

Underestimated species diversity within the *Rhacophorus rhodopus* and *Rhacophorus bipunctatus* complexes (Anura, Rhacophoridae), with a description of a new species from Hainan, China

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Abstract

Taxonomy and species boundaries within the *Rhacophorus rhodopus* and *Rhacophorus bipunctatus* complexes are very confusing. In this study, we attempt to delimit the species boundaries and test the currently accepted taxonomic assignments within these two complexes based on newly collected samples and previously published data across their distributions. Phylogenetic analyses revealed that the *R. rhodopus* and *R. bipunctatus* complexes consisted of six distinct clades (labeled A–F) that diverged from each other by genetic distances (p-distance) ranging from 5.3% to 9.2% in 16S rRNA sequences, and accordingly analyses of species delimitation placed them into six species, of which three correspond to known species (*R. rhodopus*, *R. bipunctatus*, and *R. napoensis*) and three represent different cryptic species. *Rhacophorus rhodopus* (Clade C) is distributed in southern Yunnan, China, northern Laos, and northern and central Thailand; *R. bipunctatus* (Clade F) is distributed in northeastern India and western and northern Myanmar; and *R. napoensis* (Clade B) is distributed in Guangxi, China and northern Vietnam. Based on both molecular and morphological evidence, we described the clade consisting of samples from Hainan, China and central Vietnam (Clade A) as a new species, *Rhacophorus qiongica* sp. nov. There are two cryptic species requiring additional morphological studies: one only contains samples from Motuo, Xizang, China (Clade E), and the other is distributed in western Yunnan, China, central Myanmar, central Thailand, and Malaysia (Clade D). Additionally, our results supported the idea that some old GenBank sequences of *R. reinwardtii* need to be updated with the correct species name.

Key Words

Cryptic species, Hainan, *Rhacophorus qiongica* sp. nov., Species complex, Species delimitation

Introduction

Rhacophorus Kuhl & Van Hassalt, 1822, a genus of the family Rhacophoridae that originated ca. 19.3–33.0 million years ago (O’Connell et al. 2018; Chen et al. 2020; Ellepola and Meegaskumbura 2023), is widely distributed in India, Bhutan, China (Xizang, Yunnan, Guangxi,

Hainan), Myanmar, Thailand, Laos, Cambodia, Vietnam, Indonesia (Sumatra, Sulawesi), Philippines, and Kalimantan (Frost 2023). It is characterized by medium or large body size, intercalary cartilage between the end of the finger and penultimate phalanges of digits, Y-shaped distal end of terminal phalanx, tip of digits expanded into rounded disks with circum-marginal grooves, web

between fingers, horizontal pupil, skin not co-ossified to skull, absence of upper eyelid projections and presence of tarsal projections in most species, extensive dermal folding usually on forearm and tarsus, anal folds, and brown or green dorsal color (Li et al. 2012; Pan et al. 2017; Jiang et al. 2019), and currently it contains 43 species excluding *Rhacophorus verrucopus* Huang, 1983, which was considered a synonym of *Rhacophorus tuberculatus* Anderson, 1871 by Che et al. (2020). In China, there are eight *Rhacophorus* species, namely *Rhacophorus bipunctatus* Ahl, 1927; *Rhacophorus kio* Ohler & Delorme, 2006; *Rhacophorus laoshan* Mo, Jiang, Xie & Ohler, 2008; *Rhacophorus napoensis* Li, Liu, Yu & Sun, 2022; *Rhacophorus orlovi* Ziegler & Köhler, 2001; *Rhacophorus rhodopus* Liu & Hu, 1960; *Rhacophorus translineatus* Wu, 1977; and *Rhacophorus tuberculatus* (Anderson, 1871) (AmphibiaChina 2023).

Rhacophorus rhodopus is mainly distributed in north-eastern India to Myanmar (Kachin State, Shan State), Thailand, Laos, Vietnam (Lao Cai, Ha Tinh, Bac Giang, Quang Binh, Lai Chau, Quang Tri, Thua Thien Hue, Kon Tum, Gia Lai, Lam Dong, and Dong Nai), southern China (southeast Tibet, southern Yunnan, northeastern Guangxi, Hainan), and Peninsular Malaysia (Frost 2023). This species was originally described by Liu and Hu (1960) based on specimens from Mengyang, Yunnan, China. It is characterized by reddish-brown dorsal color, pointed snout, smooth head skin, black spots at axillary region, and bright scarlet webs, resembling *R. bipunctatus*, a species originally discovered from north-eastern India (type locality: Khasi Hills) and later widely recorded from Bangladesh (e.g. Reza and Mukul 2009; Hakim et al. 2020), Cambodia (e.g. Ohler et al. 2002; Stuart and Emmett 2006; Neang and Holden 2008), Thailand (Taylor 1962; Chan-ard 2003; Chan-ard et al. 2011), Malaysia (Leong and Lim 2003; Grismer et al. 2006), Vietnam (Nguyen et al. 2005; Bain et al. 2007), Laos (Stuart 2005), Myanmar (Zug and Mulcahy 2020; Zug 2022), and China (Fei 1999; Fei et al. 2004, 2009, 2010; Che et al. 2020).

The disputes over the taxonomy of *R. rhodopus* and *R. bipunctatus* have been going on for many years. Inger et al. (1999) compared *R. bipunctatus* from northern and central Vietnam with *R. rhodopus* and concluded that the two species are synonymous. However, Bordoloi et al. (2007) considered that this conclusion is not reliable owing to the fact that Inger et al. (1999) did not compare the specimens from Vietnam with topotypes of *R. bipunctatus*, and suggested that records of *R. bipunctatus* from Thailand and Vietnam actually refer to *R. rhodopus*. Nguyen et al. (2008) also suggested that all records of *R. bipunctatus* in Vietnam should be classified as *R. rhodopus*. Fei et al. (2009) considered that *R. rhodopus* from the type locality obviously differs from *R. bipunctatus* from northern India in body size and color pattern, so they suggested maintaining the validity of *R. rhodopus* and considered that *R. bipunctatus* from Vietnam is more similar to *R. rhodopus* from China.

Analysis of molecular data can more accurately test the taxonomic hypothesis based on morphology (Jablonski and Finarelli 2009). Previous molecular phylogenetic analyses have demonstrated that the taxonomy of *R. rhodopus* and *R. bipunctatus* complexes (Chan et al. 2018) is complicated. Yu et al. (2007, 2008) found that *R. rhodopus* is not monophyletic, and *R. rhodopus* from Hainan, China is closer to *R. bipunctatus* collected from Vietnam. Li et al. (2012) found that samples of *R. rhodopus* from Hainan, China and Vietnam form a clade that did not cluster together with the clade containing *R. rhodopus* from the type locality, indicating that *R. rhodopus* from Hainan, China and Vietnam likely represents a cryptic species. Nguyen et al. (2014) also revealed that *R. rhodopus* is not monophyletic since *R. rhodopus* from Vietnam did not cluster together with the clade of samples from Yunnan and Malaysia. Dang et al. (2015) revealed two distinct lineages within *R. rhodopus* from Yunnan and considered that one of them could be a cryptic species. Moreover, Chan et al. (2018) suggested tentatively moving Malaysian *R. bipunctatus* to *R. rhodopus* and revealed that *R. rhodopus* from Vietnam is composed of two distinct clades, one only containing samples from Vietnam and one containing samples from Vietnam and Hainan, China. Over all, these previous molecular phylogenetic analyses revealed that *R. bipunctatus* and *R. rhodopus* represent two complexes (Chan et al. 2018), and at least two cryptic species may exist in the *R. rhodopus* complex: one occurs in Hainan, China and Vietnam, and one is only known from Vietnam. Recently, Li et al. (2022) described a new species resembling *R. rhodopus* from Guangxi, China (*R. napoensis*). This finding raises another question. That is, whether *R. napoensis* is conspecific with one of the two potential cryptic species within the *R. rhodopus* complex mentioned above. Additionally, Li et al. (2012) and Che et al. (2020) found that *R. bipunctatus*, which is likely restricted to northeastern India, Myanmar, and Xizang, China (Fei et al. 2009; Chan et al. 2018; Poyarkov et al. 2021), is not monophyletic since samples from Motuo, Xizang, China formed a clade whereas samples from northern Myanmar formed another clade. Because no samples from India were included in these two studies, it is necessary to investigate which of these two clades represents the true *R. bipunctatus* by employing *R. bipunctatus* samples from northeastern India.

