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New insights to the molecular phylogenetics and generic assessment in the Rhacophoridae (Amphibia: Anura) based on five nuclear and three mitochondrial genes, with comments on the evolution of reproduction

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ABSTRACT

The phylogenetic relationships among 12 genera of treefrogs (Family, Rhacophoridae), were investigated based on a large sequence data set, including five nuclear (brain-derived neurotrophic factor, proopiomelanocortin, recombination activating gene 1, tyrosinase, rhodopsin) and three mitochondrial (partial 12S and 16S ribosomal RNA and the complete valine t-RNA) genes. Phylogenetic analysis of the nuclear gene sequences resolved three major clades. The first group included *Philautus*, *Pseudophilautus*, *Kurixalus*, *Gracixalus*, and *Theloderma moloch*; *Pseudophilautus* and *Kurixalus* were sister taxa. The second group consisted of *Nyctixalus* and *Theloderma*. The third group contained *Feihyla*, *Polypedates*, *Rhacophorus*, and *Chiromantis vittatus*; *Polypedates* and *Feihyla* were sister taxa. Analyses of the nuclear and mitochondrial genes supported the following results: (1) Genus *Liuixalus* formed the sister group of all other rhacophorines. (2) *Philautus*, *Theloderma*, and *Chiromantis* were not resolved as monophyletic genera. Four groups, including *Philautus ocellatus* and *P. hainanus*, *P. longchuanensis* and *P. gryllus*, *P. banaensis*, and *P. quyeti* nested well within the genera *Liuixalus*, *Pseudophilautus*, *Kurixalus*, and *Gracixalus*, respectively. (3) *Theloderma moloch* and *Chiromantis vittatus* did not cluster with other species of *Theloderma* and *Chiromantis*, respectively. Foam nesting evolved only once, as did laying eggs in a jelly-like matrix containing some bubbles. Terrestrial direct development evolved twice in the Rhacophoridae.

1. Introduction

Tree frogs in the family Rhacophoridae, involving about 300 extant species, are distributed in Asia and Africa (Frost, 2009). Most prior studies on their systematics and taxonomy have relied primarily on morphological data (e.g., Bain and Truong, 2004; Boulenger, 1903; Channing, 1989; Duellman and Trueb, 1986; Inger, 1966; Jiang et al., 1987; Liem, 1970; Orlov et al., 2008; Wilkinson and Drewes, 2000). Recently, DNA sequences have been used for their phylogenetic reconstruction (Frost et al., 2006; Grosjean et al., 2008; Li et al., 2008; Richards and Moore, 1998; Wilkinson et al., 2002; Yu et al., 2008, 2009). Except for the study of Frost et al. (2006) with about 2300 bp, Grosjean et al. (2008), Li et al. (2008) and Yu et al. (2009) used nuclear gene sequences of relatively short fragment lengths, of about 300 base pairs (bp), ~800 bp or ~1000 bp, respectively. Although Frost et al. (2006) used the greatest quantity of data, their study suffered from incomplete taxon sampling. It included 17 species as representative rhacophorids only.

Prior phylogenies for the Rhacophoridae are not well resolved. Whereas mitochondrial DNA (mtDNA) genes are used to infer relationships among closely related taxa, nuclear DNA (nuDNA), with a slower rate of evolution, are more useful for deciphering older relationships (Simmons et al., 2002, 2004). Nuclear genes are also less influenced by base pair compositional bias (Simmons et al., 2004). Therefore, a large data set consisting of single-copy protein-coding nuclear genes is desirable to assess the phylogeny of the family Rhacophoridae.

Rhacophorid taxonomy has been controversial. Dubois (1992, 2005) considered the Rhacophoridae to be a subfamily of the Ranidae (Rhacophorinae), and to be composed of three tribes: Buergerini, Philautini, and Rhacophorini. This taxonomy was not supported by more recent studies (Frost et al., 2006; Grosjean et al., 2008; Li et al., 2008; Yu et al., 2009). Using a large data set, including anatomical characters and a mtDNA and nuDNA gene fragment of about 5000 bp, Frost et al. (2006) elevated the Rhacophorinae to the rank of family. Their study supported Channing's

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(1989) recognition of two subfamilies: Buergeriinae and Rhacophorinae. This arrangement was consistent with Grosjean et al. (2008), Li et al. (2008), and Yu et al. (2009). Within the Rhacophorinae, Grosjean et al. (2008) erected the tribe Nyctixalini, which included two genera, *Theloderma* and *Nyctixalus*. However, due to relatively weak nodal support (Bayesian posterior probability = 0.86), these divisions are far from being conclusive.

Intergeneric relationships cannot be well resolved without including the most species-rich genus in the family, Genus *Philautus* Gistel, 1848, with 146 species (Frost, 2009). *Philautus* has a wide geographic distribution occurring from India and Sri Lanka eastward through China, mainland Southeast Asia, the Greater Sunda Islands, and the Philippines (Bossuyt and Dubois, 2001; Frost, 2009). Due to their small size and high intraspecific variability, some species of *Philautus* have been confused with other genera such as '*Chirixalus*', *Kurixalus,* and *Rhacophorus* (Bossuyt and Dubois, 2001). Delorme et al. (2005) transferred *P. gracilipes* and *P. supercornutus* into *Aquixalus* (subgenus *Gracixalus*). *Philautus carinensis* and *P. odontotarsus* were also included in the genus *Aquixalus* (Delorme et al., 2005). Frost et al. (2006) erected the genus *Feihyla* for *P. palpebralis* but Grosjean et al. (2008) resolved *Feihyla* as the sister group of *Chiromantis* and retained the species within the later genus. Li et al. (2008) and Yu et al. (2009) recognized the validity of genus *Feihyla*. Yu et al. (2008) treated *Philautus albopunctatus* as a junior synonym of *Theloderma asperum* and suggested that *P. rhododiscus* be transferred to *Theloderma*. Li et al. (2008) considered *Aquixalus* to be a synonym of the genus *Kurixalus* and raised *Gracixalus* to generic rank, containing *G. gracilipes* and *G. supercornutus*. Meegaskumbura et al. (2002), Grosjean et al. (2008) and Li et al. (2008) found that species of *Philautus* from India, Sri Lanka and the Sunda Islands did not form a monophyletic group. However, due to little or no branch support, they refrained from making taxonomic changes. Consistent with Grosjean et al. (2008), Li et al. (2008) and Meegaskumbura et al. (2002), Yu et al. (2009) raised the subgenus *Philautus* (*Kirtixalus*) to generic rank and transferred *P. menglasensis, P. wynaadensis*, *P. charius* and *P. microtympanum* into it. However, the validity of the genus *Kirtixalus* needs further examination, because *Ixalus temporalis* from Sri Lanka carries an older generic name *Pseudophilautus* Laurent, 1943 than *Kirtixalus* Dubois, 1987. Findings such as these placed many Vietnamese and Chinese species of *Philautus* in *Aquixalus*, *Theloderma*, *Kurixalus, Kirtixalus* and *Gracixalus* (e.g., Li et al., 2008). It remains necessary to reassess the generic allocation of the remaining species of *Philautus*.

Another genus of interest is *Theloderma* Tschudi, 1838, which contains 14 species (Frost, 2009). It is widely distributed across Sri Lanka, northeastern India to Myanmar and southern China, through Indochina to Malaya and Sumatra (Frost, 2009). The genus is diagnosed by a collection of morphological characters, including two M. extensor digitorum communis longus slips and numerous calcified warts on the dorsum (Liem, 1970). The species of *Theloderma* usually inhabit mountain forests (elevation of 900–1000 m), where they prefer to live and breed in small karst crevices or tree hollows (Orlov, 1997). Due to their habitat preferences, cryptic coloration and relatively small population sizes, *Theloderma* is difficult to sample. Previously, only four species, *T. rhododiscus*, *T. bicolor, T. asperum*, and *T. corticale*, were included in analyses, and all studies suggested that *Theloderma* and *Nyctixalus* were sister genera (Delorme et al., 2005; Frost et al., 2006; Grosjean et al., 2008; Li et al., 2008; Wilkinson et al., 2002; Yu et al., 2008, 2009). The other species of *Theloderma* await examination.

Reproductive mode is not just an important character in taxonomy [e.g., *Philautus* is characterized by the aerial direct development of eggs into froglets (Bossuyt and Dubois, 2001; Dring, 1979)], but is critical to understand the adaptive evolution within the family. Using a mtDNA and nuDNA gene fragment length of 1676 bp, Grosjean et al. (2008) inferred the evolution of the reproductive modes. Consistent with Li et al. (2008) and Meegaskumbura et al. (2002), they suggested that *Philautus* with direct development was not a monophyletic genus. The genus is comprised of two reciprocally monophyletic clades, one from India and Sri Lanka (subgenus *Kirtixalus*), and another from the Sunda Islands (subgenus *Philautus*). Recently, Yu et al. (2009) raised the former subgenus *Kirtixalus* to full generic rank, and proposed that direct development in *Kirtixalus* evolved independently. However, the type species of *Philautus* was not included in their analyses with the complete data, thus calling into question the identity of *Philautus*. Foam nesting, another reproductive mode exhibited by many rhacophorids, may have evolved once only within the family (Grosjean et al., 2008; Wilkinson et al., 2002). However, variation occurs within the foam nesting clade, with some species, such as *Chiromantis vittatus*, laying their eggs in a jelly with some bubbles (Grosjean et al., 2008).

While providing a representative generic-level sampling of the family and more makers than earlier studies (including five nuclear genes and three mitochondrial genes), we specially test the earlier hypothesis of generic designation and intergeneric relationships: (1) the naturalness of Nyctixalini and Rhacophorini; (2) monophyly and generic allocation of *Philautus*, *Chiromantis* and *Theloderma*. Also, we discuss the evolution of reproductive modes inferred from the phylogeny.

