement of *Aquixalus odontotarsus* and the validity of genus *Aquixalus* are ongoing. According to Delorme et al. (2005) and Frost (2007), *Aquixalus* includes two subgenera (*Aquixalus* and *Gracixalus*), and *Aquixalus* (*Aquixalus*) contains most of the species of the genus *Aquixalus* whereas *Aquixalus* (*Gracixalus*) contains only *Aquixalus gracilipes* and *Aquixalus supercornutus*. Using 978 nucleotides of 12S and 16S rRNA genes, Yu et al. (2008) found that *A. odontotarsus* is more closely related

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to the genus *Kurixalus* than to the clade formed by *A. gracilipes* and *Philautus jinxiuensis*. This relationship was also obtained by Li et al. (2008) and they suggested putting *A. odontotarsus* in *Kurixalus*, transferring *Aquixalus* (*Aquixalus*) to *Kurixalus*, raising *Aquixalus* (*Gracixalus*) to the rank of genus, and placing *P. jinxiuensis* into *Gracixalus*. These changes were followed by Frost (2008), with the exception of reassignment for *P. jinxiuensis*. However, Grosjean et al. (2008) obtained a well supported clade formed by *A. odontotarsus*, *Aquixalus carinensis*, and *A. gracilipes*.

Philautus, whose species undergo direct development, is the most inclusive genus of Rhacophoridae (currently 145 species are included) and it is widely distributed in South and Southeast Asia (Frost, 2008). Throughout the history of its taxonomy, *Philautus* has been confused with many other genera such as '*Chirixalus*', *Kurixalus*, *Rhacophorus*, and even *Micrixalus* of Micrixalidae (Bossuyt and Dubois, 2001), and so far most analyses have not recovered this genus as monophyletic. Grosjean et al. (2008) showed two well supported independent clades within *Philautus*: one consists of *Philautus* from India and Sri Lanka, and another one consists of *Philautus* from Southeast Asia, although the common origin of direct development cannot be rejected. This was consistent with results of Meegaskumbura et al. (2002), who analyzed 802 bp of 12S and 16S rRNA genes. Li et al. (2008) found South Asian *Philautus* distinct from Southeast Asian *Philautus* and closely related to *Kurixalus*. Additionally, the work of Li et al. (2008) and Yu et al. (2008) indicated that the taxonomy of Chinese *Philautus* needs further examination.

Rhacophorus is the second largest genus of Rhacophoridae and contains 76 species (Frost, 2008). In China, about 24 species of *Rhacophorus* have been recorded (Fei et al., 2005). Although recent studies (Rao et al., 2006; Li et al., 2008; Yu et al., 2008) have brought new evidence in the understanding of Chinese *Rhacophorus*, disputes on the taxonomy of some species remain unresolved. For example, *Rhacophorus pingbianensis* once was considered a synonym of *Rhacophorus omeimontis* by Fei et al. (2005), and Li et al. (2008) suggested that more studies were needed on this question. Additionally, up to now no molecular evidence was provided to examine the validity of *Rhacophorus hui*, which was distinct from *Rhacophorus dugritei* karyotypically (Wu and Zeng, 1994) but was placed into the synonymy of *R. dugritei* by Fei (1999) and Fei et al. (2005).

Here, by analyzing DNA sequences (approximately 2900 bp total) of three mitochondrial (12S rRNA, 16S rRNA, and cytochrome *b*) and three nuclear genes (Rag-1, rhodopsin exon 1, and tyrosinase exon 1) crossing all known main clades of Rhacophoridae, we re-examine the evolutionary history and taxonomy of some genera and species of Rhacophoridae.

2. Materials and methods

2.1. Taxonomy and selection of taxa

With the exceptions of taxonomic changes on genus *Aquixalus*, the classification of Frost (2008) was followed mainly for convenience of discussion. A total of 57 species of Rhacophoridae were included in this study. New sequences were determined from 26 species representing all genera of Rhacophoridae except for *Kurixalus* and *Nyctixalus*. Homologous sequences of the remaining 31 species were obtained from GenBank. These retrieved sequences mainly represent *Aquixalus*, *Kurixalus*, *Nyctixalus*, and *Philautus* from South and Southeast Asia, and make our samples cover all known main clades of Rhacophoridae. Eight species of Dicroglossidae (*Occidozyga lima* and *Limnonectes kuhlii*), Mantellidae (*Mantella madagascariensis* and *Boophis xerophilus*), Microhylidae (*Kaloula taprobanica*), Petropedetidae (*Petropedetes parkeri*), Phrynobatrachidae (*Phrynobatrachus natalensis*), and Pyxicephalidae (*Pyxicephalus adspersus*) were selected as outgroups with rooting on *K. taprobanica* based on Bossuyt et al. (2006). Homologous sequences of these outgroups were also obtained from GenBank. All of the species and sequences (including those retrieved from GenBank) used in this study are listed in Table 1.

2.2. DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from muscle or liver tissue fixed in 99% ethanol. Tissue samples were digested using proteinase K, and subsequently purified following a standard phenol/chloroform isolation and ethanol precipitation. Fragments of 12S and 16S rRNA genes were amplified using primer pairs L1091 and H1478 (Kocher et al., 1989) and 16Sar-L and 16Sbr-H (Palumbi et al., 1991), respectively. Primers for fragments of cytochrome *b* (CBJ10933 and Cyt *b*B), rhodopsin exon 1 (Rhod1A and Rhod1D) and tyrosinase exon 1 (Tyr1C and Tyr1G) were from Bossuyt and Milinkovitch (2000), and the Primers for Rag-1 (Rag1B and Rag1E) were from Biju and Bossuyt (2003). PCR amplifications were performed in 50 μl reactions using the following cycling conditions: an initial denaturing step at 94 °C for 3 min; 40 cycles of denaturing at 94 °C for 60 s, annealing at 51–54 °C for 60 s (51 °C for 12S rRNA, 16S rRNA, and Rag-1, 52 °C for rhodopsin, 53 °C for cytochrome *b*, and 54 °C for tyrosinase), and extending at 72 °C for 60 s; and a final extending step of 72 °C for 10 min. PCR products were purified via spin columns. Sequencing was performed directly using the corresponding PCR primers. DNA sequences of both strands were obtained using the BigDye Terminator v3.1 on an ABI PRISM 3730 following the manufacturer's instructions. All new sequences have been deposited in GenBank under Accession Nos. EU924508-EU924629 (Table 1).