In this study, we attempt to delimit the species boundary and test the currently accepted taxonomic assignments within *R. rhodopus* and *R. bipunctatus* complexes based on newly collected and previously sequenced samples across their distributions. Our results revealed that there are probably three cryptic species, and one of them was described as a new species herein based on morphological and molecular evidence. The samples from northern Vietnam belong to *R. napoensis*, and populations from central Vietnam are conspecific with the new species described here.

Materials and methods

Sampling

This study was carried out in accordance with the ethical guidelines issued by the Ethics Committee of Guangxi Normal University (permit number: GXNU-202308-010). A total of 58 individuals of *R. rhodopus* and *R. bipunctatus* complexes collected from 33 sites across China, Vietnam, Laos, Thailand, Malaysia, Myanmar, and India (Fig. 1) were included in this study. Of the 58 samples, 18 were collected and sequenced by this study, and the homologous sequences of the other 40 individuals were downloaded from GenBank (Table 1). All newly collected specimens in this

study were deposited at Guangxi Normal University (GXNU). *Rhacophorus norhayataiae* Chan & Grismer, 2010, *Rhacophorus reinwardtii* (Schlegel, 1840), *Rhacophorus borneensis* Matsui, Shimada & Sudin, 2013, *Rhacophorus helenae* Rowley, Tran, Hoang & Le, 2012, *R. kio*, *Rhacophorus lateralis* Boulenger, 1883, and *Rhacophorus nigropalmatus* Boulenger, 1895 were included in this study, and *Leptomantis gauni* (Inger, 1966), *Zhangixalus smaragdinus* (Blyth, 1852), *Buergeria buergeri* (Temminck & Schlegel, 1838), *Kurixalus idiootocus* (Kuramoto & Wang, 1987), *Chiro-mantis rufescens* (Günther, 1869), *Nyctixalus pictus* (Peters, 1871), and *Theloderma albopunctatum* (Liu & Hu, 1962) were selected as the outgroup according to Yu et al. (2019) and Li et al. (2022).

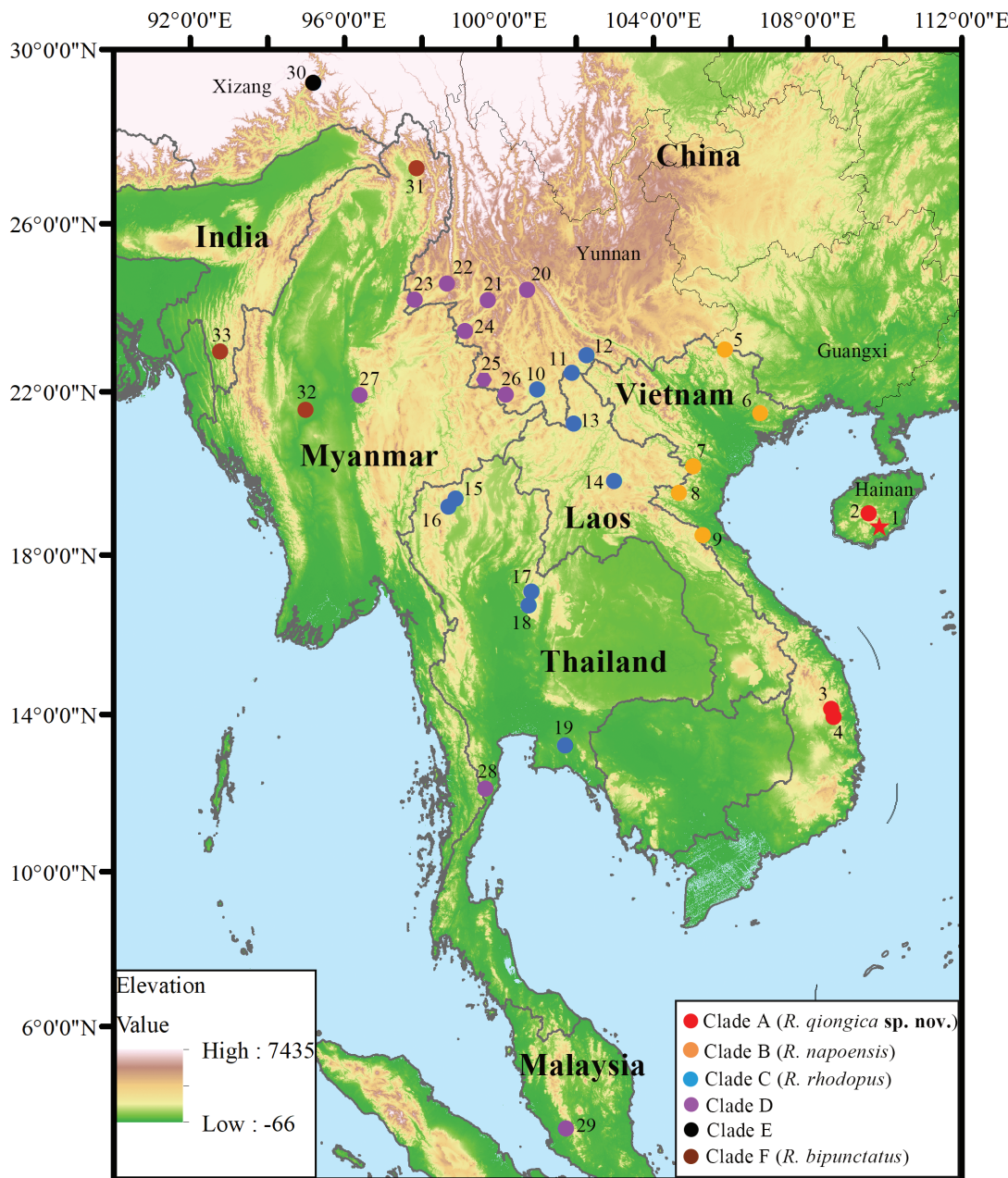


Figure 1. Map showing the collection sites of samples of the *R. rhodopus* and *R. bipunctatus* complexes used in this study. Sites are labeled as in Table 1, and the star represents the type locality of *R. qiongica* sp. nov. in Hainan, China.

Table 1. Species used in phylogenetic analyses of this study.