2. Materials and methods

2.1. Taxon sampling and data collection

For the mtDNA analyses, taxonomic sampling included all 12 genera (*Buergeria*, *Chiromantis*, *Feihyla*, *Gracixalus*, *Kirtixalus*, *Kurixalus*, *Liuixalus*, *Nyctixalus*, *Philautus*, *Polypedates*, *Rhacophorus*, *Theloderma*) within the Rhacophoridae except for the newly erected *Ghatixalus* (Biju et al., 2008; Frost, 2009; Yu et al., 2009). Whenever possible the type species or species very closely related to type species of each genus was included to evaluate generic assignments. Sequence data were also obtained from GenBank, although the data were not complete for some Indian, Sri Lankan and Vietnamese species. For the nuDNA analyses, at least one species was sampled from each genus. Three species from the family Mantellidae and one from the Ranidae were chosen as outgroup taxa (Frost et al., 2006; Li et al., 2008). The taxonomic arrangements proposed by Frost (2009) and Yu et al. (2009) were used for the purposes of discussion. GenBank Accession numbers for both the new and previously deposited data are given in Table 1.

The reproductive modes of the species were obtained by either direct observation in the field or from the literature. These data are given in Table 1.

2.2. DNA extraction, amplification, and sequencing

Genomic DNA was extracted from toe clips, muscle, or liver tissues preserved in 95% or 100% ethanol. Tissue samples were digested using proteinase K, and then followed a standard 3-step phenol/chloroform extraction procedure (Hillis et al., 1996; Sambrook et al., 1989). Five nuclear protein-coding gene fragments were PCR-amplified and cycle-sequenced on both strands as follows: (i) a region from brain-derived neurotrophic factor (BDNF); (ii) a region from proopiomelanocortin (POMC); (iii) a region from the recombination activating gene 1 (RAG-1); (iv) a region from exon 1 of rhodopsin (RHOD), and (v) a region from exon 1 of tyrosinase (TYR). A sixth fragment involved mtDNA sequences including most of the 12S and 16S rRNA together with the complete t-RNA for valine. The primers are given in Table 2. Double stranded poly-

Table 1
Samples and sequences used in this study.

Specific epithet	Frost (2009)	Present genus	Specimen voucher No.	Locality	GenBank No. (partial 12S and 16S and complete t-RNA for valine)	GenBank No. (brain-derived neurotrophic factor, proopiomelanocortin, recombination activating gene 1, rhodopsin tyrosinase)	Reproductive mode data (DD: direct development, FA: foam nesting, and EJ: laying eggs in a jelly with some bubbles)
	Rhacophoridae Buergeriinae	**Rhacophoridae Buergeriinae**					
japonica	*Buergeria*	*Buergeria*	UMFS 5821	China: Taiwan	DQ283055	–	
japonica	*Buergeria*	*Buergeria*	SCUM061101	China: Lianhuachi, Taiwan		GQ285691[a] GQ285722[a] GQ285754[a] GQ285783[a] GQ285801[a]	
oxycephala	*Buergeria*	*Buergeria*	SCUM 050267YJ	China: Hainan	EU215524	GQ285695[a] GQ285726[a] GQ285758[a] EU215556 EU215585	
	Rhacophorinae	**Rhacophorinae**					
eiffingeri	*Kurixalus*	*Kurixalus*	UMFS 5969	China: Nantou, Taiwan	DQ283122	–	
idiootocus	*Kurixalus*	*Kurixalus*	SCUM 061107L	China: Lianhuachi, Taiwan	EU215547	GQ285688[a] GQ285719[a] GQ285751[a] EU215577 EU215607	
odontotarsus	*Kurixalus*	*Kurixalus*	SCUM 060688L	China: Mengyang, Jinghong	EU215549	GQ285687[a] GQ285718[a] GQ285750[a] EU215579 EU215609	
hainanus	*Kurixalus*	*Kurixalus*	HNNU A1180	China: Mt. Diaoluo, Hainan	EU215548	GQ285686[a] GQ285717[a] GQ285749[a] EU215578 EU215608	
banaensis	*Philautus*	*Kurixalus*	ROM32986	Vietnam: Krong Pa, Gia Lai	GQ285667[a]	GQ285689[a] GQ285720[a] GQ285752[a] GQ285781[a] GQ285799[a]	
gracilipes	*Gracixalus*	*Gracixalus*	AMNH A163897	Vietnam	DQ283051	–	
gracilipes	*Gracixalus*	*Gracixalus*	060821196Rao	China: Mt. Dawei, Yunnan	GQ285668[a]	GQ285701[a] GQ285732[a] GQ285764[a] GQ285789[a] GQ285807[a]	
quyeti	*Philautus*	*Gracixalus*	VNUH160706	Vietnam: Quang Binh	EU871428	–	
jinxiuensis	*Philautus*	*Gracixalus*	KIZ 061210YP	China: Mt. Dayao, Guangxi	EU215525	GQ285700[a] GQ285731[a] GQ285763[a] EU215557 EU215587	
jinxiuensis	*Philautus*	*Gracixalus*	IEBR2351	Vietnam: Lai Chau	EU871425	–	
sp.	–	*Gracixalus*	03320Rao	China: Wenshang, Yunnan	GQ285669[a]	–	
carinensis	*Kurixalus*	*Gracixalus*	ROM39660	Vietnam: Sa Pa, Lao Cai	GQ285670[a]	GQ285699[a] GQ285730[a] GQ285762[a] GQ285788[a] GQ285806[a]	
"odontotarsus"	*Kurixalus*	*Gracixalus*	MNHN1999.5942	Vietnam	AY550593AY550507	–	
romeri	*Liuixalus*	*Liuixalus*	KIZ 061205YP	China: Mt. Shiwan, Guangxi	EU215528	GQ285693[a] GQ285724[a] GQ285756[a] EU215559 EU215589	
sp.	–	*Liuixalus*	KIZ 061209YP	China: Mt. Dayao, Guangxi	EU215526	–	
ocellatus	*Philautus*	*Liuixalus*	HN0806045	China: Mt. Wuzhi, Hainan	GQ285672[a]	GQ285692[a] GQ285723[a] GQ285755[a] GQ285784[a] GQ285802[a]	
hainanus	*Philautus*	*Liuixalus*	060401L	China: Mt. Diaoluo, Hainan	GQ285671[a]	GQ285694[a] GQ285725[a] GQ285757[a] GQ285785[a] GQ285803[a]	
acutirostris	*Philautus*	*Philautus*	–	–	AF458137	–	DD (Meegaskumbura et al., 2002)
surdus	*Philautus*	*Philautus*	CAS 219932	Philippine	AF458138	–	DD (Grosjean et al., 2008)
abditus	*Philautus*	*Philautus*	ROM33145	Vietnam: Krong Pa, Gia Lai	GQ285673[a]	GQ285712[a] GQ285743[a] GQ285775[a] GQ285794[a] GQ285812[a]	DD (Bossuyt and Dubois, 2001)
microtympanum	*Philautus*	*Pseudophilautus*	Genbank	Sri Lanka	DQ346974	– – DQ019506 DQ019566 AF249189	DD (Bahir et al., 2005)
wynaadensis	*Philautus*	*Pseudophilautus*	Genbank	India	DQ346966	–	DD (Meegaskumbura et al., 2002)
charius	*Philautus*	*Pseudophilautus*	Genbank	India	DQ346967	–	DD (Meegaskumbura et al., 2002)
gryllus	*Philautus*	*Pseudophilautus*	ROM30288	Vietnam: Pac Ban, Tuyen Quang	GQ285674[a]	GQ285714[a] GQ285745[a] GQ285777[a] GQ285796[a] GQ285814[a]	DD (Bossuyt and Dubois, 2001)
longchuanensis	*Philautus*	*Pseudophilautus*	5Rao	China: Longchuan, Yunnan	GQ285675[a]	GQ285713[a] GQ285744[a] GQ285776[a] GQ285795[a] GQ285813[a]	DD (direct observation on field)

(*continued on next page*)

Table 1 (*continued*)