2.3. Sequence alignment and analyses

DNA sequences were aligned using ClustalX v1.83 (Thompson et al., 1997) with the default parameters, and the alignments were revised by eye in an effort to maximize the positional homology. As recommended by Swofford et al. (1996), we excluded hypervariable regions of the 12S and 16S rRNA genes from subsequent analyses because of uncertain alignment. To test for the possible saturation of substitution types, we plotted the number of transitions (Ti) and transversions (Tv) against the uncorrected pairwise distances. Saturation plots were also examined separately for the first, second, and third positions of protein-coding genes. The saturated positions were excluded from further analyses.

2.4. Phylogenetic reconstructions

Fragments of Cyt *b*, Rag-1, rhodopsin, and tyrosinase are not complete for some species on GenBank (see Table. 1), and therefore the complete supermatrix included numerous missing data. Hence, we performed two separate analyses, one with all taxa (dataset I) and one with only the species for which all individual genes are available (dataset II).

For dataset I, owing to the absence of homologous nuDNA for some species, the mtDNA and nuDNA were combined into a single partition in all phylogenetic analyses. As to dataset II, prior to phylogenetic analyses, the degree of heterogeneity between the mtDNA and nuDNA was investigated using the partition homogeneity test (Farris et al., 1994) in PAUP* v4.0b10 (Swofford, 2002) with 1000 replicates and 10 random sequence additions.

The datasets were subjected to three different phylogenetic analyses using: maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI). Unweighted MP and ML analyses were performed with PAUP*. The MP method was performed using heuristic search with 1000 random-addition sequence replicates

Table 1
Species used in this study and GenBank Accession Nos.