Species	Voucher	Locality (ID)	Accession No.
<i>Buergeria buergeri</i>	TTU-R-11759	Japan	AF458122
<i>Nyctixalus pictus</i>	FMNH 231094	Lahad Datu, Sabah, Malaysia	AF458135
<i>Theloderma albopunctatum</i>	ROM 30246	Vietnam	AF458148
<i>Chiromantis rufescens</i>	CAS 207601	Bioko Norte Province, Equatorial Guinea	AF458126
<i>Kurixalus idiootocus</i>	CAS 211366	Taipei, Taiwan, China	AF458129
<i>Zhangixalus smaragdinus</i>	HMO5292	Xima, Yingjiang, Yunnan, China	MN613221
<i>Leptomantis gauni</i>	FMNH 273928	Bintulu, Sarawak, Malaysia	JX219456
<i>Rhacophorus borneensis</i>	BORN 22411	Sabah, Maliau Basin, Malaysia	AB781694
<i>R. helenae</i>	UNS 00451	Binh Thuan, Vietnam	JQ288090
<i>R. kio</i>	KUHE 55165	Xuan Lien, Than Hoa, Vietnam	AB781695
<i>R. lateralis</i>	-	Mudigere, India	AB530548
<i>R. nigropalmatus</i>	Rao081204	Malaysia	JX219437
<i>R. norhayatieae</i>	NNRn	Johor, Endau Rompin, Malaysia	AB728191
<i>R. reinwardtii</i>	NMBE 1056517	Batang Ai NP, Sarawak, Malaysia	JN377366
<i>R. reinwardtii</i>	Rao081205	Malaysia	JX219443
<i>R. reinwardtii</i>	ENS 16447 (UTA)	Sumatra, Bandung, Indonesia	KY886335
<i>R. reinwardtii</i>	ENS 16179 (UTA)	Java, Patuha, Indonesia:	KY886328
<i>R. qiongica</i> sp. nov.	GXNU HN110501	Diailuo Mountain, Hainan, China (1)	OP740711
<i>R. qiongica</i> sp. nov.	GXNU HN110502	Diailuo Mountain, Hainan, China (1)	OP740712
<i>R. qiongica</i> sp. nov.	GXNU HN110503	Diailuo Mountain, Hainan, China (1)	OP740713
<i>R. qiongica</i> sp. nov.	GXNU YU000691	Yinggeling, Hainan, China (2)	PP115440
<i>R. qiongica</i> sp. nov.	GXNU YU000693	Yinggeling, Hainan, China (2)	PP115441
<i>R. qiongica</i> sp. nov.	GXNU YU000696	Yinggeling, Hainan, China (2)	PP115442
<i>R. qiongica</i> sp. nov.	GXNU YU000697	Yinggeling, Hainan, China (2)	PP115443
<i>R. qiongica</i> sp. nov.	GXNU YU000698	Yinggeling, Hainan, China (2)	PP115444
<i>R. qiongica</i> sp. nov.	VNMN:4117	K' Bang, Gia Lai, Vietnam (3)	LC010604
<i>R. qiongica</i> sp. nov.	FMNH253114	Ankhe Dist, Gia Lai, Vietnam (4)	GQ204716
<i>R. napoensis</i>	GXNU YU000171	Napo, Guangxi, China (5)	ON217796
<i>R. napoensis</i>	GXNU YU000173	Napo, Guangxi, China (5)	ON217798
<i>R. rhodopus</i>	VNMN:4118	Yen Tu, Bac Giang, Vietnam (6)	LC010605
<i>R. bipunctatus</i>	AMNHA 161418	Huon Son Reserve, Ha Tinh, Vietnam (7)	AY843750
<i>R. rhodopus</i>	VNMN:4120	Pu Huong, Nghe An, Vietnam (8)	LC010609
<i>R. rhodopus</i>	VNMN:4121	Thanh Hoa, Vietnam (9)	LC010608
<i>R. rhodopus</i>	clone 5	Mengyang, Yunnan, China (10)	EF646366
<i>R. rhodopus</i>	SCUM 060692L	Mengyang, Yunnan, China (10)	EU215531
<i>R. rhodopus</i>	GXNU HP018	Jiangcheng, Yunnan, China (11)	OP740717
<i>R. rhodopus</i>	KIZ060821229	Lvchun, Yunnan, China (12)	EF564574
<i>R. rhodopus</i>	clone 3	Lvchun, Yunnan, China (12)	EF646364
<i>R. rhodopus</i>	2004.0409	Long Nai Khao, Phongsali, Laos (13)	KR828049
<i>R. rhodopus</i>	2006.2519	Ban Yang Thong, Louangphrabang, Laos (14)	KR828069
<i>R. rhodopus</i>	K3353	Ban Keng Koung, Louangphrabang, Laos (14)	KR828071
<i>R. rhodopus</i>	K3046	Doi Chiang Dao, Chiang Mai, Thailand (15)	KR828066
<i>R. rhodopus</i>	K3085_1	Mae Lao-Mae Sae Wildlife Sanctuary, Chiang Mai, Thailand (16)	KR828067
<i>R. rhodopus</i>	0909Y3	Phu Hin Rong Kla NP, Phitsanulok, Thailand (17)	KR828052
<i>R. rhodopus</i>	0906Y5	Phu Hin Rong Kla NP, Phitsanulok, Thailand (17)	KR828078
<i>R. rhodopus</i>	0954Y	Thung Salaeng Luang NP, Phetchabun, Thailand (18)	KR828061
<i>R. rhodopus</i>	0955Y	Thung Salaeng Luang NP, Phetchabun, Thailand (18)	KR828062
<i>R. rhodopus</i>	1000Y	Khao Ang Rui Ni wildlife sanctuary, Chachoengsao, Thailand (19)	KR828065
<i>R. rhodopus</i>	clone 4	Jingdong, Yunnan, China (20)	EF646365
<i>R. rhodopus</i>	KIZ060821248	Jingdong, Yunnan, China (20)	EF564575
<i>R. rhodopus</i>	KIZ060821175	Yongde, Yunnan, China (21)	EF564573
<i>R. rhodopus</i>	clone 2	Yongde, Yunnan, China (21)	EF646363
<i>R. rhodopus</i>	KIZ587	Longling, Yunnan, China (22)	EF564577
<i>R. rhodopus</i>	KIZ589	Longling, Yunnan, China (22)	EF564578
<i>R. rhodopus</i>	GXNU 039927	Longchuan, Yunnan, China (23)	OP740718
<i>R. rhodopus</i>	GXNU 039928	Longchuan, Yunnan, China (23)	OP740719
<i>R. rhodopus</i>	GXNU YU20160263	Mengding, Yunnan, China (24)	PP106375
<i>R. rhodopus</i>	GXNU YU20160264	Mengding, Yunnan, China (24)	PP106376
<i>R. rhodopus</i>	GXNU YU000492	Menglian, Yunnan, China (25)	OP740720
<i>R. rhodopus</i>	GXNU YU000493	Menglian, Yunnan, China (25)	OP740721

Species	Voucher	Locality (ID)	Accession No.
<i>R. rhodopus</i>	GXNU YU000485	Xiding, Menghai, Yunnan, China (26)	OP740714
<i>R. rhodopus</i>	GXNU YU000486	Xiding, Menghai, Yunnan, China (26)	OP740715
<i>R. rhodopus</i>	GXNU YU000487	Xiding, Menghai, Yunnan, China (26)	OP740716
<i>R. rhodopus</i>	USNM:Herp:587063	Kandawgyi National Gardens, Mandalay, Myanmar (27)	MG935991
<i>R. rhodopus</i>	0937Y1	Kui Buri NP, Prachuap Khiri Khan, Thailand (28)	KR828056
<i>R. rhodopus</i>	0937Y4	Kui Buri NP, Prachuap Khiri Khan, Thailand (28)	KR828058
<i>R. bipunctatus</i>	KUHE:53375	Genting, Pahang, Malaysia (29)	LC010569
<i>R. bipunctatus</i>	KIZ016380	Motuo, Xizang, China (30)	MW111517
<i>R. bipunctatus</i>	YPX40427	Motuo, Xizang, China (30)	MW111518
<i>R. rhodopus</i>	L06245	Motuo, Xizang, China (30)	JX219441
<i>R. rhodopus</i>	L062456	Motuo, Xizang, China (30)	JX219442
<i>R. bipunctatus</i>	CAS229913	Nagmung Township, Putao District, Kachin State, Myanmar (31)	JX219445
<i>R. bipunctatus</i>	CAS235303	Mindat Township, Mindat District, Chin State, Myanmar (32)	JX219444
<i>R. bipunctatus</i>	PUCZM/IX/SL360	Mizoram, India (33)	MH087073
<i>R. bipunctatus</i>	PUCZM/IX/SL612	Mizoram, India (33)	MH087076

Molecular analyses and species delimitation

The total genomic DNA of the specimens was extracted from liver tissue preserved in 99% ethanol. Tissue samples were digested with proteinase K and purified using standard phenol/chloroform separation and ethanol precipitation. A fragment encoding partial 12S rRNA, complete tRNA^{val}, and partial 16S rRNA (16S) was amplified using the protocol of Yu et al. (2019) and the primer pair L1091 (Kocher et al. 1989)/16H1 (Hedges 1994). Sequencing was conducted using the corresponding PCR primers and the internal primer Rh-int (Yu et al. 2019). All new sequences have been deposited in GenBank under accession Nos. **OP740711–OP740721**, **PP106375–PP106376**, and **PP115440–PP115442** (Table 1).