Specific epithet	Frost (2009)	Present genus	Specimen voucher No.	Locality	GenBank No. (partial 12S and 16S and complete t-RNA for valine)	GenBank No. (brain-derived neurotrophic factor, proopiomelanocortin, recombination activating gene 1, rhodopsin tyrosinase)	Reproductive mode data (DD: direct development, FA: foam nesting, and EJ: laying eggs in a jelly with some bubbles)
menglaensis	*Philautus*	*Pseudophilautus*	060821286Rao	China: Lvchun, Yunnan	GQ285676[a]	GQ285715[a] GQ285746[a] GQ285778[a] GQ285797[a] GQ285815[a]	DD (direct observation on field)
rhododiscus	*Theloderma*	*Theloderma*	SCUM 061102L	China: Mt. Dayao, Guangxi	EU215530	GQ285696[a] GQ285727[a] GQ285759[a] EU215555 EU215586	
asperum	*Theloderma*	*Theloderma*	060821203Rao	China: Jinping, Yunnan	GQ285677[a]	GQ285697[a] GQ285728[a] GQ285760[a] GQ285786[a] GQ285804[a]	
asperum	*Theloderma*	*Theloderma*	HN0806100	China: Mt. Yinggeling. Hainan	GQ285678[a]	–	
corticale	*Theloderma*	*Theloderma*	AMNH A161499	Vietnam	DQ283050	–	
moloch	*Theloderma*	*Theloderma*	6255Rao	China: Motuo, Xizang	GQ285679[a]	GQ285690[a] GQ285721[a] GQ285753[a] GQ285782[a] GQ285800[a]	
spinosus	*Nyctixalus*	*Nyctixalus*	ACD 1043	Philippine Islands: Mindanao	DQ283114	–	
pictus	*Nyctixalus*	*Nyctixalus*	FMNH 231095	Malaysia	DQ283133	–	
pictus	*Nyctixalus*	*Nyctixalus*	R081203	Malaysia	–	GQ285698[a] GQ285729[a] GQ285761[a] GQ285787[a] GQ285805[a]	
leucomystax	*Polypedates*	*Polypedates*	CAS 219931	Philippines	AF458140	–	FA (Grosjean et al., 2008)
megacephalus	*Polypedates*	*Polypedates*	–	–	AF458141	–	FA (direct observation on field)
megacephalus	*Polypedates*	*Polypedates*	6212Rao	China: Motuo, Xizang	GQ285685[a]	GQ285706[a] GQ285737[a] GQ285769[a] GQ285791[a] GQ285809[a]	FA (direct observation on field)
megacephalus	*Polypedates*	*Polypedates*	SCUM 050508C	China: Mt. Daiyun, Fujian	EU215552	GQ285708[a] GQ285739[a] GQ285771[a] EU215582 EU215612	FA (direct observation on field)
mutus	*Polypedates*	*Polypedates*	SCUM 37940C	China: Xishuangbanna, Yunnan	EU215551	GQ285707[a] GQ285738[a] GQ285770[a] EU215581 EU215611	FA (direct observation on field)
maculatus	*Polypedates*	*Polypedates*	WHT 3432	Sri Lanka	AY880607 AY880520	–	FA (Grosjean et al., 2008)
cruciger	*Polypedates*	*Polypedates*	VUB0125	Sri Lanka	DQ346973	–	FA (Grosjean et al., 2008)
kio	Rhacophorus	Rhacophorus	SCUM 37941C	China: Xishuangbanna, Yunnan	EU215532	GQ285703[a] GQ285734[a] GQ285766[a] EU215562 EU215592	FA (direct observation on field)
rhodopus	Rhacophorus	Rhacophorus	SCUM 060692L	China: Mengyang, Jinghong	EU215531	–	FA (direct observation on field)
sp.	Rhacophorus	Rhacophorus	03308 Rao	China: Wenshan, Yunnan	GQ285680[a]	GQ285702[a] GQ285733[a] GQ285765[a] GQ285790[a] GQ285808[a]	FA (direct observation on field)
orlovi	Rhacophorus	Rhacophorus	AMNH A161405	Vietnam	DQ283049	–	FA
calcaneus	Rhacophorus	Rhacophorus	AMNH A163749	Vietnam	DQ283380	–	FA
dugritei	Rhacophorus	Rhacophorus	SCUM 051001L	China: Baoxing, Sichuan	EU215541	GQ285705[a] GQ285736[a] GQ285768[a] EU215571 EU215601	FA (direct observation on field)
moltrechti	Rhacophorus	Rhacophorus	SCUM 061106L	China: Lianhuachi, Taiwan	EU215543	–	FA (direct observation on field)
nigropunctatus	Rhacophorus	Rhacophorus	SCUM 070657L	China: Weining, Guizhou	EU215533	GQ285704[a] GQ285735[a] GQ285767[a] EU215563 EU215593	FA (direct observation on field)
dennysi	Rhacophorus	Rhacophorus	SCUM 060401L	China: Shaoguan, Guangdong	EU215545	–	FA (direct observation on field)
feae	Rhacophorus	Rhacophorus	SCUM 050642W	China: Hekou, Yunnnan	EU215544	–	FA (direct observation on field)
palpebralis	*Feihyla*	*Feihyla*	SCUM 0606132L	China: Mt. Dawei, Yunnan	EU215546	GQ285710[a] GQ285741[a] GQ285773[a] EU215576 EU215606	EJ (direct observation on field)

Table 1 (*continued*)

palpebralis	*Feihyla*	*Feihyla*	712	Vietnam: Lam Dong	GQ285681[a]	GQ285709[a] GQ285740[a] GQ285772[a] GQ285792[a] GQ285810[a]	EJ (direct observation on field)
doriae	Chiromantis	Chiromantis	SN 030051	China: Hainan	EU215527	GQ285716[a] GQ285747[a] GQ285779[a] EU215554 EU215584	FA (direct observation on field)
doriae	Chiromantis	Chiromantis	KIZ 005Rao	China: Simao, Yunnan	GQ285682[a]	–	FA (direct observation on field)
doriae	Chiromantis	Chiromantis	1056	Vietnam: Binh Thuan	GQ285683[a]	–	FA
rufescens	Chiromantis	Chiromantis	CAS 207601	Equatorial Guinea	AF458126	–	FA (Schiotz, 1999)
rufescens	Chiromantis	Chiromantis	CAS 207599	Equatorial Guinea	–	– – DQ347237 DQ347356 DQ347139	FA (Schiotz, 1999)
xerampelina	Chiromantis	Chiromantis	–	–	AF458132	–	FA (Schiotz, 1999)
vittatus	Chiromantis	Chiromantis	KIZ 0001Rao	China: Simao, Yunnan	GQ285684[a]	GQ285711[a] GQ285742[a] GQ285774[a] GQ285793[a] GQ285811[a]	EJ (direct observation on field)
vittatus	Chiromantis	Chiromantis	FMNH 254444	Vietnam: Gia Lai	DQ283134	–	EJ
vittatus	Chiromantis	Chiromantis	ZFMK 65463	Myanmar	–	– – DQ019497 DQ019556 –	EJ
Outgroup							
	Mantellidae	**Mantellidae**					
tephraeomystax	*Boophis*	*Boophis*	AMNH A168144	Madagascar	DQ283032	–	
madagascariensis	*Aglyptodactylus*	*Aglyptodactylus*	UMMZ 198472	Madagascar	DQ283056	–	
aurantiaca	*Mantella*	*Mantella*	UMMZ 201411	Madagascar	DQ283035	–	
labrosum	*Laliostoma*	*Laliostoma*	GenBank	Madagascar	–	– – AY571652 DQ283786 AF249169	
wittei	*Blommersia*	*Blommersia*	ZSM 405/2000	Madagascar	–	EF396018 – AY323774 AY323743 AY341751	
madagascariensis	*Mantella*	*Mantella*	GenBank	GenBank	–	– – DQ019500 AY263284 AF249164	
	Ranidae	**Ranidae**					
poilani	*Limnonectes*	*Limnonectes*	AMNH A163717	Vietnam	DQ283378	–	
kukunoris	*Rana*	*Rana*	KIZ 0152	China: Qinghai	–	– GQ285748[a] GQ285780[a] GQ285798[a] GQ285816[a]	

ROM, Royal Ontario Museum, Toronto, Canada; HNNU, Hainan Normal University; KIZ, Kunming Institute of Zoology, the Chinese Academy of Sciences; SCUM, Sichuan University Museum; SN =field numbers of Shunqing Lu. "–", unknown data.

[a] Sequences new to this study.

Table 2
Primers used in PCR and sequencing.

Locus	Primer	Primer sequence	Size (bp)	Cited source
The recombination activating gene 1	L6300	5′-CTG GTC GTC AGA TCT TTC AGC-3′	1164	This study
	H6301	5′-GCA AAA CGT TGA GAG TGA TAA C-3′		This study
	L6302	5′-GGA AAT TGG TGG AAT CCT CAG-3		This study
	H6303	5′-ATA TAG ATA GAG CCT GAG GC-3′		This study
	L7501	5′-AGA AAG CCT CNT TCC AGG-3′		This study
	H5406	5′-TCG CGT TCG ATG ATC TCT GG-3′		This study
	L4935	5′-ACA GGA TAT GAT GAR AAG CTT GT-3′		Hoegg et al. (2004)
	H4936	5′-GGT GYT TYA ACA CAT CTT CCA TYT CRT A-3′		Hoegg et al. (2004)
	L7119	5′-GAA TGT ATY AAA GMM TGC AAG ATG GWC CT-3′		Wiens et al. (2005)
Proopiomelanocortin	R7120	5′-TAY TGR CCC TTY TTG TGG GCR TT-3′	601	Wiens et al., (2005)
	L7121	5′-GGA RCA CTT YCG ATG GGG YAA ACC-3′		Wiens et al. (2005)
	R7122	5′-GGT TTR CCC CAT CGR AAG TGY TCC-3′		Wiens et al. (2005)
Brain-derived neurotrophic factor	L7150	5′-ACC ATC CTT TTC CTK ACT ATG G-3′	614	Vieites et al. (2007)
	R7151	5′-CTA TCT TCC CCT TTT AAT GGT C-3′		Vieites et al. (2007)
	L7152	5′-ACC ATC CTT TTC CTT ACT ATG G-3′		Van der Meijden et al. (2007)
Exon 1 of rhodopsin	L2903	5′-ACC ATG AAC GGA ACA GAA GGY CC-3′	315	Bossuyt and Milinkovitch (2000)
	H2904	5′-GTA GCG AAG AAR CCT TCA AMG TA-3′		Bossuyt and Milinkovitch (2000)
Exon 1 of tyrosinase	L2976	5′-TGC TGG GCR TCT CTC CAR TCC CA-3′	531	Bossuyt and Milinkovitch (2000)
	H2977	5′-AGG TCC TCY TRA GGA AGG AAT G-3′		Bossuyt and Milinkovitch, 2000
	F0001	5′-AGA TAC CCC ACT ATG CCT ACC C-3′		Wilkinson et al. (2002)
Partial 12S and 16S ribosomal genes and the complete valine t-RNA	R1169	5′-GTG GCT GCT TTT AGG CCC ACT-3′	2041	Wilkinson et al. (2002)
	F0483	5′-GAA GAG GCA AGT CGT AAC ATG G -3′		Wilkinson et al. (2002)
	R0483	5′-CCA TGT TAC GAC TTG CCT CTT C-3′		Wilkinson et al. (2002)
	F0937	5′-TGG GAT GAT TTT CAA GTA G-3′		Wilkinson et al. (2002)
	F1624	5′-GTA TCA ACG GCA TCA CGA GGG-3′		Wilkinson et al. (2002)
	R1624	5′-CCC TCG TGA TGC CGT TGA TAC-3′		Wilkinson et al. (2002)
	R end	5′-GAC CTG GAT TAC TCC GGT CTG A-3′		Wilkinson et al. (2002)
	L7270	5′-AGA TAC CCC ACT ATG CCA AGT C-3′		This study
	L7271	5′-AGA TAC CCC ACT ATG CCT AGC C-3′		This study