Species	Voucher number	Locality	GenBank Accession Nos. (12S, 16S, Cyt *b*, Tyrosinase, Rhodopsin, Rag-1)					
Outgroup								
Limnonectes kuhlii	–	–	AF249020[a]	AF249034[a]	AF249065[a]	AF249179[a]	AF249116[a]	DQ347232[b]
Occidozyga lima	–	–	DQ347025[b]	DQ347316[b]	–	DQ347159[b]	DQ347375[b]	DQ347255[b]
Petropedetes parkeri	–	–	AY341628[c]	AY341724[c]	AY341738[c]	AY341757[c]	AY341813[c]	AY571656[d]
Kaloula taprobanica	–	–	AF249004[a]	AF249057[a]	AF249085[a]	AF249163[a]	AF249100[a]	AY948915[e]
Phrynobatrachus natalensis	–	–	DQ347012[b]	DQ347303[b]	–	DQ347145[b]	DQ347362[b]	DQ347242[b]
Pyxicephalus adspersus	–	–	DQ347013[b]	DQ347304[b]	–	DQ347146[b]	DQ347363[b]	DQ347242[b]
Mantella madagascariensis	–	–	AF249005[a]	AF249049[a]	AF249076[a]	AF249164[a]	AF249101[a]	DQ019500[f]
Boophis xerophilus	–	–	AF249008[a]	AF249038[a]	AF249069[a]	AF249167[a]	AF249104[a]	AY364209[g]
Ingroup								
Buergeria buergeri	–	Japan	AY880478[h]	AY880504[h]	AB127977[i]	–	AY880623[h]	AY948921[e]
Buergeria oxycephala	SN030031	Limu Mt, Hainan, China	EF564442[j]	EF564514[j]	EU924592	EU924564	EU924536	EU924508
Aquixalus carinensis	MNHN1999.5961	Vietnam	AY880589[h]	AY880503[h]	–	–	AY880635[h]	–
Aquixalus gracilipes	KIZ060821196	Pingbian, Yunnan, China	EF564451[j]	EF564523[j]	EU924593	EU924565	EU924537	EU924509
	MNHN1999.592	Vietnam	AY880590[h]	AY880504[h]	–	–	AY880636[h]	–
Aquixalus odontotarsus	KIZ060821030	Simao, Yunnan China	EF564455[j]	EF564527[j]	EU924594	EU924566	EU924538	EU924510
	MNHN1999.5942	Vietnam	AY880593[h]	AY880507[h]	–	–	AY880638[h]	–
Chiromantis doriae	KIZ060821034	Simao, Yunnan, China	EF564444[j]	EF564516[j]	EU924595	EU924567	EU924539	EU924511
Chiromantis rufescens	–	Africa	AY341622[c]	AY341721[c]	AY341729[c]	DQ347139[b]	AY341807[c]	DQ347237[b]
Chiromantis vittatus	KIZ060821090	Simao, Yunnan, China	EF564447[j]	EF564519[j]	EU924596	EU924568	EU924540	EU924512
Chiromantis xerampelina	–	Africa	AF458132[k]	AF458132[k]	–	–	DQ284012[l]	–
Feihyla palpebralis	KIZ080177	Pingbian, Yunnan, China	EU924625	EU924620	EU924597	EU924569	EU924541	EU924513
Liuixalus romeri	KIZ060821245	Jinxiu, Guangxi, China	EF564463[j]	EF564535[j]	EU924598	EU924570	EU924542	EU924514
Kurixalus eiffingeri	UMFS 5969	Taiwan, China	DQ283122[l]	DQ283122[l]	–	DQ282931[l]	DQ283830[l]	–
Kurixalus idiootocus	SCUM 061107L	Taiwan, China	EU215547[m]	EU215547[m]	–	EU215607[m]	EU215577[m]	–
Nyctixalus pictus	MNHN1999.7718	Thailand	AY880587[h]	AY880502[h]	–	–	AY880634[h]	–
Nyctixalus spinosus	ACD 1043	Mindanao, Philippine	DQ283114[l]	DQ283114[l]	–	–	DQ283827[l]	–
Philautus acutirostris	TNHC 59857	Philippines	AY326059[n]	AY326059[n]	–	–	–	–
Philautus aurifasciatus	–	Java, Indonesia	AY141804[o]	AY141850[o]	–	–	–	–
Philautus charius	–	India	AF249032[a]	AF249062[a]	AF249095[a]	AF249191[a]	AF249128[a]	DQ347208[b]
Philautus femoralis	WHT 2779	Sri Lanka	AY141787[o]	AY141833[o]	–	–	–	–
Philautus ingeri	FMNH239280	Borneo	AY880581[h]	AY880496[h]	–	–	AY880629[h]	–
Philautus jinxiuensis	KIZ060821013	Jinxiu, Guangxi, China	EF564452[j]	EF564524[j]	EU924599	EU924571	EU924543	EU924515
Philautus menglaensis	KIZ060821286	Yunnan, China	EU924626	EU924621	EU924600	EU924572	EU924544	EU924516
Philautus microtympanum	–	Sri Lanka	AF249030[a]	AF249046[a]	AF249088[a]	AF249189[a]	AF249126[a]	DQ019506[f]
Philautus mjobergi	–	Malaysia	AF026348[p]	AF026365[p]	–	–	–	–
Philautus petersi	–	Malaysia	AF026349[p]	AF026366[p]	–	–	–	–
Philautus schmarda	WHT 5404	Sri Lanka	AY880617[h]	AY880530[h]	–	–	AY880660[h]	–
Philautus signatus	–	India	AY141795[o]	AY141841[o]	AY708169[q]	–	–	–
Philautus sp.	DNP Sarawak	Borneo	AY880595[h]	AY880509[h]	–	–	AY880640[h]	–
Philautus sp.	TBGRI Keralia	South India	AY882564[h]	AY882567[h]	–	–	AY882570[h]	–
Philautus sp.	TBGRI 2001.0090	South India	AY880596[h]	AY880510[h]	–	–	AY880641[h]	–
Philautus sp.	WHT 3419	Sri Lanka	AY880597[h]	AY880511[h]	–	–	AY880642[h]	–
Philautus sp.	WHT 3424	Sri Lanka	AY880598[h]	AY880512[h]	–	–	AY880643[h]	–
Philautus sp.	WHT 3421	Sri Lanka	AY880602[h]	AY880516[h]	–	–	AY880645[h]	–
Philautus surdus	CAS 219932	Philippines	AF458138[k]	AF458138[k]	–	–	–	–
Philautus temporalis	WHT5382	Sri Lanka	AY880603[h]	AY880517[h]	–	–	AY880646[h]	–
Philautus wynaadensis	–	India	AF249031[a]	AF249059[a]	AF249087[a]	AF249190[a]	AF249127[a]	AY364199[g]
Polypedates cruciger	–	Sri Lanka	AF249028[a]	AF249045[a]	AF249089[a]	AF249187[a]	AF249124[a]	DQ347212[b]
Polypedates eques	–	Sri Lanka	AY880489[h]	AY920531[r]	–	–	AY880647[h]	–
Polypedates megacephalus	KIZ060821040	Simao, Yunnan, China	EF564478[j]	EF564550[j]	EU924601	EU924573	EU924545	EU924517
Rhacophorus bipunctatus	SN030035	Limu Mt, Hainan, China	EF564507[j]	EF564579[j]	EU924602	EU924574	EU924546	EU924518
Rhacophorus chenfui	KIZ060821073	Xichang, Sichuan, China	EF564465[j]	EF564537[j]	EU924603	EU924575	EU924547	EU924519
Rhacophorus dennysi	KIZ060821050	Jinxiu, Guangxi, China	EF564467[j]	EF564539[j]	EU924604	EU924576	EU924548	EU924520
Rhacophorus dugritei	KIZ060821003	Yongde, Yunnan, China	EF564469[j]	EF564541[j]	EU924605	EU924577	EU924549	EU924521
Rhacophorus feae	KIZ060821197	Pingbian, Yunnan, China	EF564474[j]	EF564546[j]	EU924606	EU924578	EU924550	EU924522
Rhacophorus hui	KIZ07052101	Zhaojue, Sichuan, China	EU924627	EU924622	EU924607	EU924579	EU924551	EU924523
Rhacophorus malabaricus	–	India	AF249029[a]	AF249050[a]	AF249094[a]	AF249188[a]	AF249125[a]	AY948912[i]
Rhacophorus maximus	KIZ060821140	Simao, Yunnan, China	EF564476[j]	EF564548[j]	EU924608	EU924580	EU924552	EU924524
Rhacophorus minimus	KIZ060821020	Jinxiu, Yunnan, China	EF564489[j]	EF564561[j]	EU924609	EU924581	EU924553	EU924525
Rhacophorus nigropunctatus	KIZ060821199	Pingbian, Yunnan, China	EF564490[j]	EF564562[j]	EU924610	EU924582	EU924554	EU924526
	KIZ07061001	Weining, Guizhou, China	EU924628	EU924623	EU924611	EU924583	EU924555	EU924527
Rhacophorus omeimontis	KIZ060821282	Hongya, Sichuan, China	EF564492[j]	EF564564[j]	EU924612	EU924584	EU924556	EU924528
Rhacophorus pingbianensis	KIZ060821289	Jinping, Yunnan, China	EF564495[j]	EF564567[j]	EU924613	EU924585	EU924557	EU924529
	KIZ080158	Pingbian, Yunnan, China	EU924629	EU924624	EU924614	EU924586	EU924558	EU924530
Rhacophorus reinwardtii	KIZ060821224	Lvchun, Yunnan, China	EF564498[j]	EF564570[j]	EU924615	EU924587	EU924559	EU924531
Rhacophorus rhodopus	KIZ060821037	Simao, Yunnan, China	EF564500[j]	EF564572[j]	EU924616	EU924588	EU924560	EU924532
Rhacophorus taronensis	KIZ1039	Gongshan, Yunnan, China	EF564496[j]	EF564568[j]	EU924617	EU924589	EU924561	EU924533

(*continued on next page*)

Table 1 (*continued*)