Sequences were aligned in MEGA v. 7 (Kumar et al. 2016) using the MUSCLE option with the default parameters. The uncorrected pairwise distances (p-distances) between species were calculated in MEGA v. 7. The best substitution model (GTR + I + G) was selected in jMODELTEST v. 2.1.10 (Darriba et al. 2012) based on the corrected Akaike Information Criterion (AICc). Bayesian phylogenetic inference and maximum likelihood analysis were performed based on the best model. Bayesian phylogenetic inference was performed using MrBayes v. 3.2.6 (Ronquist et al. 2012). Two runs were performed simultaneously with four Markov chains starting from a random tree. The chain was run for 3,000,000 generations and sampled every 100 generations. When the average standard deviation of the split frequency was less than 0.01, the first 25% of the sampled trees were discarded as burn-in, and the remaining trees were used to create a consensus tree and estimate the Bayesian posterior probabilities (BPPs). The maximum likelihood analysis was conducted using raxmlGUI 2.0 (Edler et al. 2020) with 1000 bootstrap replicates.

We used Assemble Species by Automatic Partitioning (ASAP; Puillandre et al. 2021) and multirate PTP (mPTP; Kapli et al. 2017) to delineate the species boundary within the *R. rhodopus* and *R. bipunctatus* complexes based on 16S rRNA sequences. For the ASAP method, the substitution model of p-distances was used to compute the distances under the default values for other parameters. We

selected the partition with the lowest ASAP-score as the best partition, according to Puillandre et al. (2021). The mPTP analysis was conducted in mPTP v. 0.2.5 using a maximum likelihood tree generated from 16S sequences by raxmlGUI 2.0. For this analysis, 10 different runs were performed with the following settings: mcmc run of 50 million generations, samples every 1000 generations, and the first 10 million generations were discarded as burn-in.

Morphology

As the molecular phylogenetic and species delimitation analyses revealed that species diversity in the *R. rhodopus* and *R. bipunctatus* complexes was underestimated and Hainan populations represent one of the three putative species (see below), we further conducted morphological analyses to confirm its taxonomic status and to officially describe it. The other two putative species were not included in morphological analyses owing to the fact that not enough morphometric data on them is available for the time being.

Morphometric data were taken using electronic digital calipers to the nearest 0.1 mm. The terminology followed Fei (1999). Measurements included the following: snout-vent length (SVL); head length (HL); head width (HW); snout length (SL); internarial distance (IND); interorbital distance (IOD); upper eyelid width (UEW); diameter of eye (ED); diameter of tympanum (TD); distance from nostril to eye (DNE); length of forearm and hand (FHL); tibia length (TL); length of tarsus and foot (TFL); and foot length (FL). Comparative morphological data of congeneric species were obtained from published literature (Liu and Hu 1960; Ohler and Delorme 2006; Bordoloi et al. 2007; Fei et al. 2009, 2012; Chan and Grismer 2010; Rowley et al. 2012; Matsui et al. 2013; Li et al. 2022).

Measurements were corrected for size (measurements divided by SVL). We used the *t*-test in SPSS v. 17.0 (SPSS Inc., Chicago, IL, USA) to evaluate the differences in quantitative characters of adult males between Hainan populations and its two relatives (*R. rhodopus* and *R. napoensis*) because the Hainan populations were once placed in *R. rhodopus* and both the clade containing Hain-

an populations and the clade of *R. napoensis* occur in Vietnam (see below). Principal component analyses (PCA) were conducted based on a correlation matrix of size-corrected measurements of males using SPSS v. 17.0. Scatter plots of the first two PCA factors were used to examine the morphological differentiation between specimens from Hainan, *R. rhodopus*, and *R. napoensis*. Females were not included as a separate analysis for both the *t*-test and PCA analysis owing to the small sample size ($n = 2$; one female from Hainan and one female of *R. rhodopus*).

Results

Phylogeny and species delimitation

The BI and ML analyses yielded similar topologies, and both analyses revealed that there are six distinct clades in the *R. rhodopus* and *R. bipunctatus* complexes (Clades A–F; Fig. 2): Clade A contains samples from Hainan, China (sites 1 and 2) and Gia Lai, Vietnam (sites 3 and 4); Clade B contains types of *R. napoensis*

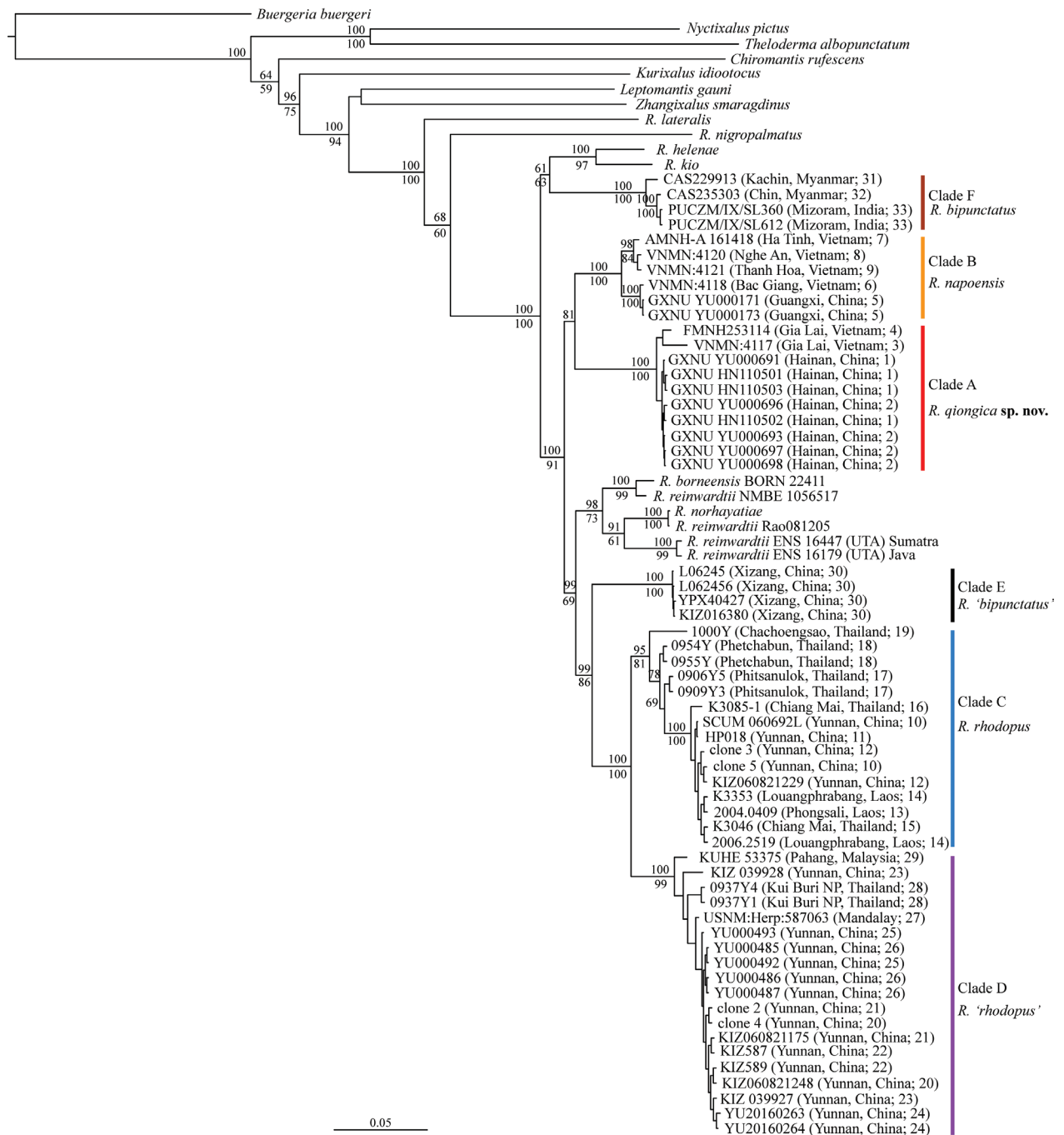


Figure 2. Bayesian phylogenetic tree of *R. rhodopus* and *R. bipunctatus* complexes and related species inferred from 12S rRNA, tRNA^{Val}, and 16S rRNA genes. The numbers above and below the branches are Bayesian posterior probabilities (BPP) and maximum likelihood (ML) bootstrap values, respectively (only values greater than 50% are shown).