merase chain reaction (PCR) amplification was carried out in a 25–50 μl volume reaction using the following procedures. For RAG-1 cycle conditions were adapted from a long range PCR protocol (Barnes, 1994), with an initial denaturing step of 5 min at 94 °C, followed by 10 cycles of 30 s at 94 °C, annealing temperatures increasing by 0.5 °C per cycle from 52 to 57 °C and extending for 3 min at 68 °C. Additionally, 20 cycles were performed for 10 s at 94 °C, 57 °C for 40 s, and for 3 min at 68 °C. The final extension was done for 5 min at 68 °C. Cycle conditions for BDNF were an initial denaturation of 5 min at 94 °C, followed by 94 °C denaturation for 1 min, 50 °C annealing for 1 min, and for 1 min extension at 72 °C. Final extension at 72 °C was conducted for 10 min. For POMC, RHOD, TYR, and the mitochondrial DNA, the same procedure as for BDNF was used, but with annealing at 54, 52, 54, and 55 °C, respectively. The amplified DNA fragments were purified via spin columns and sequenced with an ABI 3730 automated DNA sequencer following the manufacturer's protocol. 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 Clustal X 1.81 (Thompson et al., 1997) with default parameters (gap opening 15.00, gap extension 6.66), and subsequently adjusted by eye for all the gene fragments (RAG-1, POMC, BDNF, TYR, RHOD, and mtDNA). Nucleotide sites having ambiguous alignments were removed from the analyses to increase the reliability of the phylogenetic analysis (Swofford et al., 1996). The aligned sequences were submitted to TREEBASE http://www.treebase.org under accession number (SN 4501). We included the secondary structure for aligning rRNA and t-RNA to the analysis. Gaps resulting from the alignment were treated as missing data. Because all mtDNA gene sequences are effectively inherited as one locus, they were concatenated into a single fragment for analyses. For the mtDNA and each nuDNA fragment, possible saturation of substitution types was checked by plotting the number of transitions (Ti) and transversions (Tv) versus F84 distance using DAMBE (Xia, 2000). Saturation plots were examined separately for the first, second and the third positions of each nuDNA gene. Pairwise comparisons of sequence divergences (*P* distance) were calculated using PAUP* 4.0b 10a (Swofford, 2003).

2.4. Phylogenetic analyses

Missing data were coded as "N" in the analyses. The inclusion of a limited amount of missing data was unlikely to distort the phylogenetic results in Bayesian Inference (BI) and Maximum Parsimony (MP) analyses (Wiens et al., 2005; Wiens and Moen, 2008).

To examine possible incongruence in the combined data, we used an incongruence length difference (ILD) test (Farris et al., 1994), often referred to as a partition homogeneity test in PAUP*. One hundred replicates of the ILD test with 10 random-addition sequences were implemented.

Phylogenetic hypotheses were also inferred using BI as implemented in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003), incorporating both unpartitioned and partitioned strategies. Each data set followed its own best-fit model in the analysis of the combined data set. For the five nuDNA fragments, we combined codon positions 1 and 2 into a single partition and treated the third positions separately. The best-fitting nucleotide substitution models for each of the 10 nuDNA partitions and the unpartitioned mtDNA data set were selected by using the Akaike Information Criterion as imple-

mented in Modeltest 3.7 (Posada and Crandall, 1998). The model GTR+I+G was selected for the mtDNA sequences. The following models were selected for analysis of the nuDNA data: TrN+I for codon positions 1 and 2, and SYM+G for codon position 3 in the BDNF partition; GTR+G for codon positions 1, 2, and 3 in the POMC partition; TVM+I+G for codon positions 1 and 2, and TrN+G for codon position 3 in the RAG-1 partition; TrN+G for codon positions 1 and 2, and TVM+G for codon position 3 in the RHOD partition; TVM+I+G for codon positions 1 and 2, and GTR+G for codon position 3 in the TYR partition. For BI and Bayesian posterior probabilities (BPP), the following settings were applied: number of Markov chain Monte Carlo (MCMC) generations = 5,000,000 and sampling frequency = 100. The first 12,500 sampled trees 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.

Maximum parsimony (MP) analyses were conducted using PAUP*. All characters were weighted equally and treated as unordered. Only potentially phylogenetically informative (PPI) sites were retained for tree searching. MP analysis was performed using a heuristic search with 1000 random stepwise additions followed by TBR branch swapping. Bootstrap branch support (BBS) values were calculated with 1000 replicates with 10 random-addition sequences performed in each replication.

In the analysis of the nuDNA, partitioned maximum likelihood (ML) analyses were performed using RAxML Web-Servers (Stamatakis et al., 2008). We combined codon positions 1 and 2 into a single partition and treated third positions separately. RAxML searches were executed in 100 rapid bootstrap inferences and thereafter in a thorough ML search on partitioned datasets. All free model parameters were estimated by RAxML.

Alternative phylogenetic topologies were tested using the MP-based Templeton Test (TT) (Templeton, 1983), the likelihood-based Kishino–Hasegawa (KH) test (Kishino and Hasegawa, 1989), and the approximately unbiased (AU) test (Shimodaira, 2002). To perform the KH and AU tests, site-wise log-likelihoods for all trees were estimated using PAUP* as follows: heuristic searches under a GTR+I+G model and incorporating a topological constraint were conducted so as to find the highest-likelihood topology that satisfied a given hypothesis. Subsequently, a log file was produced for the site-wise log-likelihoods of alternative trees given the concatenated data set with a GTR+I+G model. The generated log file was then used as input for CONSEL (Shimodaira and Hasegawa, 2001) to calculate the *P*-value for each alternative topology. TT was performed using 1000 RELL bootstrap replicates with a similar search strategy for finding the alternative topology.

3. Results

3.1. Sequence characteristics

The aligned mtDNA gene fragments consisted of 2041 sites, corresponding to sites 726 through 2666 of the *P. megacephalus* mitochondrial genome (AY458598), with 748 constant characters (CC) and 1074 PPI characters. In the case of the five nuDNA sequence data sets, transitions and transversions were accumulating linearly and gave no indication of a saturation effects. Consequently, all substitutions in these genes were used for phylogenetic reconstructions. These plots are available from the authors upon request. The concatenated nuclear data set consisted of a matrix of 3225 base pairs (bp). Of these, 2143 bp were CC and 726 bp were PPI. The aligned data set was assembled from five gene fragments: BDNF gene data set (614 amplified bp/54 PPI sites/522 CC); POMC (601 bp/131 PPI sites/393 CC); RAG-1 (1164 bp/295 PPI sites/751 CC); RHOD (315 bp/72 PPI sites/181 CC), and TYR (531 bp/174 PPI sites/296 CC).

3.2. Phylogenetic analysis

3.2.1. Mitochondrial genes

Analysis of mtDNA data under equally weighted MP yielded six trees with a length of 8990 steps, consistency index (CI) = 0.259, retention index (RI) = 0.519, and rescaled consistency index (RC) = 0.134. The strict consensus tree is shown in Fig. 2. For BI, the likelihood values of the 50% majority consensus tree was lnL = −39550.70. The standard deviation of split frequencies among the four BI runs (Fig. 1) was 0.002716. Although the BI and MP trees differed for some taxa, such as the placement of *Theloderma moloch*, nodes highly supported by BBS and BPP were almost identical. Among well support nodes, only the positions of the clade of *T. asperum* from Hainan and Yunnan, and a clade consisting of *T. rhododiscus* and *T. corticale* differed. In the BI tree, these two clades formed a sister group (BPP = 99). In the MP tree, the clade consisting of *T. rhododiscus* and *T. corticale* formed a sister group with a clade consisting of *N. pictus* and *N. spinosus* (BBS = 82), and together they formed a sister group to a clade of *T. asperum* from Hainan and Yunnan (BBS = 87). Because the MP analysis did not well resolve basal dichotomies between deep clades, we used the BI tree (Fig. 1) for discussion.

The family Rhacophoridae was corroborated as being monophyletic, and with high support (BPP = 100; BBS = 92). A strongly supported dichotomy between the Buergeriinae and Rhacophorinae was obtained (BPP = 100; BBS = 99). A total of 12 major clades (A–L) were recovered in all analyses (Figs. 1 and 2) and these can be summarized in seven major groupings as follows:

(1) Well-supported clade K (Fig. 2) (BPP = 100; BBS = 100) contained *Philautus ocellatus*, *P. hainanus*, and the type species of *Liuixalus*, *L. romeri*, which was the sister clade to the remaining rhacophorids (BPP = 96), excluding *Buergeria*.
(2) A well-supported clade (A+B) consisting of *Nyctixalus* (Clade A) and *Theloderma* (Clade B) was the sister clade to all other rhacophorines except for those in clade (K) (BPP = 96).
(3) Clade C included *Philautus banaensis*, *K. hainanus*, *K. odontotarsus*, *K. idiootocus*, and the type species of *Kurixalus*, *K. eiffingeri* (BPP = 100; BBS = 100). Clade C was the sister clade to Clade D (BPP = 100; BBS = 94) consisting of *Philautus longchuanensis, P. gryllus, Ki. charius, Ki. menglaensis, Ki. wynaadensis,* and the type species of *Kirtixalus, Ki. microtympanum* (BPP = 99).
(4) *Theloderma moloch* was the sister taxon (BPP = 93) to Clade E (BPP = 100; BBS = 100) consisting of *P. abditus*, *P. acutirostris*, and *P. surdus*. These clades were united with Clade C and Clade D but without nodal support. In turn, Clade E, Clade C, Clade D and *T. moloch* formed the sister group of Clade F, Clade G, Clade H, Clade I, and Clade J (BPP = 99).
(5) Clade F contained *Philautus quyeti*, *G. carinensis*, *G. jinxiuensis*, *Gracixalus* sp. and the type species of *Gracixalus*, *G. gracilipes* (BPP = 100; BBS = 96). Clade F was resolved as the sister group of a united Clade G, Clade H, Clade I and Clade J (BPP = 99). Within Clade F, "*Aquixalus odontotarsus*", *G. carinensis* and *G. jinxiuensis* from Vietnam were united (BPP = 100; BBS = 100), and together they constituted a clade with *G. jinxiuensis* from the type locality (Jiuxiu, Guangxi) and a new species (BPPv100; BBS = 100). *Philautus quyeti* formed a sister taxon relationship (BPP = 100; BBS = 97) with the clade (BPP = 100; BBS = 100) consisting of *G. gracilipes* from China and Vietnam.
(6) The Chinese and Vietnamese populations of *Feihyla palpebralis* formed Clade I (BPP = 100; BBS = 92).