Species	Voucher number	Locality	GenBank Accession Nos. (12S, 16S, Cyt *b*, Tyrosinase, Rhodopsin, Rag-1)					
Theloderma asperum	KIZ060821201	Jinping, Yunnan, China	EF564449[j]	EF564521[j]	EU924618	EU924590	EU924562	EU924534
Theloderma bicolor	MNHN1999.5986	Vietnam	AY880616[h]	AY880529[h]	—	—	AY880659[h]	—
Theloderma rhododiscus	KIZ060821063	Jinxiu, Guangxi, China	EF564461[j]	EF564533[j]	EU924619	EU924591	EU924563	EU924535

SN is from the field number of Shunqing Lv; KIZ: Kunming Institute of Zoology, the Chinese Academy of Sciences.
Sources of sequences retrieved from GenBank:
[a] Bossuyt and Milinkovitch (2000).
[b] Bossuyt et al. (2006).
[c] Vences et al. (2003).
[d] Van der Meijden et al. (2004).
[e] Roelants et al. (2007).
[f] Van der Meijden et al. (2005).
[g] Biju and Bossuyt (2003).
[h] Delorme (2004).
[i] Sano et al. (2004).
[j] Yu et al. (2008).
[k] Wilkinson et al. (2002).
[l] Frost et al. (2006).
[m] Li et al. (2008).
[n] Darst and Cannatella (2004).
[o] Meegaskumbura et al. (2002).
[p] Richards and Moore (1998).
[q] Bossuyt et al. (2004).
[r] Delorme et al. (2004).

and tree bisection reconnection (TBR) branch swapping. ML analysis was performed using heuristic search with 10 random-addition sequence replicates based on the best substitution model, which was selected by Modeltest v3.7 (Posada and Crandall, 1998) using the Akaike information criterion (AIC, Akaike, 1973). Support for nodes of the resulting MP trees was assessed by analyses of 1000 non-parametric bootstrap replicates (Felsenstein, 1985). Owing to computational constraints, clade support for the ML tree of dataset I was assessed by non-parametric bootstrap analyses using the fast-heuristic search with 200 replicates. Clade support for the ML tree of dataset II was assessed by analyses of 100 non-parametric bootstrap replicates with standard heuristic search. BI analysis was performed using MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001). For the dataset II, we implemented a mixed model approach using 'unlink' option to account for the potential difference in the evolutionary model parameters between mtDNA and nuDNA. To ensure that the BI analysis was not trapped in local optima (Huelsenbeck and Bollback, 2001; Leaché and Reeder, 2002), two runs were performed simultaneously with four Markov chains starting from random trees. The Markov chains were run for 2,000,000 generations and sampled every 100 generations, yielding 20,000 parameter point estimates. The program Tracer v1.3 (Rambaut and Drummond, 2003) was used to determine when the log likelihood ($\ln L$) of sampled trees reached a stationary distribution. Generations sampled before the chain reached stationarity were discarded as burn-in, and the remaining trees were used to create 50% majority-rule consensus tree and to estimate Bayesian posterior probabilities (BPPs).

3. Results

3.1. Sequence and tree statistics

3.1.1. Dataset I

Alignments of the 12S rRNA, 16S rRNA, and cytochrome *b* genes yielded 419, 573, and 564 sites, respectively. After the removal of the highly variable regions of rRNA genes (12S rRNA: 153–161, 226–239; 16S rRNA: 41–51, 249–293), and exclusion of the third positions of cytochrome *b* gene that obviously showed saturation (data not shown), mtDNA contained 1277 characters. For nuclear genes, no obvious saturation was found and total nuDNA consisted of 1358 characters (rhodopsin, 314 bp; tyrosinase, 528 bp; Rag-1, 516 bp). Indels were observed in the alignments of Rag-1 and tyrosinase genes. Of 2635 bp in the concatenation of mtDNA and nuDNA, 1265 characters were variable and 939 positions were phylogenetically informative (48.0% and 35.6%, respectively). Sequence statistics for each gene fragment and combination are given in Table 2.

The GTR + I + G model was selected as best-fit model of nucleotide substitution. Settings for this model were as follows: *R*-matrix = (3.0308, 7.9748, 2.8103, 1.2066, 16.2244, and 1.0000); base frequencies = (A = 0.2918, C = 0.2421, G = 0.1994, and T = 0.2667); Proportion of invariable sites = 0.3537; and the shape-parameter of the γ distribution = 0.5750.

Thirty-two most parsimonious trees (MPTs) were obtained with 5743 evolutionary steps, a consistency index (CI) of 0.335 and a retention index (RI) of 0.495. The likelihood value of the ML tree

Table 2
Alignment statistics of datasets I and II. The high-variable regions of rRNA and the saturated third positions of Cyt *b* were excluded.

Fragments	Mt				Nu				Mt + Nu
	12S	16S	Cyt *b*	Total	Rag-1	Tyrosinase	Rhodopsin	Total	
Dataset I									
bp	396	517	364	1277	516	528	314	1358	2635
vs	240	288	152	680	215	263	107	585	1265
pi	196	218	119	533	153	184	69	406	939
Dataset II									
bp	393	521	364	1278	516	528	314	1358	2636
vs	208	240	151	599	203	253	74	530	1129
pi	157	172	117	446	134	169	46	349	795

Abbreviations: bp, base pairs; vs, variable sites; pi, parsimony informative sites.

was ln L = −29,515.44, and the likelihood values of the consensus tree in the Bayesian approach were −29,566.50 and −29,573.04 for the cold chain of runs 1 and 2, respectively. The first 2000 samples were discarded as burn-in and the remaining trees were used to construct the consensus tree.

3.1.2. Dataset II

Alignments of 12S rRNA, 16S rRNA, and cytochrome *b* gene fragments yielded 412, 567, and 547 sites, respectively. After removing the highly variable regions of rRNA genes (12S rRNA: 153–159, 221–232; 16S rRNA: 43–49, 248–286) and the saturated third positions of the cytochrome *b* gene, mtDNA contained 1278 sites. The length of nuDNA was 1358 bp. Of 2636 bp in the combination of mtDNA and nuDNA, 1129 characters were variable and 795 positions were phylogenetically informative (Table 2).