(site 5) and samples from northern Vietnam (Bac Giang, Ha Tinh, Nghe An, and Thanh Hoa; sites 6–9); Clade C contains *R. rhodopus* from the type locality (Mengyang, Yunnan, China; site 10) and samples from southern Yunnan (Jiangcheng and Lvchun; sites 11 and 12), northern Laos (Phongsali and Louangphrabang; sites 13 and 14), and northern and southeastern Thailand (Chiang Mai, Phitsanulok, Phetchabun, and Chachoengsao; sites 15–19); Clade D contains samples from western and southwestern Yunnan, China (Jingdong, Yongde, Longling, Longchuan, Mengding, Menglian, and Xiding; sites 20–26), Myanmar (Mandalay; site 27), Thailand (Prachuap Khiri Khan; site 28), and Malaysia (Pahang; site 29); Clade E is consisted of Xizang population (site 30) that was previously identified as *R. rhodopus* or *R. bipunctatus*. Clade F is consisted of *R. bipunctatus* from northern (Kachin State; site 31) and western (Chin State; site 32) Myanmar and northeastern India (Mizoram; site 33). All phylogenetic analyses supported that clades C, D, and E form a monophyly, and clade C is sister to clade D. Clade F was recovered as sister to the clade of *R. helenae* and *R. kio* with moderate support, and clade A was recovered as sister to clade B with weak support.

The sequences of specimens under the name *R. reinwardtii* in GenBank did not form monophyly. The two specimens that came from Malaysia (NMBE 1056517 and Rao081205) clustered together with *R. borneensis* and *R. norhayatiae*, respectively, and the two specimens that came from Indonesia (Java and Sumatra) formed a clade.

Genetically, the pairwise distances between the six clades in *R. rhodopus* and *R. bipunctatus* complexes ranged from 5.3% to 9.2%, which is greater than the distance between *R. kio* and *R. helenae* (4.5%), the distance between *R. helenae* and *R. borneensis* (4.9%), and the distances between *R. norhayatiae*, *R. borneensis*, and *R. reinwardtii* (4.0%–4.8%; Table 2).

The best partition (score = 2.50) obtained by the ASAP species delimitation analysis grouped all samples of *R. rhodopus* and *R. bipunctatus* complexes used in this study into six species completely corresponding to

the six distinct clades (A–F) mentioned above (Fig. 3a), with a distance threshold of about 4% (Fig. 3b). The clade consisting of *R. borneensis* and the specimen under the name *R. reinwardtii* (NMBE 105617), the clade containing *R. norhayatiae* and the specimen under the name *R. reinwardtii* (Rao081205), and the clade comprising of *R. reinwardtii* from Indonesia were identified as three different species (Fig. 3a). These operational taxonomic units were also supported by the mPTP analysis (Fig. 4).

Morphological study

Morphological data are summarized in Table 3. The *t*-tests revealed that the male specimens from Hainan differ significantly ($p < 0.05$) from male topotypes of *R. rhodopus* in tympanum diameter (TD), upper eyelid width (UEW), and distance between nostril and eye (DNE; Table 4), and differ from *R. napoensis* in body size (SVL), head length (HL), internarial distance (IND), and distance between nostril and eye (Table 5). PCA analysis on Hainan populations and *R. rhodopus* revealed that the first two principal components accounted for 66.13% of the total variance (Table 6), and loadings for PC2 were heavily loaded on TD and UEW, which separated Hainan samples from *R. rhodopus* along the PC2 axis (Fig. 5a). PCA analysis on Hainan populations and *R. napoensis* showed that loadings for PC2 were heavily loaded on HL, which can effectively separate Hainan populations from *R. napoensis* along the PC2 axis (Fig. 5b). Additionally, morphological comparison indicated that specimens from Hainan can be distinguished from known members of *R. rhodopus* and *R. bipunctatus* complexes and other related species of *Rhacophorus* by a series of characters (see below).

Based on the above molecular and morphological evidence, we considered that misidentifications were involved in the *R. rhodopus* and *R. bipunctatus* complexes, and herein, the clade consisting of specimens from Hainan and central Vietnam (Clade A) is described as a new species.

Table 2. Mean uncorrected pairwise distances (%) between clades of *Rhacophorus rhodopus* and *R. bipunctatus* complexes and related species based on 16S rRNA sequences.

ID	Species	1	2	3	4	5	6	7	8	9	10
1	Clade A (<i>R. qiongica</i> sp. nov.)										
2	Clade B (<i>R. napoensis</i>)	6.0									
3	Clade C (<i>R. rhodopus</i>)	6.7	7.6								
4	Clade D (<i>R. 'rhodopus'</i>)	8.0	7.3	5.3							
5	Clade E (<i>R. 'bipunctatus'</i>)	7.4	6.0	7.9	6.7						
6	Clade F (<i>R. bipunctatus</i>)	9.2	7.0	8.7	8.3	9.1					
7	<i>R. helenae</i>	7.9	7.1	9.6	7.3	6.8	7.4				
8	<i>R. kio</i>	8.2	7.7	9.9	8.4	8.2	8.7	4.5			
9	<i>R. norhayatiae</i>	7.4	7.0	7.5	6.5	7.7	9.5	7.3	8.3		
10	<i>R. borneensis</i>	6.1	5.8	8.0	6.2	6.2	8.6	4.9	6.8	4.8	
11	<i>R. reinwardtii</i>	5.7	5.3	7.1	5.9	5.2	6.8	6.3	7.5	4.0	4.5

Table 3. Measurements (mm) of *Rhacophorus qiongica* sp. nov., *R. rhodopus*, and *R. napoensis*.

Species	Voucher	SEX	SVL	HL	HW	SL	IND	IOD	UEW	ED	TD	DNE	FHL	TL	TFL	FL
<i>Rhacophorus qiongica</i> sp. nov.	GXNU YU000690	M	35.5	11.4	13.1	5.4	3.7	4.6	3.7	4.7	2.3	2.8	17.3	18.1	25.3	16.5
<i>Rhacophorus qiongica</i> sp. nov.	GXNU YU000691	M	37.8	12.0	13.2	5.5	3.8	4.2	3.6	4.7	2.3	2.6	18.2	18.6	25.6	16.7
<i>Rhacophorus qiongica</i> sp. nov.	GXNU YU000693	M	37.8	12.3	13.4	5.6	3.9	4.4	3.9	4.7	2.2	2.8	18.5	21.0	27.5	17.6
<i>Rhacophorus qiongica</i> sp. nov.	GXNU YU000696	M	36.1	11.1	12.7	5.4	3.9	4.1	3.7	4.8	2.0	2.8	17.8	18.7	25.8	16.6
<i>Rhacophorus qiongica</i> sp. nov.	GXNU YU000697	M	35.1	11.1	12.7	5.2	3.8	4.0	3.8	4.7	1.9	2.7	17.2	17.2	24.3	15.6
<i>Rhacophorus qiongica</i> sp. nov.	GXNU YU000698	F	49.3	14.6	16.8	7.4	4.8	5.5	4.2	5.4	2.6	3.7	23.6	24.9	34.3	23.1
<i>Rhacophorus qiongica</i> sp. nov.	GXNU HN110501	M	38.2	11.5	13.6	5.4	3.6	4.4	3.5	4.8	2.3	2.7	17.3	17.9	24.5	16.2
<i>Rhacophorus qiongica</i> sp. nov.	GXNU HN110502	M	38.1	11.0	13.6	5.2	3.7	4.2	3.5	4.7	2.2	2.8	18.1	19.2	25.5	16.8
<i>Rhacophorus qiongica</i> sp. nov.	GXNU HN110503	M	37.8	11.7	13.7	5.1	3.6	4.3	3.8	4.7	2.1	2.7	18.0	18.4	25.1	16.8
<i>Rhacophorus rhodopus</i>	GXNU YU090185	M	33.1	10.7	11.5	5.0	3.5	3.8	2.6	4.0	2.1	2.1	16.4	16.5	22.4	14.8
<i>Rhacophorus rhodopus</i>	GXNU YU090186	M	33.6	11.2	12.4	4.8	3.8	4.1	2.8	4.2	2.3	2.3	17.0	17.1	23.2	15.5
<i>Rhacophorus rhodopus</i>	GXNU YU090187	M	33.4	10.6	12.0	4.6	3.6	4.0	3.0	4.3	2.0	2.1	16.4	16.5	22.8	14.8
<i>Rhacophorus rhodopus</i>	GXNU YU090188	M	38.7	11.8	13.8	5.5	3.9	4.3	3.2	4.9	2.5	2.5	18.0	18.3	25.4	16.8
<i>Rhacophorus rhodopus</i>	GXNU YU090189	F	50.2	14.9	17.6	7.5	5.4	5.9	4.1	5.6	3.3	3.3	25.6	24.4	35.4	23.6
<i>Rhacophorus rhodopus</i>	GXNU YU090190	M	37.4	11.7	12.8	5.2	4.0	4.4	2.6	4.0	2.4	2.5	18.2	18.5	26.5	17.2
<i>Rhacophorus rhodopus</i>	GXNU YU090194	M	35.5	10.8	12.5	4.9	3.8	4.1	2.8	4.0	2.5	2.4	17.0	16.4	23.4	15.6
<i>Rhacophorus napoensis</i>	GXNU YU000169	M	39.9	12.8	14.1	5.7	4.3	4.4	4.0	4.9	2.1	2.8	19.9	19.2	28.4	18.7
<i>Rhacophorus napoensis</i>	GXNU YU000170	M	44.2	15.3	16.2	6.9	4.7	4.7	4.5	5.3	2.5	3.0	20.1	20.8	29.2	19.2
<i>Rhacophorus napoensis</i>	GXNU YU000171	M	41.2	14.5	15.3	6.4	4.6	4.6	4.3	5.2	2.5	2.8	20.7	19.9	28.7	19.0
<i>Rhacophorus napoensis</i>	GXNU YU000172	M	39.7	13.1	14.2	5.9	4.3	4.3	4.1	5.2	2.3	2.8	18.9	19.1	26.7	17.4
<i>Rhacophorus napoensis</i>	GXNU YU000173	M	41.4	13.9	15.2	6.3	4.5	4.5	4.0	4.9	2.3	2.9	20.6	20.5	28.3	18.7