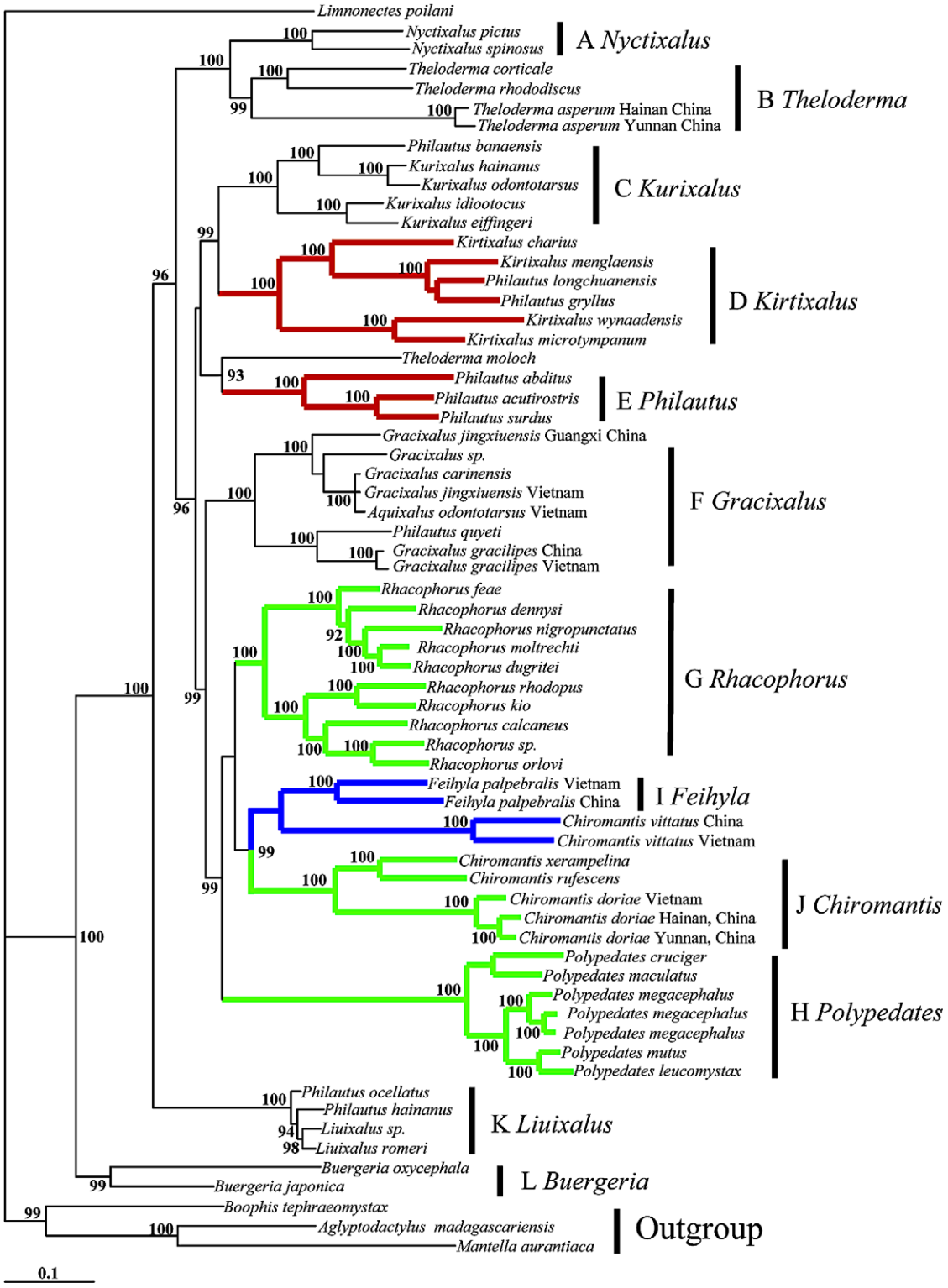

Fig. 1. Bayesian inference tree derived from partial fragments of 12S and 16S ribosomal DNA genes together with the complete t-RNA for valine. Numbers above the lines or besides the nodes are given as Bayesian posterior probabilities (⩾90 retained)/bootstrap support for maximum likelihood analyses (⩾50 retained); "- -" represents Bayesian posterior probabilities and bootstrap values lower than 90% and 50%, respectively. Species with terrestrial direct development, foam nesting, or laying eggs in a jelly with some bubbles are marked with a thick red bar, a thick green bar, or a thick blue bar, respectively. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

(7) *Chiromantis doriae* from China and Vietnam formed a monophyletic clade (BPP = 100; BBS = 100), and together formed a strongly supported Clade J with a clade consisting of *C. rufescens* and *C. xerampelina* (BPP = 100; BBS = 98). *Chiromantis vittatus* from China and Vietnam constituted a monophyletic clade (BPP = 100; BBS = 100), and together clustered with Clade I (*F. palpebralis*) but with low nodal support (BBS = 62).

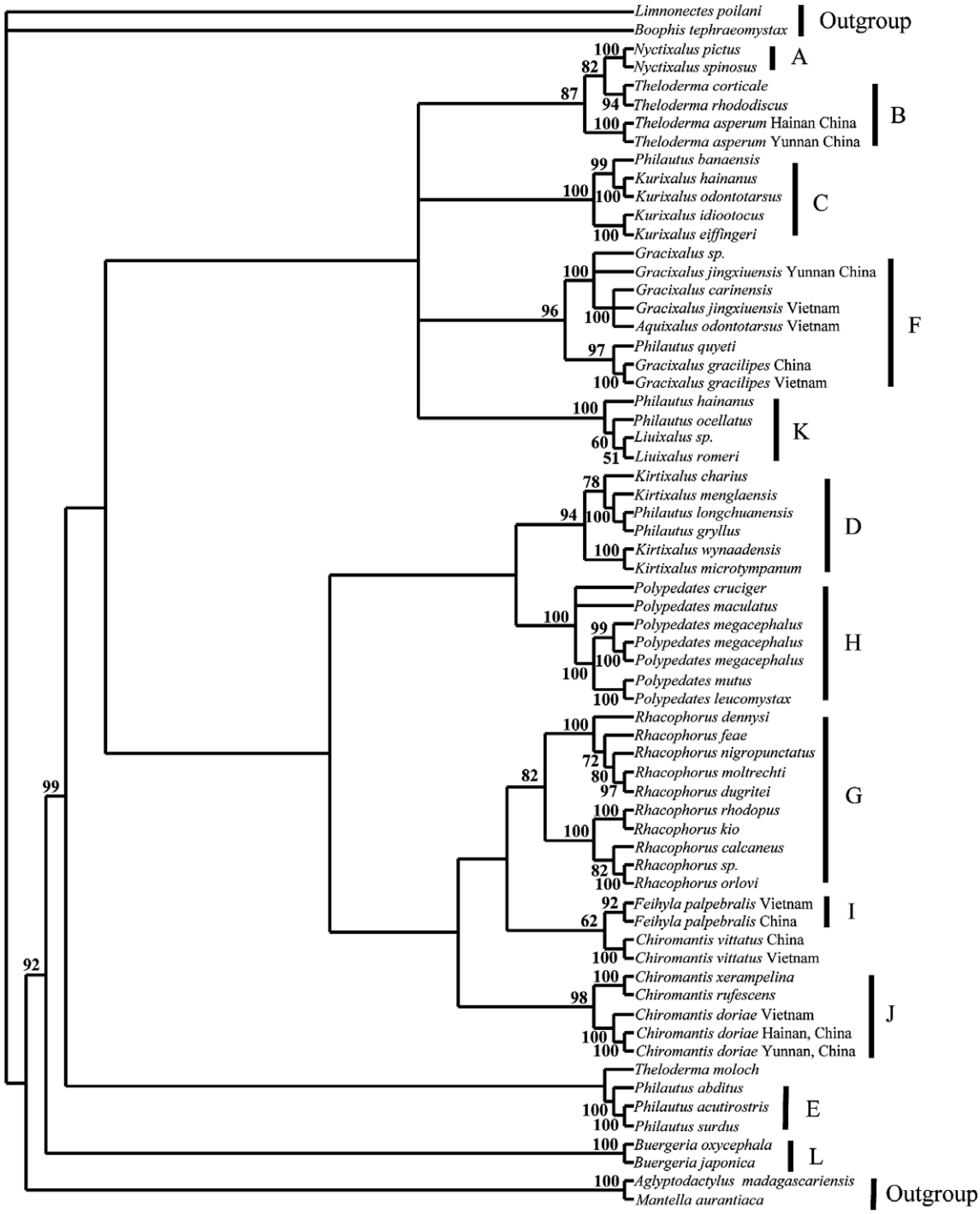

Fig. 2. Maximum parsimony tree derived from partial fragments of 12S and 16S ribosomal DNA genes together the complete t-RNA for valine. Numbers above the lines or besides the nodes are bootstrap support for maximum parsimony analyses (1000 replicates, ⩾50 retained) and "- -" represents a bootstrap proportion lower than 50%.