The partition homogeneity test revealed no significant conflicting phylogenetic signals between mtDNA and nuDNA (P = 0.247), so only the combination of mtDNA and nuDNA was used to perform constructions of phylogeny. The GTR + I + G model was selected as the best-fit model of nucleotide substitution. Settings for this model were as follows: R-matrix = (2.9118, 7.2109, 2.3547, 1.1617, 13.9728, and 1.0000); base frequencies = (A = 0.2852, C = 0.2392, G = 0.2030 and T = 0.2726); Proportion of invariable sites = 0.3882; and the shape-parameter of the γ distribution = 0.5741. Additionally, Modeltest concluded that GTR + I + G and TVM + I + G fit for mtDNA and nuDNA, respectively. These models were applied to their respective partitions when BI analysis was performed. Seven most parsimonious trees (MPTs) were obtained with 4091 steps, a CI of 0.407 and a RI of 0.472. The likelihood value of the ML tree was ln$L$ = −22,226.42, and the likelihood values of the consensus tree in the Bayesian approach were −22,037.11 and −22,040.68 for the cold chain of runs 1 and 2, respectively.

3.2. Phylogenetic relationships

3.2.1. Phylogeny based on dataset I

The BI and ML analyses of dataset I yielded highly similar topologies, and the only difference between these two solutions was the placement of the clade consisting of *Philautus ingeri* and *Philautus sp* (DNP Sarawak): in the BI tree this clade was the sister group to the clade formed by *Philautus petersi*, *Philautus mjobergi*, *Philautus surdus*, and *Philautus acutirostris*, whereas in ML tree it was the sister group to *Philautus aurifasciatus*. The MP analysis did not well resolve the basal dichotomy between deep clades, but most relationships within these deep clades were resolved and the topologies within these deep clades were almost same as those of ML and BI analyses. Thus, only the BI tree is presented here (Fig. 1). The following relationships among rhacophorids are noteworthy:

(1) The dichotomy between Buergeriinae and Rhacophorinae was reconstructed with strong support values.
(2) *Liuixalus romeri* was recovered as the sister taxon to all other Rhacophorinae frogs by all analyses.
(3) The clade consisting of *Nyctixalus* and *Theloderma* was supported by all analyses (100%, 73%, and 94% for BI, ML, and MP, respectively), and all analyses recovered it as the sister clade to all other Rhacophorinae frogs except for *L. romeri*, although the support values of ML and MP analyses were not strong.
(4) The monophyly of *A. odontotarsus* was not recovered by all analyses. *A. odontotarsus* from the type locality (Caiyanghe, Simao, Yunnan Province) was the sister taxon to *Kurixalus* (100%, 100%, and 100%, respectively), whereas *A. odontotarsus* from Vietnam was clustered together with *A. carinensis* (100%, 99%, and 100%, respectively).
(5) The monophyly of *Philautus* was not recovered. *P. jinxiuensis* was the sister taxon to the clade consisting of *A. odontotarsus* from Vietnam and *A. carinensis* (100%, 95%, and 100%, respectively), and other species of *Philautus* were grouped into two main clades; one consisted of species from Southeast Asia (SA clade) (100%, 91%, and 84%, respectively), and another one consisted of species from South Asia and China (SAC clade) (100%, 93%, and 99%, respectively). Furthermore, all analyses found that the SAC clade is closer to the clade consisting of *Kurixalus* and *A. odontotarsus* from China than to the SA clade (98%, -, and 54%, respectively).
(6) The clade consisting of *Chiromantis*, *Feihyla*, *Polypedates*, and *Rhacophorus* was strongly supported by Bayesian inference (98%), although bootstrap values of ML and MP were weak, and *Chiromantis* was the sister taxon to the clade formed by *Feihyla*, *Polypedates* and *Rhacophorus*.
(7) The monophyly of *Rhacophorus* was recovered by all analyses (100%, 51%, and 84%, respectively), and it can be divided into two main clades.
(8) The monophyly of *Rhacophorus nigropunctatus* was not supported and *R. nigropunctatus* from the type locality (Weining County, Guizhou Province) was clustered together with *Rhacophorus chenfui* (100%, 91%, and 97%, respectively).
(9) *Rhacophorus pingbianensis* from Jinping County was closer to *R. omeimontis* than to *R. pingbianensis* from Pingbian County (100%, 91%, and 90%, respectively).
(10) *Rhacophorus hui* was recovered as the sister taxon to *Rhacophorus minimus* (100%, 66%, and 60%, respectively).

3.2.2. Phylogeny based on dataset II

The ML and BI analyses of dataset II yielded identical topologies. Like the MP analysis of dataset I, the MP analysis of dataset II also did not completely resolve the basal dichotomy within Rhacophoridae. Compared with analyses of dataset I, the BI analysis of dataset II strongly supported the clade of *Feihyla*, *Polypedates*, and *Rhacophorus* (98%), and that *Chiromantis* is the sister taxon to it (Fig. 2).

4. Discussion

The controversies about the phylogeny among rhacophorid frogs have been ongoing for long time (Liem, 1970; Channing, 1989; Richards and Moore, 1998; Wilkinson and Drewes, 2000; Wilkinson et al., 2002; Delorme et al., 2005; Frost et al., 2006; Grosjean et al., 2008; Li et al., 2008; Yu et al., 2008). We expand previous assessments of rhacophorid phylogeny by examining three mitochondrial and three nuclear genes. Analyses of these data provide new evidence for the phylogeny and taxonomy of some genera and species of Rhacophoridae.