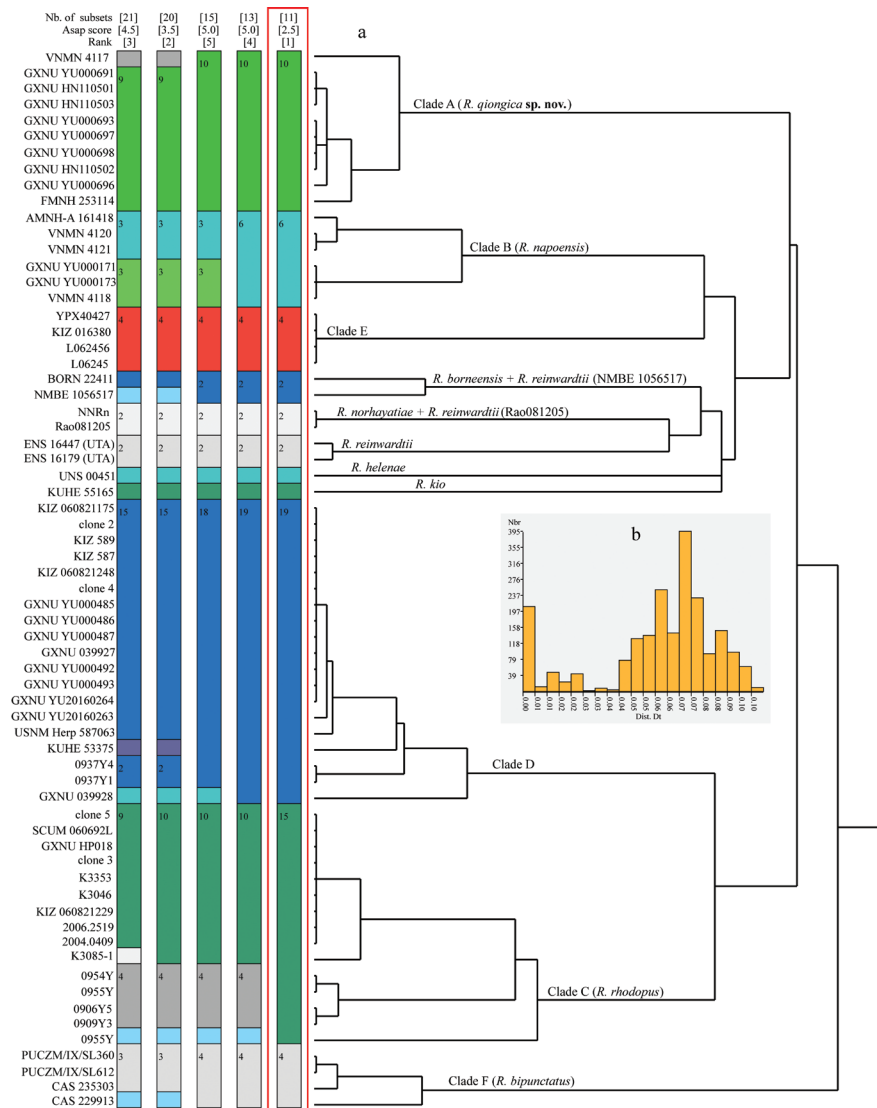
**Figure 3.** ASAP species delimitation based on 16S rRNA sequences used in this study. The best partition with the lowest score is highlighted with a red frame.

Table 4. Results of the t-test between male specimens of *Rhacophorus qiongica* sp. nov. and *R. rhodopus* based on the size-adjusted data except SVL.

Character	Mean ± SD (n = 8)	Mean ± SD (n = 6)	Levene's test		t-test	
	<i>R. qiongica</i> sp. nov.	<i>R. rhodopus</i> (clade C)	F	p-value	t	p-value
SVL	37.1 ± 1.3	35.3 ± 2.3	4.016	0.068	1.825	0.093
HL	0.311 ± 0.012	0.316 ± 0.011	0.022	0.883	−0.816	0.430
HW	0.358 ± 0.006	0.354 ± 0.009	0.871	0.369	0.774	0.454
SL	0.144 ± 0.006	0.142 ± 0.005	1.036	0.329	0.872	0.400
IND	0.101 ± 0.005	0.107 ± 0.004	2.199	0.164	−2.094	0.058
IOD	0.115 ± 0.006	0.117 ± 0.004	0.143	0.712	−0.470	0.647
UEW	0.100 ± 0.006	0.080 ± 0.007	0.019	0.891	5.581	0.000*
ED	0.128 ± 0.005	0.120 ± 0.009	3.321	0.093	2.127	0.055
TD	0.058 ± 0.004	0.065 ± 0.004	0.021	0.887	−3.488	0.004*
DNE	0.074 ± 0.004	0.066 ± 0.002	1.212	0.293	4.948	0.000*
FHL	0.481 ± 0.013	0.487 ± 0.014	0.034	0.857	−0.892	0.390
TL	0.503 ± 0.026	0.488 ± 0.018	0.392	0.543	1.181	0.261
TFL	0.687 ± 0.030	0.679 ± 0.020	1.908	0.192	0.601	0.559
FL	0.448 ± 0.014	0.447 ± 0.011	0.539	0.477	0.106	0.917