3.2.2. Nuclear genes (BDNF+POMC+RAG-1+RHOD+TYR)

A total evidence strategy (Kluge, 1989), involving all nuDNA sequence data combined unconditionally, was used in the analysis of the nuDNA sequence data due to the limited number of potentially informative characters in each gene. Phylogenetic reconstructions were performed using partitioned BI and ML analyses. The topologies of the ML and BI trees were almost identical. For BI, the likelihood value was lnL = −17554.61. The standard deviation of split frequencies among the four BI runs (Fig. 3) was 0.003262. Both bootstrap values from partitioned ML and the BPPs are represented on the BI tree (Fig. 3).

Consistent with the results of the mtDNA data, monophyly of the Rhacophoridae and the dichotomy between the Buergeriinae and Rhacophorinae were strongly supported. *Liuixalus* (Clade K) was strongly recovered as the sister group to all remaining rhacophorines (BPP = 100/BBS = 100). Within the Rhacophorinae, we recovered three additional major groups. Group I included the genera *Gracixalus*, *Philautus*, *Kurixalus* and *T. moloch* (BPP = 95/BBS = 50). *Kurixalus* (Clade C) and *Kirtixalus* (Clade D) were sister groups (BPP = 100/BBS = 79), and together formed a sister clade to *T. moloch* (BPP = 100/BBS = 57). In the BI tree, this clade formed a polytomy with *P. abditus* and the representative species of *Gracixalus*. In con-

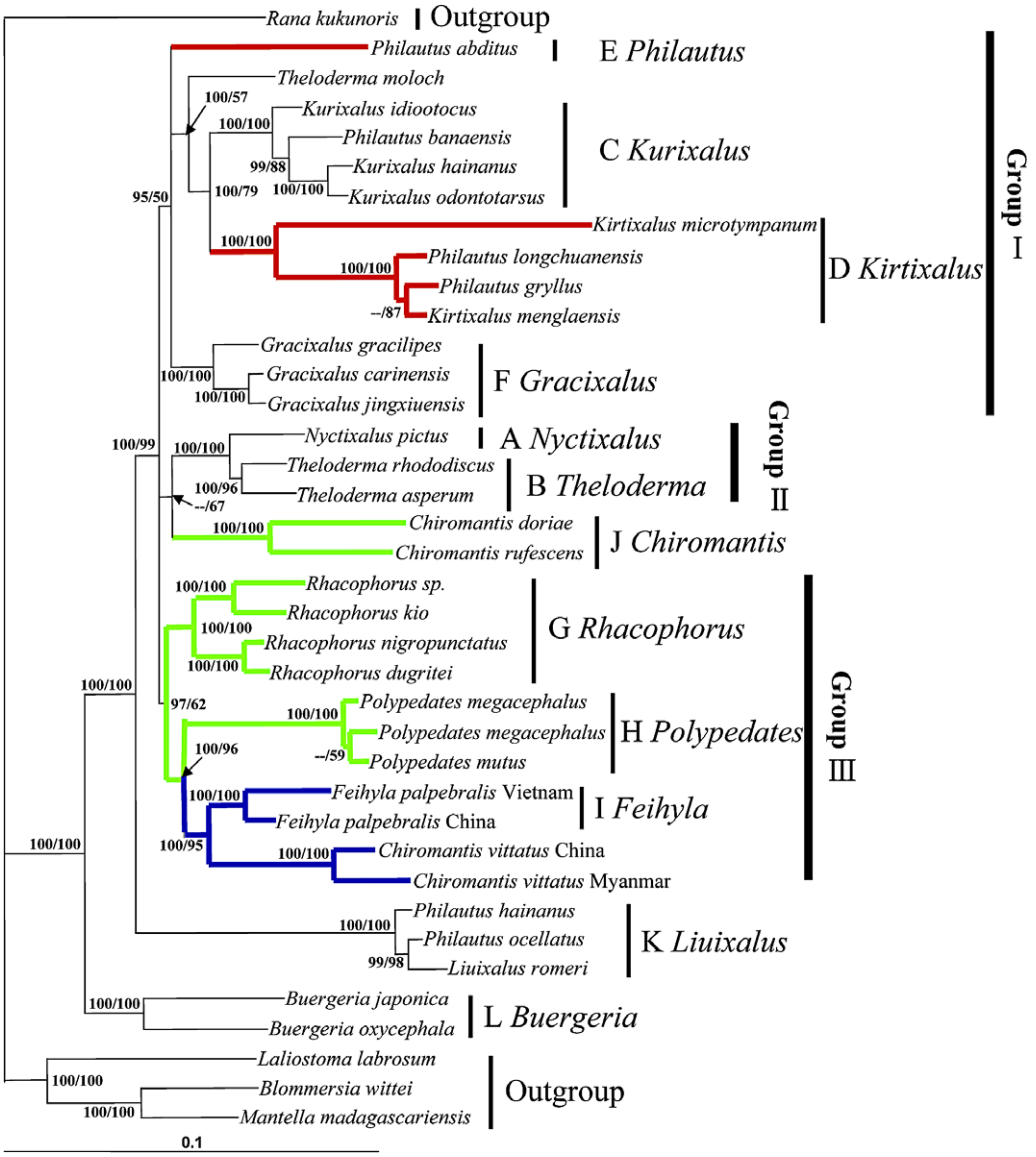

Fig. 3. Partitioned Bayesian inference tree derived from five combined nuclear fragments (BDNF+POMC+RAG-1+RHOD+TYR). Bayesian posterior probabilities (⩾90 retained) and bootstrap support from partitioned maximum likelihood (100 replicates) (⩾50 retained) are shown at the nodes, respectively; "- -" represents Bayesian posterior probabilities and bootstrap values from partitioned ML lower than 90% and 50%, respectively. Species with terrestrial direct development, foam nesting or laying eggs in a jelly with some bubbles are marked with a thick red bar, a thick green bar or a thick blue bar, respectively. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

trast, the ML analysis united *T. moloch*, *Kurixalus* (Clade C) and *Kirtixalus* as the sister group of *P. abditus* (BBS = 63), and together they formed with a sister group of *Gracixalus*.

Group II contained most species of *Theloderma* (Clade B) and its sister group *Nyctixalus* (Clade A) (BPP = 100/BBS = 100). Group III included representatives of *Rhacophorus*, *Polypedates*, *Feihyla* and *Chiromantis vittatus* (BPP = 97/BBS = 62). *Feihyla* (Clade I) was sister to a clade comprised of *C. vittatus* from China and Myanmar (BPP = 100/BBS = 95). Together they formed a clade with *Polypedates* (Clade H) (BPP = 100/BBS = 96). *Rhacophorus* (Clade G) formed the sister group of a large clade consisting of *C. vittatus*, *Feihyla* (Clade I) and *Polypedates* (Clade H) (BPP = 97/BBS = 62).

3.2.3. The combined mitochondrial and nuclear genes

The mtDNA and nuDNA data were combined to reconstruct the phylogeny of the family Rhacophoridae. We only used the complete mtDNA and nuDNA data for analysis, and the results are largely similar between mtDNA (Figs. 1 and 2) and nuDNA (Fig. 3) trees (data not shown). Uniquely, *Chiromantis doriae* and *C. vittatus* were recovered as sister species in the combined analyses, which was not consistent with either the nuDNA or the mtDNA analyses. In addition, Gracixalus formed a well-supported sister group relationship to a clade consisting of *Rhacophorus*, *Polypedates*, *Feihyla* and *Chiromantis*, and with a high BPP, which was congruent with the mtDNA result but conflicted with the nuDNA analysis.

The partition homogeneity test discovered significant incongruence between the mtDNA and nuDNA data ($P = 0.01$). Therefore, we considered the nuDNA and mtDNA trees separately.

4. Discussion

Our study contained, for the first time, a large data set of single-copy protein-coding nuclear genes to assess the phylogeny of the family Rhacophoridae. Our taxon sampling included all relevant

clades identified by our current mtDNA data and previous studies (Grosjean et al., 2008; Li et al., 2008; Wilkinson et al., 2002; Yu et al., 2008, 2009), except for the newly described *Ghatixalus* by Biju et al. (2008).

4.1. Phylogeny of the Rhacophoridae

Our newly collected nuDNA data better resolved the basal relationships among rhacophorids than either our mtDNA data alone or previous studies (Frost et al., 2006; Grosjean et al., 2008; Li et al., 2008; Wilkinson and Drewes, 2000; Wilkinson et al., 2002; Yu et al., 2008, 2009). The nuDNA tree strongly supported the monophyly of the Rhacophoridae with two inclusive subfamilies: Buergeriinae and Rhacophorinae. This finding was concordant with prior molecular and morphological studies (Biju et al., 2008; Channing, 1989; Frost et al., 2006; Grosjean et al., 2008; Li et al., 2008; Wilkinson and Drewes, 2000; Wilkinson et al., 2002; Yu et al., 2009) and our mtDNA data. Both the nuDNA and mtDNA data confirmed that *Liuixalus*, elevated by Li et al. (2008), was the sister group to the remaining nine rhacophorine genera, as previously proposed (Li et al., 2008; Yu et al., 2009). Our nuDNA data resolved three additional major groups of rhacophorines (Fig. 3: Group I, II, III). Group I was not resolved in previous studies (Frost et al., 2006; Grosjean et al., 2008; Li et al., 2008; Wilkinson et al., 2002; Yu et al., 2008, 2009). Regardless, many sets of relationships within these three groups were not resolved. The possibility of a rapid species radiation should be investigated in future studies.