4.1. Phylogeny of Feihyla and Chiromantis

Feihyla is a new genus erected by Frost et al. (2006) and currently it only includes the type species *F. palpebralis*. Recently, Grosjean et al. (2008) found that *F. palpebralis* is closely related to *Chiromantis*, and they placed *F. palpebralis* in *Chiromantis*. In the present study, the monophyletic group consisting of *Feihyla*, *Rhacophorus*, and *Polypedates* is recovered by BI and ML analyses of datasets I and II, and *Chiromantis* is the sister taxon to this clade (Figs. 1 and 2), which is consistent with Li et al. (2008). Furthermore, both BI and ML analyses based on datasets I and II indicate that *Feihyla* is the sister taxon to genus *Polypedates*, although the support values for it are not strong (Figs. 1 and 2). According to Delorme (2004), the synapomorphies for the genus *Chiromantis* are the apposition of fingers I and II with fingers III and IV, the pres-

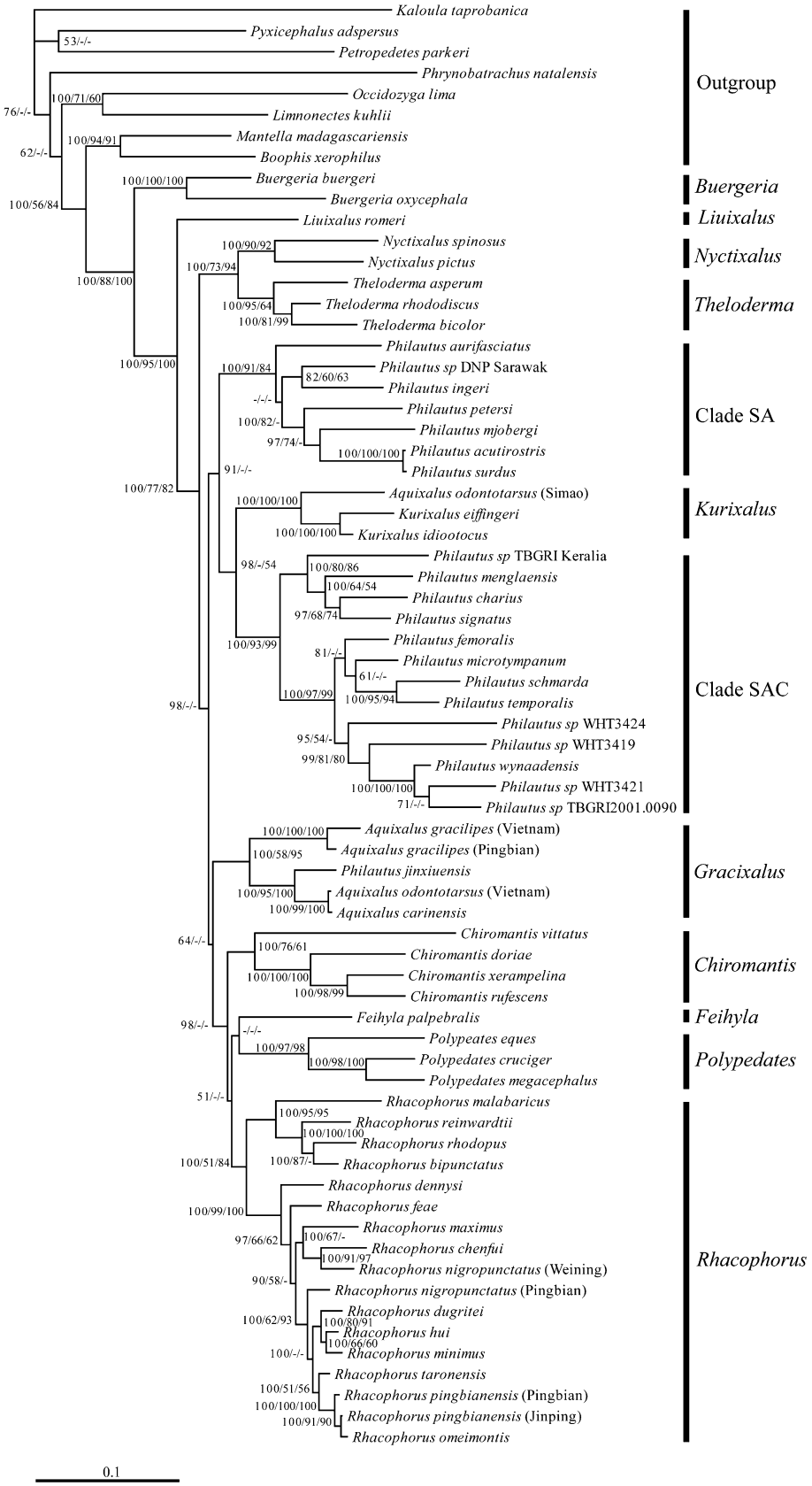

Fig. 1. Bayesian inference tree inferred from dataset I (2635 bp of 12S rRNA, 16S rRNA, Cyt *b*, rhodopsin exon 1, tyrosinase exon 1, and Rag-1) with all species. *Kaloula taprobanica* is defined as the root. The nodal numbers are BPP, ML, and MP bootstrap values, respectively. Only values above 50% are given. Locality sites for individuals from different populations are given in parentheses.