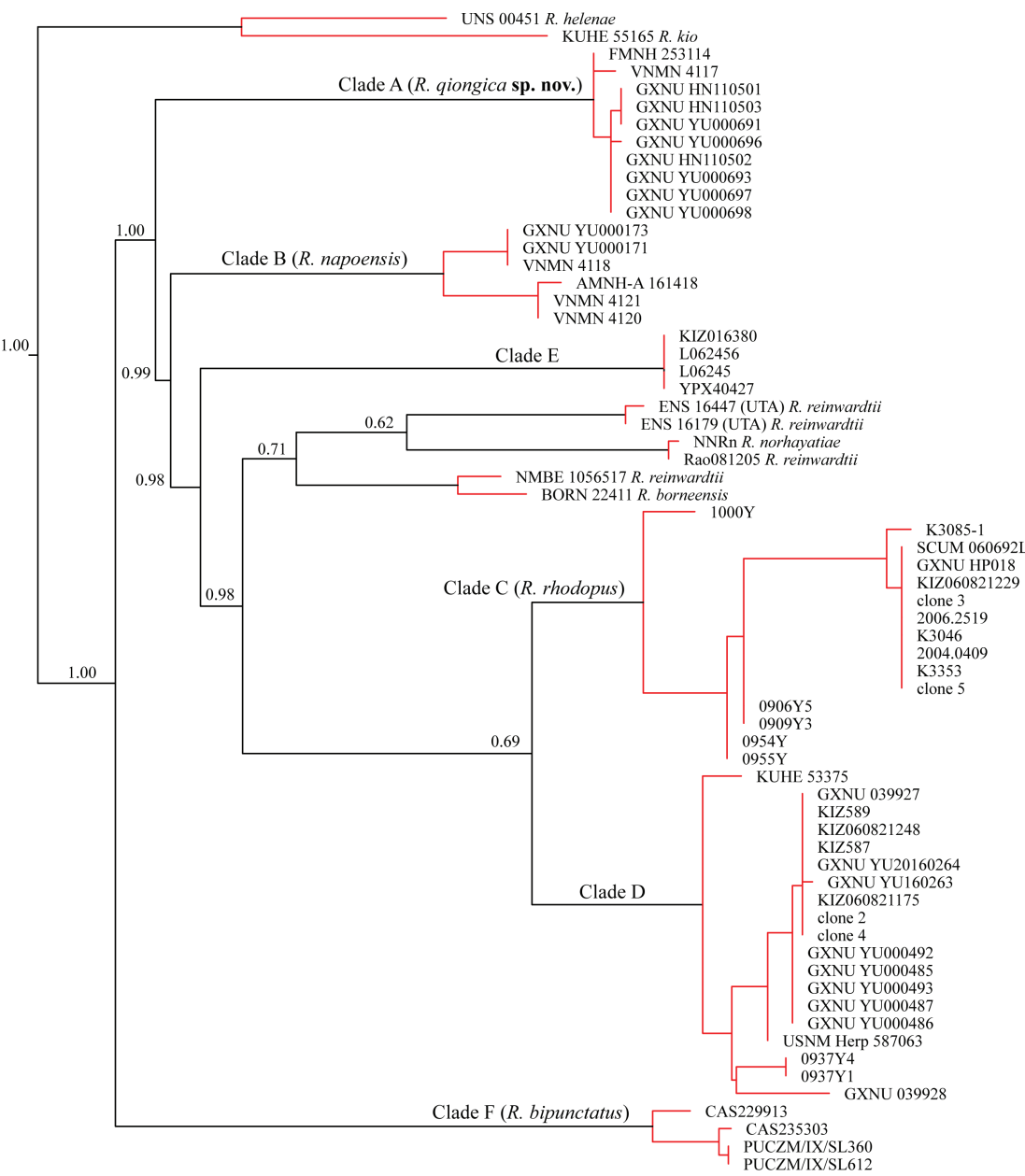


Figure 4. mPTP species delimitation based on ML tree generated from 16S rDNA sequences. The support values above the branches indicate the fraction of sampled delimitations in which a node was part of the speciation process.

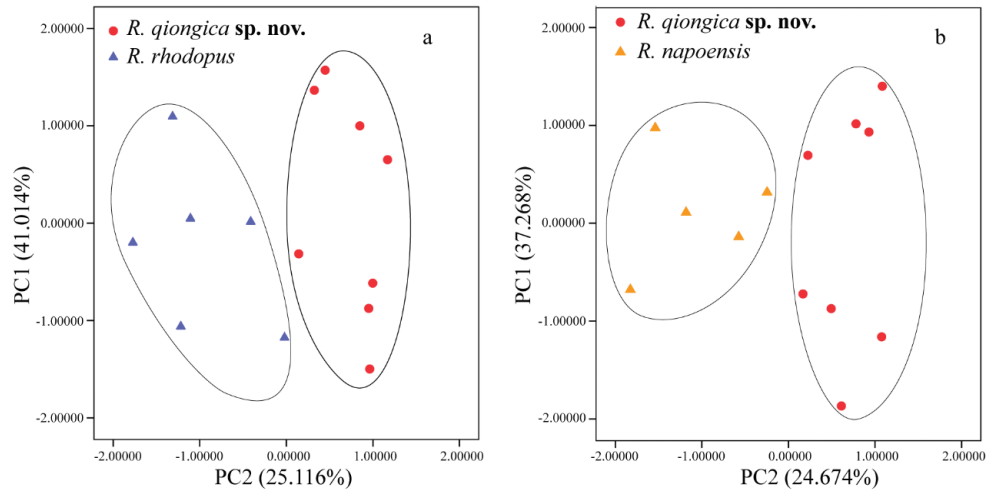


Figure 5. The scatter plot of the principal component analysis based on size-adjusted morphological data from males of the new species plus *R. rhodopus* (a) and data from males of the new species plus *R. napoensis* (b).

Table 5. Results of the t-test between male specimens of *Rhacophorus qiongica* sp. nov. and *R. napoensis* based on the size-adjusted data except SVL.

Character	Mean \pm SD (n = 8)	Mean \pm SD (n = 5)	Levene's test		t-test	
	<i>R. qiongica</i> sp. nov.	<i>R. napoensis</i> (clade B)	F	p-value	t	p-value
SVL	37.1 \pm 1.3	41.3 \pm 1.8	0.053	0.823	-5.006	0.000*
HL	0.311 \pm 0.012	0.337 \pm 0.012	0.019	0.892	-3.778	0.003*
HW	0.358 \pm 0.006	0.363 \pm 0.007	0.364	0.559	-1.420	0.183
SL	0.144 \pm 0.006	0.151 \pm 0.005	0.365	0.558	-1.897	0.084
IND	0.101 \pm 0.005	0.109 \pm 0.002	7.240	0.021	-3.387	0.008*
IOD	0.115 \pm 0.006	0.109 \pm 0.002	1.146	0.307	2.267	0.045*
UEW	0.100 \pm 0.006	0.101 \pm 0.003	4.071	0.069	-0.536	0.603
ED	0.128 \pm 0.005	0.124 \pm 0.005	0.012	0.916	1.480	0.167
TD	0.058 \pm 0.004	0.057 \pm 0.003	0.282	0.606	0.892	0.391
DNE	0.074 \pm 0.004	0.069 \pm 0.001	4.361	0.061	2.756	0.019*
FHL	0.481 \pm 0.013	0.486 \pm 0.020	2.125	0.173	-0.572	0.579
TL	0.503 \pm 0.026	0.482 \pm 0.009	2.803	0.122	1.705	0.116
TFL	0.687 \pm 0.030	0.685 \pm 0.020	1.570	0.236	0.156	0.879
FL	0.448 \pm 0.014	0.451 \pm 0.015	0.003	0.957	-0.317	0.757

Table 6. Factor loadings of first two principal components of 13 size-adjusted morphometric characteristics of male specimens of *Rhacophorus qiongica* sp. nov., *R. rhodopus*, and *R. napoensis*.

Character	<i>R. qiongica</i> sp. nov. and <i>R. rhodopus</i>		<i>R. qiongica</i> sp. nov. and <i>R. napoensis</i>	
	PC1	PC2	PC1	PC2
Eigenvalue	5.332	3.265	4.845	3.208
% variation	41.014%	25.116%	37.268%	24.674%
HL	0.724	-0.399	0.425	-0.852
HW	0.354	0.351	0.270	-0.506
SL	0.682	0.113	0.590	-0.597
IND	0.604	-0.577	0.729	-0.573
IOD	0.603	-0.269	0.437	0.615
UEW	0.459	0.853	0.785	-0.185
ED	0.419	0.715	0.458	0.460
TD	-0.111	-0.790	0.063	0.186
DNE	0.507	0.675	0.500	0.738
FHL	0.791	-0.409	0.775	-0.029
TL	0.794	0.112	0.544	0.529
TFL	0.891	-0.079	0.907	0.217
FL	0.885	-0.246	0.849	0.108

Taxonomy

Rhacophorus qiongica sp. nov.

<https://zoobank.org/182E48F4-9743-4B7F-A825-FA63499F15F2>

Figs 6–9

Rhacophorus rhodopus — Fei 1999; Fei et al. 2004, 2009, 2010, 2012; Shi 2011; Nguyen et al. 2014.