4.1.1. Phylogeny of Philautus, Kirtixalus, Kurixalus and Gracixalus

Wilkinson et al. (2002) suggested that *Philautus* and *Kurixalus* were sister taxa, but this association received weak support. Their suggestion was consistent with the recent study by Biju et al. (2008). Including genus *Kirtixalus*, Yu et al. (2009) supported a clade appearing as (*Philautus* (*Kurixalus*, *Kirtixalus*)) but with only a moderate BPP. Li et al. (2008) found that *Philautus* and the clade (*Kurixalus*, *Kirtixalus*) formed a polytomy. Our nuDNA phylogeny supported the monophyly of Group I, consisting of *Philautus*, *Kirtixalus, Kurixalus*, *Gracixalus* and *Theloderma moloch*. Based on similar results from others (Grosjean et al., 2008; Li et al., 2008; Meegaskumbura et al., 2002), Yu et al. (2009) raised the subgenus *Kirtixalus* to the level of genus. Herein, the nuDNA phylogeny strongly supported that *Kirtixalus* was more closely related to *Kurixalus* than to *Philautus*. Our nuDNA tree depicted that *Gracixalus* and *Philautus* formed the sister group to the clade consisting of *Kirtixalus, Kurixalus* and *Theloderma moloch* (Group I) with moderate BPP and ML BBS support. This result conflicted with our mtDNA phylogeny (Figs. 1 and 2) and previous studies (Frost et al., 2006; Grosjean et al., 2008; Li et al., 2008; Yu et al., 2009). Without the representatives of *Philautus* (the only representative of *Philautus* turns out to be a *Theloderma*) and *Kirtixalus*, Frost et al. (2006) considered that *Gracixalus* was the sister taxon to all other rhacophorines. Grosjean et al. (2008), Li et al. (2008), and Yu et al. (2009) considered that *Gracixalus* was the sister group of a clade formed by *Rhacophorus*, *Polypedates*, *Feihyla*, and *Chiromantis*. Owing to the absence of strong support, Yu et al. (2008) suggested that further study was required before committing to its phylogenetic placement. We prefer the nuDNA results (Fig 3) for the following reasons. Both *G. gracilipes* and *G. jinxiuensis* have been long placed in *Philautus* (Group I), and were recently transferred to *Gracixalus* (Delorme et al., 2005; Li et al., 2008; Yu et al. (2009)). Their placement in *Philautus* indicated similar morphological characters, including body size (Group I: 10–30 mm; Group II: 40–100 mm, habitat (Group I: usually live in undershrub or shrub; Group II: usually live in the tree canopy), dorsal color pattern (Ziegler et al., 2006 considered that the color pattern of *G. jinxiuensis* was present in other members of the genera *Philautus* and "*Aquixalus*", but never in *Rhacophorus*), and reproductive mode (see below).

Presently, *Philautus*, containing approximately 150 species, is widely distributed in South and Southeast Asia (Frost, 2009). Because this genus is a species-rich group of small frogs sometimes with highly variable coloration (Bossuyt and Dubois, 2001; Inger et al., 1984), it is considered as a particularly difficult group for taxonomists (Biju and Bossuyt, 2006). Our nuDNA and mtDNA data suggest that *Philautus* is polyphyletic, forming four clades (C, D, E, and K) and five clades (C, D, E, K and F), respectively. Clade E is likely to be the *Philautus* sensu stricto. It contains *P. acutirostris*, which is considered closely related to the type species, *P. aurifasciatus* (Dring, 1987; Inger, 1966). This finding is consistent with previous studies (Li et al., 2008; Wilkinson et al., 2002; Yu et al., 2009). In our mtDNA study, *P. abditus* from Vietnam nests well within Clade E. Our nuDNA and mtDNA data strongly support that *P. ocellatus* and *P. hainanus* form well supported Clade K with the type species of *Liuixalus*, *L. romeri*. However, *Liuixalus* is distantly related to Clade E (*Philautus*) (Fig. 3; Li et al., 2008; Yu et al., 2008, 2009). *Philautus banaensis* nests well within *Kurixalus* (Clade C), as strongly supported by our mtDNA and nuDNA data. Our phylogenies strongly support Clade D as containing *P. gryllus, P. longchuanensis* and *Kirtixalus menglaensis*, all within *Kirtixalus*. Our mtDNA phylogenies strongly support that the newly described *P. quyeti* (Truong et al., 2008) clusters in the genus *Gracixalus* (Clade F).

Recently, the subgenus *Kirtixalus* was raised to full genus level by Yu et al. (2009). According to the study by Grosjean et al. (2008), *Ixalus temporalis*, the type species of *Pseudophilautus* Laurent, 1943, and *Polypedates microtympanum*, the type species of *Kirtixalus* Dubois, 1987, formed a monophyletic clade with strong support. *Pseudophilautus* is an older generic name than *Kirtixalus*. Therefore, genus *Kirtixalus* is a junior synonym of *Pseudophilautus* Laurent, 1943.

In order to avoid polyphyly within *Philautus*, we recommend the following taxonomic transfers: (1) *Philautus ocellatus* and *P. hainanus* to be placed within *Liuixalus* as *L. ocellatus* (new combination) and *L. hainanus* (new combination), respectively. (2) *Philautus banaensis* be treated as a member of *Kurixalus* as *K. banaensis* (new combination). (3) *Philautus longchuanensis* and *P. gryllus* be transferred to the genus *Pseudophilautus* as *Ps. longchuanensis* (new combination) and *Ps. gryllus* (new combination), respectively. (4) *Philautus quyeti* be transferred to *Gracixalus* as *G. quyeti* (new combination). According to the phylogenies of Grosjean et al. (2008), Gururaja et al. (2007) and Meegaskumbura et al. (2002), the genus *Pseudophilautus* should include the species *P. schmarda* as *Ps. schmarda* (new combination), *P. femoralis* as *Ps. femoralis* (new combination), *P. signatus* as *Ps. signatus* (new combination), *P. annandali* as *Ps. annandali* (new combination), *P. parvulus* as *Ki. parvulus* (new combination), *P. griet* as *Ps. griet* (new combination), *P. temporalis* as *Ps. temporalis* (new combination), *P. nanus* as *Ps. nanus* (new combination) and *P. luteolus* as *Ps. luteolus* (new combination), in addition to *Ps. charius*, *Ps. wynaadensis, Ps. microtympanum* and *Ps. menglaensis* as proposed by Yu et al. (2009). Recently, many species of *Philautus* have been described from Sri Lanka and the Western Ghats of India (e.g., Gururaja et al., 2007; Manamendra-Arachchi and Pethiyagoda, 2005; Meegaskumbura and Manamendra-Arachchi, 2005). They may have close phylogenetic relationships (Grosjean et al., 2008; Meegaskumbura et al., 2002). Indian and Sri Lankan species of *Philautus* were considered to have experienced rapid morphological differentiation (Grosjean et al., 2008). Therefore, we provisionally suggest that these newly described species of *Philautus* should likely be transferred to the genus *Pseudophilautus*, pending more data.

Most Chinese species of *Philautus* have already been transferred to other genera, such as *Liuixalus, Gracixalus*, and *Pseudophilautus* (Li et al., 2008; Yu et al., 2008, 2009), except for *P. medogensis*

and *P. andersoni* (Fei et al., 2005; Frost, 2009; Zhao and Adler, 1993). Morphologically, *P. medogensis* is very similar to *Gracixalus jinxiuensis* (Ye and Hu, 1984) [as *P. jinxiuensis*], and they were assigned to the same species group (*P. jinxiuensis* group) (Fei, 1999; Fei et al., 2005). Therefore, we transfer *P. medogensis* to the genus *Gracixalus* as *G. medogensis* (new combination). *Philautus andersoni* was recognized as *P. tuberculatus* by Fei (1999) and Fei et al. (2005). However, the latter taxon has been treated as a junior synonym of *P. andersoni* (Dutta, 1997). *Philautus andersoni* is morphologically very similar to *P. albopunctatus* (Fei, 1999), and *P. albopunctatus* is now a junior synonym of *T. asperum* (Yu et al., 2008). Therefore, *P. andersoni* should be placed within the genus *Theloderma* as *T. andersoni* (new combination), although confirmation is desirable. Accordingly, the genus *Philautus* does not occur in China.

The following species of *Philautus* are currently known from Vietnam: *P. abditus*; *P. banaensis*; *P. gryllus*; *P. jinxiuensis*; *P. maosonensis*; *P. parvulus*; *P. quyeti* and *P. truongsonensis* (Frost, 2009; Orlov et al., 2008). Yu et al. (2009) transferred *P. jinxiuensis* into *Gracixalus*. All of these species have been transferred to either *Kurixalus* or *Pseudophilautus*, except for *P. abditus*, *P. maosonensis* and *P. truongsonensis*. We provisionally suggest retaining these three species in *Philautus* pending additional data. Therefore, in Vietnam the genus *Philautus* contains only three species: *P. abditus*, *P. maosonensis*, and *P. truongsonensis*.

The accuracy of a phylogeny depends upon the identification of the taxa. The sample of *K. odontotarsus* (as *Aquixalus odontotarsus*) from Vietnam used by Delorme et al. (2005) and Grosjean et al. (2008) was not found to be the same species as *K. odontotarsus* from the type locality (Yu et al., 2009). Our mtDNA trees suggest that the Vietnamese *K. odontotarsus, G. carinensis,* and *G. jinxiuensis* form a strongly supported clade, and the genetic distances (uncorrected *P* distances) of *G. carinensis* from *K. odontotarsus* and *G. jinxiuensis* are 0.8% and 0.4%, respectively. The average interspecific *P*-distance within the Rhacophoridae is 18.8%. Therefore, we suggest that the Vietnamese sample of *K. odontotarsus* used by Delorme et al. (2005) and Grosjean et al. (2008) is *G. carinensis*, as is *G. jinxiuensis* of Truong et al. (2008).

4.1.2. Phylogeny of Nyctixalus and Theloderma

Theloderma was shown to be paraphyletic in both the mtDNA and nuDNA results. Clade B included *T. corticale*, *T. rhododiscus*, and *T. asperum*, all of which have long been recognized as *Theloderma* sensu stricto (Frost et al., 2006; Grosjean et al., 2008; Li et al., 2008; Wilkinson et al., 2002; Yu et al., 2008, 2009). The monophyly of *Theloderma corticale*, *T. rhododiscus*, and *T. asperum* was strongly recovered in mtDNA BI and nuDNA analyses, but not in the mtDNA MP analysis. The artifact of long-branch attraction might result in the artificial clustering of *T. asperum*, *T. corticale*, *T. rhododiscus*, *N. pictus,* and *N. spinosus* in the mitochondrial MP analysis. This possibility needs to be investigated in future analyses.