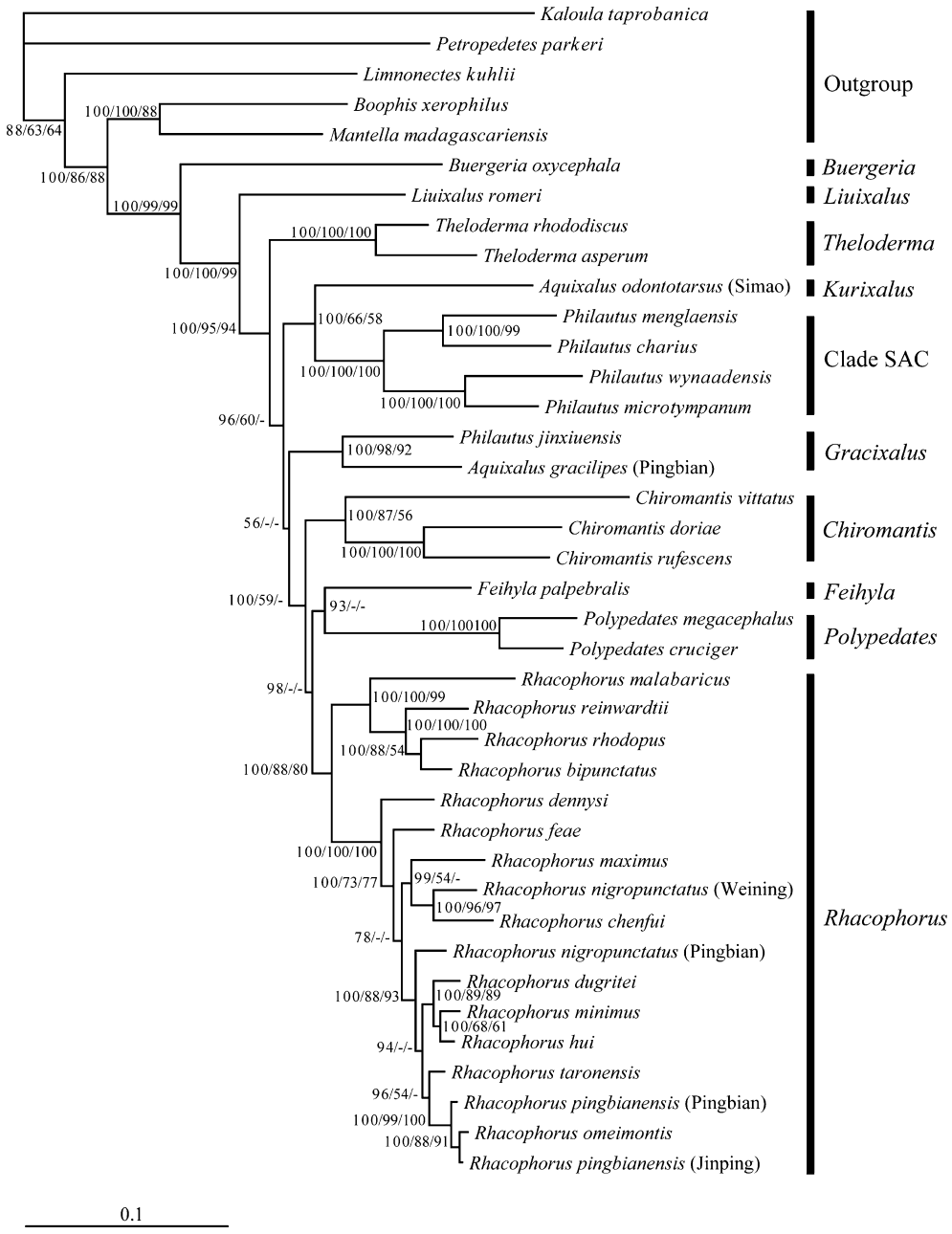

Fig. 2. Bayesian inference tree derived from dataset II (2636 bp of 12S rRNA, 16S rRNA, Cyt *b*, rhodopsin exon 1, tyrosinase exon 1, and Rag-1) with species for which we had all portions of the six genes. *Kaloula taprobanica* is defined as the root. The nodal numbers are BPP, ML, and MP bootstrap values, respectively. Only values above 50% are given. Locality sites for individuals from different populations are given in parentheses.

ence of anal glands in a semi-circle above the cloaca, and the presence of webbing between fingers not extending more than half-length of fingers, but these characters are not observed on *F. palpebralis* (Fei, 1999). Based on this molecular and morphological evidence, we recognize the validity of genus *Feihyla*. The placement of *Feihyla* relative to *Polypedates* and *Rhacophorus* needs further examination because of the weak support.

Before the genus *Chirixalus* was put in the synonymy of *Chiromantis* by Frost et al. (2006), Wilkinson et al. (2002) recovered '*Chirixalus*' *vittatus* as the sister taxon to *Polypedates*, whereas '*Chirixalus*' *doriae* was recovered as the sister taxon to the clade formed by *Chiromantis rufescens* and *Chiromantis xerampelina*. In the present study, the monophyly of *Chiromantis* is obtained by all analyses of datasets I (Fig. 1) and II (Fig. 2). This is consistent with Delorme et al. (2005), Frost et al. (2006), and the Bayesian analysis of Yu et al. (2008). Moreover, the Asian species *Chiromantis vittatus* and *Chiromantis doriae* are paraphyletic with respect to the African species *C. rufescens* and *C. xerampelina* (Figs. 1 and 2), which agrees with Richards and Moore (1998), Kosuch et al. (2001) and Vences et al. (2003) that *Chiromantis* was originally an Asian genus and it dispersed westward overland into Africa.

4.2. Phylogeny of Aquixalus, Kurixalus, and Philautus

Aquixalus odontotarsus is the type species of genus *Aquixalus* of Delorme et al. (2005). We infer that the incongruence on the phylogenetic placement of *A. odontotarsus* among previous studies (Grosjean et al., 2008; Li et al., 2008; Yu et al., 2008) results from the sampling of *A. odontotarsus*. The type locality of *A. odontotarsus* is Caiyanghe, Simao County, Yunnan Province (Ye and Fei, 1993). In

Li et al. (2008) and Yu et al. (2008), the specimens of *A. odontotarsus* came from the type locality and/or adjacent area, whereas specimens of *A. odontotarsus* in Delorme et al. (2005) and Grosjean et al. (2008) were collected from Vietnam. In the present study, *A. odontotarsus* from both the type locality and Vietnam are examined, and all analyses of dataset I support the clade consisting of *A. odontotarsus* from Vietnam, *A. carinensis*, *P. jinxiuensis*, and *A. gracilipes*, whereas *A. odontotarsus* from the type locality is clustered together with the genus *Kurixalus* (Fig. 1). These novel findings indicate that the specimens of *A. odontotarsus* from Vietnam in Delorme et al. (2005) and Grosjean et al. (2008) do not belong to this species. We agree with Li et al. (2008) in placing *A. odontotarsus* into *Kurixalus* and putting *Aquixalus* in the synonymy of *Kurixalus*, but we suggest placing the remaining species of *Aquixalus* and *P. jinxiuensis* into the new genus *Gracixalus* raised by Li et al. (2008).

As to the phylogenetic position of *Gracixalus*, BI, and ML analyses of datasets I and II indicate that it is the sister taxon to the clade formed by *Feihyla*, *Rhacophorus*, *Polypedates*, and *Chiromantis* (Figs. 1 and 2), which is consistent with Li et al. (2008) and Grosjean et al. (2008). However, considering the weak support values for this hypothesis in current and previous studies, more studies using broader sampling of DNA data will be needed to resolve the placement of *Gracixalus*.