Our study is the first to include *Theloderma moloch* in a phylogenetic analysis, a taxon whose generic placement is uncertain. Liem (1970) stated that *T. moloch* probably belongs to *Hazelia* (=*Nyctixalus*), but notes that further study is required. Dubois (1981) provisionally transfers *T. moloch* to *Nyctixalus*. However, this transfer is not recognized by Chinese taxonomists (Fei, 1999; Fei et al., 2005; Zhao and Adler, 1993). Our mtDNA results suggest that *T. moloch* is the sister group of *Philautus* but this association only received moderate BPP support and no support in MP; *T. moloch* did not nest with *Theloderma* sensu stricto. In contrast, our nuDNA phylogeny places *T. moloch* as the sister taxon of *Pseudophilautus* and *Kurixalus*, which is nested well within the clade consisting of *Philautus*, *Pseudophilautus, Kurixalus*, and *Gracixalus*. This association is strongly supported. Therefore, both mtDNA and nuDNA phylogenies indicate that the phylogenetic position of *T. moloch* is only remotely related to *Theloderma* sensu stricto (Clade B). However, the determination of whether *T. moloch* is a *Philautus* or represents a new genus will require further analysis, with greater taxon sampling and the inclusion of morphological characters.

Based on breeding behavior, Liem (1970) suggested that *Theloderma* may be closely related to *Nyctixalus*. These frogs lay small clutches of eggs above water-filled holes in tree trunks. Channing (1989), in a reanalysis of Liem's study, considered *Theloderma* and *Nyctixalus* to be sister taxa. Wilkinson and Drewes (2000) compared the two previous studies and produced a third phylogenetic hypothesis. They considered *Nyctixalus* to be the sister group of *Philautus*, which was relatively remotely related to *Theloderma*. Our nuDNA results strongly support the hypothesis of Channing (1989). *Theloderma* is distantly related to *Philautus*, as is consistent with our present mtDNA analysis and previous studies (Delorme et al., 2005; Frost et al., 2006; Grosjean et al., 2008; Li et al., 2008; Wilkinson et al., 2002; Yu et al., 2008).

4.1.3. Phylogeny of Rhacophorus, Polypedates, Feihyla, and Chiromantis

Both molecular (Frost et al., 2006; Wilkinson et al., 2002) and morphological (Wilkinson and Drewes, 2000) studies concluded that *Rhacophorus*, *Polypedates* and *Chiromantis* formed a monophyletic group. However, the representative species of *Chiromantis*, *C. rufescens* and *C. doriae*, were excluded from this group (Group III) in our nuDNA tree. Consequently, we tested the strength of the alternative arrangement. Monophyly of Group III including the genus *Chiromantis* cannot be rejected (AU, $P = 0.441$; KH, $P = 0.446$; TT, $P = 0.5127$).

We included the monotypic genus *Feihyla* in our analyses and our mtDNA and nuDNA BI analyses confirmed previous findings that *Feihyla* was a member of Group III (Delorme et al., 2005; Grosjean et al., 2008; Li et al., 2008; Yu et al., 2009). Our mtDNA analyses did not strongly resolve relationships among the genera in Group III, which was also consistent with previous studies (Grosjean et al., 2008; Li et al., 2008; Wilkinson et al., 2002). Grosjean et al. (2008) considered that the type species of *Feihyla*, *F. palpebralis* was part of *Chiromantis*, which was not consistent with the studies by Biju et al. (2008), Li et al. (2008) and Yu et al. (2009). The latter studies recognized the validity of *Feihyla*. Our nuDNA data suggested *Feihyla* was more closely related to *Polypedates* than to *Chiromantis* and this association had strong nodal support. This result was recovered with weak support by Yu et al. (2009). The grouping of *Feihyla* and *Polypedates* did not conflict with the results of Li et al. (2008), although they resolved *Polypedates*, *Rhacophorus*, and *Feihyla* as a polytomy.

The phylogenetic position of *Chiromantis vittatus* may be unique. Frost et al. (2006) placed *Chirixalus* into synonymy with *Chiromantis*, and as a result, *Chirixalus vittatus* and *Chirixalus doriae* were transferred to *Chiromantis* (Frost, 2009; Frost et al., 2006). However, this relocation of *C. vittatus* was uncertain (Frost et al., 2006; Grosjean et al., 2008; Wilkinson et al., 2002; Yu et al., 2008, 2009). Wilkinson et al. (2002) recovered *C. vittatus* as the sister taxon to *Polypedates*. Yu et al. (2008) considered it as the sister taxon of either *Feihyla palpebralis* (as *Philautus palpebralis*) or the *Rhacophorus* (=*Polypedates*) *leucomystax* group in their MP and ML analyses, respectively. Frost et al. (2006), Grosjean et al. (2008) and Yu et al. (2009) found that *C. vittatus* nested within *Chiromantis*. Herein, our analysis of the mtDNA data removed *C. vittatus* from *Chiromantis* with moderate support in MP yet low nodal support in BI (Figs. 1 and 2). Our nuDNA data strongly supported *C. vittatus* as the sister taxon of *Feihyla*, remotely from the representatives of *Chiromantis* (Fig. 3). Liem's (1970) morphological study found that *C. vittatus* was externally very similar to *C. doriae*, and the latter has been consistently placed within *Chiromantis* (Frost et al., 2006; Li et al., 2008; Yu et al., 2009). In contrast, Wilkinson and Drewes (2000) found that *C. vittatus* differed from *C. doriae* in five morpho-

logical characters; they stated that the inclusion of *C. vittatus* could collapse the "*Chirixalus*" node. Significantly, the reproductive mode of *C. vittatus* differs from other species of *Chiromantis* (see below). Thus, morphological, molecular, and reproductive characters indicate that *C. vittatus* should not be placed in genus *Chiromantis* and that it may represent either a new genus or a member of *Feihyla*. However, we currently refrain from making any transfer, awaiting further study with greater taxon sampling and morphological character coding.

4.2. Validity of the tribes Nyctixalini and Rhacophorini

Based on fragments of about 1300 bp of mtDNA and 300 bp nuDNA, Grosjean et al. (2008) erected a new tribe Nyctixalini, consisting of *Theloderma* and *Nyctixalus*. The tribe was diagnosed by one morphological synapomorphy, the presence of numerous dense glands of varying sizes on the eyelids. Our mtDNA and nuDNA trees strongly supported the monophyly of this clade, the tribe Nyctixalini. Consistent with the studies by Biju et al. (2008), Grosjean et al. (2008) and Yu et al. (2009), our mtDNA BI tree also supported the recognition of the tribe Rhacophorini with moderate Bayesian support. Although monophyly of the Rhacophorini was not supported by the nuDNA data alone (Fig. 3), the total evidence tree (not shown) supported the tribe, exclusive of *Liuixalus*. In addition to the genera *Rhacophorus*, *Polypedates*, *Philautus*, *Kurixalus* (*Aquixalus* as a junior synonym of *Kurixalus*) and *Chiromantis*, referred to by Grosjean et al. (2008), the Rhacophorini should also include *Feihyla, Gracixalus*, *Pseudophilautus*, and new erected *Ghatixalus* (not studied by us). Within the Rhacophorinae, our nuDNA and mtDNA BI trees strongly place *Liuixalus* as the sister group to all other genera, and thus the sister group to the Nyctixalini and Rhacophorini. Because taxonomy should reflect historical relationships, the genus *Liuixalus* must be placed in a new tribe, herein named as Liuixalini new taxon, with the type genus *Liuixalus* (Li et al., 2008).

4.3. The evolution of reproduction in the Rhacophoridae

Direct development, i.e. embryonic and larval development taking place within the eggs without a free larval stage in water, is a remarkable reproductive feature. Traditionally, within the Rhacophoridae, it served to diagnose the genus *Philautus* (e.g., Bossuyt and Dubois, 2001; Dring, 1979). Yu et al. (2009) suggested that direct development has evolved independently within the genus. Based on nuDNA and mtDNA results, we further confirm that the species of *Pseudophilautus*, which undergo direct development (Bahir et al., 2005), is the sister group to species of *Kurixalus*, which have a typical aquatic larval tadpole (Kam et al., 1996; Kuramoto and Wang, 1987). Direct development also occurs in *Philautus*, but this genus is not the sister group of *Pseudophilautus*. Furthermore, all species within the Rhacophoridae except for *Philautus* and *Pseudophilautus* do not undergo direct development, such as *Theloderma moloch* (Liem, 1970) and *Liuixalus romeri* (Smith, 1953). Therefore, the phylogenetic relationships suggest that terrestrial direct development evolved twice in the Rhacophoridae.

Another interesting reproductive mode within the Rhacophoridae is the deposition of eggs in self-produced foam nests. This character is shared by three genera *Rhacophorus*, *Polypedates*, and *Chiromantis* (Grosjean et al., 2008; Wilkinson et al., 2002). Wilkinson et al. (2002) and Grosjean et al. (2008) suggested that foam nesting evolved only once in this family. However, Grosjean et al. (2008) found that *C. vittatus*, which does not make foam nests, nested within the foam nesting clade. *Feihyla palpebralis* lays eggs in a jelly containing some bubbles (direct observation in the field), which is similar to *C. vittatus*. Our phylogeny recovered a clade with both *C. vittatus* and Chinese and Vietnamese *F. palpebralis*. This clade formed the sister group to the foam nesting clade. Therefore, we consider that foam nesting evolved only once in the family Rhacophoridae, and laying eggs in a jelly containing some bubbles is another specialized method of foam nesting. Significantly, if *C. vittatus* is a member of *Feihyla*, this reproductive mode likely serves as a behavioral synapomorphy to diagnose *Feihyla*, and future behavioral observations alone may serve to transfer additional species into the genus.

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