Philautus is clearly heterogeneous in terms of morphology, and possibly also life-history (Bossuyt and Dubois, 2001). Dubois (1987) proposed three subgenera within *Philautus* including *Kirtixalus* (type species: *Philautus microtympanum*), *Gorhixalus* (type species: *Philautus hosii*) and the nominotypical *Philautus* (type species: *P. aurifasciatus*), but Bossuyt and Dubois (2001) pointed out that particularly the subgenus *Kirtixalus* probably deserves the rank of genus. In the present study, after the placement of *P. jinxiuensis* into *Gracixalus* as discussed above, all analyses of dataset I indicate that *Philautus* can be divided into two well supported clades (Clades SA and SAC, Fig. 1). The SAC clade comprises subgenus *Kirtixalus*, which is closer to *Kurixalus* than it is to the SA clade, which comprises the complex of *Philautus* (*Philautus*) and *Philautus* (*Gorhixalus*) (Fig. 1). These are consistent with Meegaskumbura et al. (2002) and Li et al. (2008), and indicate that the subgenus *Kirtixalus* does not belong to *Philautus*. It was known that eggs of *P. microtympanum*, the type species of *Philautus* (*Kirtixalus*), undergo terrestrial direct development (Bahir et al., 2005), whereas members of *Kurixalus* experience a typical aquatic larval life (Kuramoto and Wang, 1987; Kam et al., 1996; Ziegler and Vences, 2002). Based on our phylogeny and the difference in reproductive mode between *Philautus* (*Kirtixalus*) and *Kurixalus*, we consider that *Philautus* (*Kirtixalus*) deserves the rank of genus, and the direct development has evolved independently. Broader sampling of molecular markers is needed to resolve the phylogenetic position of true *Philautus* represented by the Clade SA.

The division of species groups within Bornean *Philautus* needs further revision. According to Dring (1987), in which the species of Bornean *Philautus* were reviewed and divided into five species groups, *P. acutirostris*, *P. aurifasciatus*, *P. mjobergi*, and *P. petersi* belong to *P. aurifasciatus* group, and *P. surdus* belongs to *P. surdus* group. However, in the present study, the *P. aurifasciatus* species group does not form a clade. All analyses of dataset I strongly support the clade of *P. acutirostris* and *P. surdus*, and both BI and ML analyses indicate that *P. petersi* and *P. mjobergi* are paraphyletic with respect to it (Fig. 1).

4.3. Systematics of Rhacophorus

The monophyly of *Rhacophorus* is supported well and *Rhacophorus* can be divided into two main clades (Figs. 1 and 2). These are in keeping with Li et al. (2008) and Grosjean et al. (2008). *Rhacophorus hui* was described by Liu (1945) based on the specimens from Zhaojue County, Sichuan Province. However, Liu and Hu (1961) treated *R. hui* as a synonym of *R. dugritei*. Although karyotypic evidence (Wu and Zeng, 1994) indicated that *R. dugritei* from Zhaojue and Lichuan County is very distinct from other populations, the validity of *R. hui* was still not recognized by Fei (1999) and Fei et al. (2005), and Fei et al. (2005) considered that it is more reasonable to treat the differences in karyotypes between *R. hui* and *R. dugritei* as polymorphism within *R. dugritei* until contrary evidence is provided. Here, the monophyly of *R. dugritei* and *R. hui* is rejected by our molecular evidence, and *R. hui* is closely related to *R. minimus* (Figs. 1 and 2). This result indicates that *R. hui* is a valid species.

Fei et al. (2005) suggested a division of species groups among Chinese *Rhacophorus*, and placed *Rhacophorus maximus*, *Rhacophorus feae*, *Rhacophorus dennysi*, and *Rhacophorus tuberculatus* into the *R. maximus* group based on the appearance of full webbing between fingers (at least between the third and the fourth finger). In this study, monophyly of *R. maximus* group is not supported. All analyses of datasets I and II find that *R. dennysi* is the sister taxon to the clade of *R. maximus*, *R. nigropunctatus*, *R. chenfui*, *R. feae*, *Rhacophorus taronensis*, *R. pingbianensis*, *R. omeimontis*, *R. dugritei*, *R. minimus*, and *R. hui* (Figs. 1 and 2). This result indicates that full webbing between fingers is a plesiomorphy, and the division of species groups among *Rhacophorus* suggested by Fei et al. (2005) needs further revision.

Rhacophorus nigropunctatus has a wide distribution in China including Yunan, Guizhou, Anhui and Hunan Province (Fei et al., 2005). In the current study, two specimens of *R. nigropunctatus* from different populations are examined, and the monophyly of this species is not recovered by all analyses. *Rhacophorus nigropunctatus* from the type locality (Weining County, Guizhou Province) is the sister taxon to *R. chenfui*, whereas *R. nigropunctatus* from Pingbian County, Yunnan Province is clustered together with *R. dugritei*, *R. hui*, *R. minimus*, *R. taronensis*, *R. pingbianensis*, and *R. omeimontis* (Figs. 1 and 2). We consider the specimens of *R. nigropunctatus* from Pingbian a cryptic species of *Rhacophorus*, and more studies are needed to unveil the general phylogenetic structure within *R. nigropunctatus* because of its discontinuous distribution in China. Additionally, Fei et al. (2005) placed *R. nigropunctatus* into the *R. dugritei* species group together with *R. dugritei*, *R. chenfui*, *Rhacophorus yaoshanensis* and *Rhacophorus hungfuensis*. However, in the present study, *R. dugritei* obviously is not related to the clade formed by *R. nigropunctatus* and *R. chenfui* (Figs. 1 and 2), which indicates that the *R. dugritei* species group of Fei et al. (2005) needs modification.

Rhacophorus pingbianensis is morphologically similar with *R. omeimontis* (Kou et al., 2001) and recent molecular studies (Li et al., 2008; Yu et al., 2008) have recovered the sister relationship between these two species. In the present study, the clade consisting of *R. pingbianensis* and *R. omeimontis* is recovered and *R. pingbianensis* from the type locality (Pingbian) and Jinping County is paraphyletic with respect to *R. omeimontis* (Figs. 1 and 2). Based on this topology and the short branch length within this clade, we agree with Fei et al. (2005) that *R. pingbianensis* is the synonymy of *R. omeimontis*.

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