Rhacophorus bipunctatus — Orlov et al. 2002.

Type material. *Holotype*. GXNU YU000691, adult male, collected on 14 July 2023 by Lingyun Du from Diaoluo Mt., Lingshui, Hainan, China (18°43'28"N, 109°52'12"E, ca 914 m a.s.l.).

Paratypes. GXNU YU000690, an adult male, collected at the same time as the holotype from the type locality by Lingyun Du and Jiaqi Luo; GXNU HN110501–HN110503, three adult males, collected on 20 July 2021 by Fanrong Xiao from the type locality; and three adult males (GXNU YU000693, GXNU YU000696, GXNU

YU000697) and an adult female (GXNU YU000698) collected on 11 July 2023 by Qiumei Mo and Chunyi Pang from Yinggeling, Hainan, China (19°2'24"N, 109°34'12"E, ca 670 m a.s.l.).

Etymology. The specific name *qiongica* is derived from Qiong (琼), referring to Hainan, China, and meaning good and beautiful in Chinese. The specific name means that this species is very beautiful, and in China, it is distributed in Hainan. We suggest the English common name “Hainan flying frog” and the Chinese common name “琼树蛙 (Qióng Shù Wā)”.

Diagnosis. The new species is assigned to *Rhacophorus* by the presence of intercalary cartilage between terminal and penultimate phalanges of digits, terminal phalanges of fingers and toes Y-shaped, the tip of the digits expanded into disks with circummarginal grooves, fingers webbed, tarsal projections present, dermal folds along the forearm and tarsus present, and a horizontal pupil (Jiang et al. 2019). *Rhacophorus qiongica* sp. nov. differs from its congeners by a combination of the following characters: 1) medium body size (adult males SVL 35.1–38.2 mm); 2) dorsal surface red brown; 3) entire web between fingers and toes; 4) webbing between toes purely scarlet; 5) small black blotches on flank; 6) bands on limbs distinct; 7) throat smooth; 8) absence of dermal appendage on snout tip; 9) absence of small black spots on head sides; 10) palm smooth without small tubercles; and 11) tibiotarsal articulation reaching beyond eye.

Description of holotype. Adult male, body size medium (SVL 37.8 mm); head width (HW 13.2 mm) longer than head length (HL 12.0 mm); snout blunt pointed, sloping in profile, protruding beyond the margin of lower jaw in ventral view; snout length (SL 5.5 mm) longer than diameter of eye (ED 4.4 mm); canthus rostralis distinct, curved; loreal region oblique, concave; nostril oval, lateral, slightly protuberant, slightly closer to tip of snout than to eye; internarial space (IND 3.8 mm) slightly smaller than interorbital distance (IOD 4.2 mm), nearly equal to the width of the upper eyelid (UEW 3.6 mm); pupil horizontal; pineal ocellus absent; tympanum distinct (TD 2.3 mm), rounded, about half eye diameter (ED 4.4 mm); supratympanic fold narrow, flat; tongue cordiform, attached anteriorly, notably notched posteriorly; choanae oval; vomerine teeth

present in two series, touching the inner front edges of the choanae; an internal single subgular vocal sac; a vocal sac opening on the floor of the mouth at each corner.

Forelimbs thin, length of forearm and hand (FHL 18.2 mm) is about half snout-vent length; relative length of fingers $I < II < IV < III$; tips of all fingers expand into discs with circummarginal and transverse ventral grooves, disc of finger I smaller than discs of other fingers; entire web between fingers, webbing formula: $I2-2III1-1.5III1-1IV$; subarticular tubercles rounded and prominent, formula 1, 1, 2, 2, proximal one smaller than distal one on the third and fourth fingers; supernumerary tubercles below the base of finger absent; metacarpal tubercle single, inner, oval and prominent (Fig. 7).

Hindlimbs slender and long, heels overlapping when legs at right angle to body, tibiotarsal articulation reaching beyond eye; tibia length (TL 18.6 mm) nearly equal to length of forearm and hand (FHL 18.2 mm), longer than foot length (FL 16.7 mm), and shorter than length of tarsus and foot (TFL 25.6 mm); relative length of toes $I < II < III < V < IV$; tips of all toes expanded into discs with circummarginal and transverse ventral grooves; entire web between toes, webbing formula $II-1III1-1III1-1IV1-1V$; subarticular tubercles rounded and prominent, formula 1, 1, 2, 3, 2; supernumerary tubercles absent; single inner metatarsal tubercle, oval (Fig. 7).

Dorsal skin smooth with very fine granules; throat and ventral surface of forelimbs smooth; chest, belly, and ventral surface of thighs granular (Figs 6, 7); dermal folds on forearm, tarsus, heels, and vent present.

Coloration in life. Iris light brown; dorsal surface red brown, mottled with two discontinued rows of dark patches and scattered with small black spots on dorsum; dark brown bands and small black spots on dorsal surface of limbs; upper part of flank orange red and lower part of flank orange yellow, scattered with a few small black blotches; skin of ventral surface semi-transparent, mottled with orange yellow on throat and belly; ventral, anterior, and posterior surfaces of limbs orange yellow; discs of fingers and dorsal surface of fingers I–III orange yellow; discs of toes and toes I–IV red; web between fingers yellow, mottled with red; web between toes completely red.



Figure 6. Lateral, dorsal, and ventral views of the holotype of *R. qiongica* sp. nov. (GXNU YU000691) in life.



Figure 7. Dorsal, ventral, and lateral views of the holotype of *R. qiongica* sp. nov. (a–c) in preservative and ventral views of its hand (d) and foot (e).

Color of holotype in preservative. The color faded, but the pattern remained the same as in life. Dorsal surface brown, with dark patches and spots; dorsal side of limbs barred with dark brown; throat, chest, belly, webbing, ventral surface of limbs, and anterior and posterior parts of thighs faded to yellowish; a few small black blotches on flank.

Sexual dimorphism. The body size of males is smaller than that of female; adult males have an internal single subgular vocal sac with a pair of slit-like openings on the floor of the mouth at each corner. Additionally, adult males have a milk-white nuptial pad on the inner side of the base of finger I.

Morphological variation. The number of small black spots on the flank varied among specimens. The holotype GXNU YU000691 and two paratypes (GXNU YU000698 and GXNU HN110502) have multiple small black spots on flank; paratypes GXNU YU000690 and GXNU YU000697 have no black spots on flank; and paratypes GXNU YU000693 and GXNU YU000696 have two small black spots on flank (Fig. 8). Additionally, dorsal color pattern also varied among specimens, as the two paratypes GXNU YU000696 and GXNU YU000698 have yellowish-brown blotches on dorsal surfaces of the body and limbs (Fig. 9).

Distribution and ecology. The species is distributed in Hainan, China and Gia Lai, Vietnam. In Hainan, the species was found usually in shrubs and small arbors at elevations ranging from 600 to 850 m (Shi 2011; as *R. rhodopus*) and called from 19:00 to 03:00 every night during the breeding season (from May to July), with a peak at about 22:00 (Sun et al. 2017; as *R. rhodopus*). The types in this study were found in roadside bushes ca. 1–2 m above the ground (Fig. 10). There were temporary puddles under the bushes, and there is a lake (Tianchi) and a stream nearby the road in the type locality. *Chirixalus doriae* Boulenger, 1893, *Kurixalus hainanus* (Zhao, Wang & Shi, 2005), and *Polypedates megacephalus* Hallowell, 1861 were also found in sympatry at the type locality.

Comparisons. Currently, there are three known species in the *R. rhodopus* and *R. bipunctatus* complexes, namely *R. bipunctatus*, *R. napoensis*, and *R. rhodopus*. The new species differs from *R. bipunctatus* by smaller body size (male SVL 35.1–38.2 mm, $n = 8$ vs. 37.8–50.4 mm, $n = 28$; Table 7), dorsal surface red brown (vs. green; Fig. 11), spots on flanks small (vs. large; Fig. 11b), bands on limbs distinct (vs. indistinct), and throat smooth (vs. granular; Bordoloi et al. 2007); from *R. napoensis* by smaller body size (male SVL 35.1–38.2 mm [37.1 ± 1.3 , $n = 8$] vs.

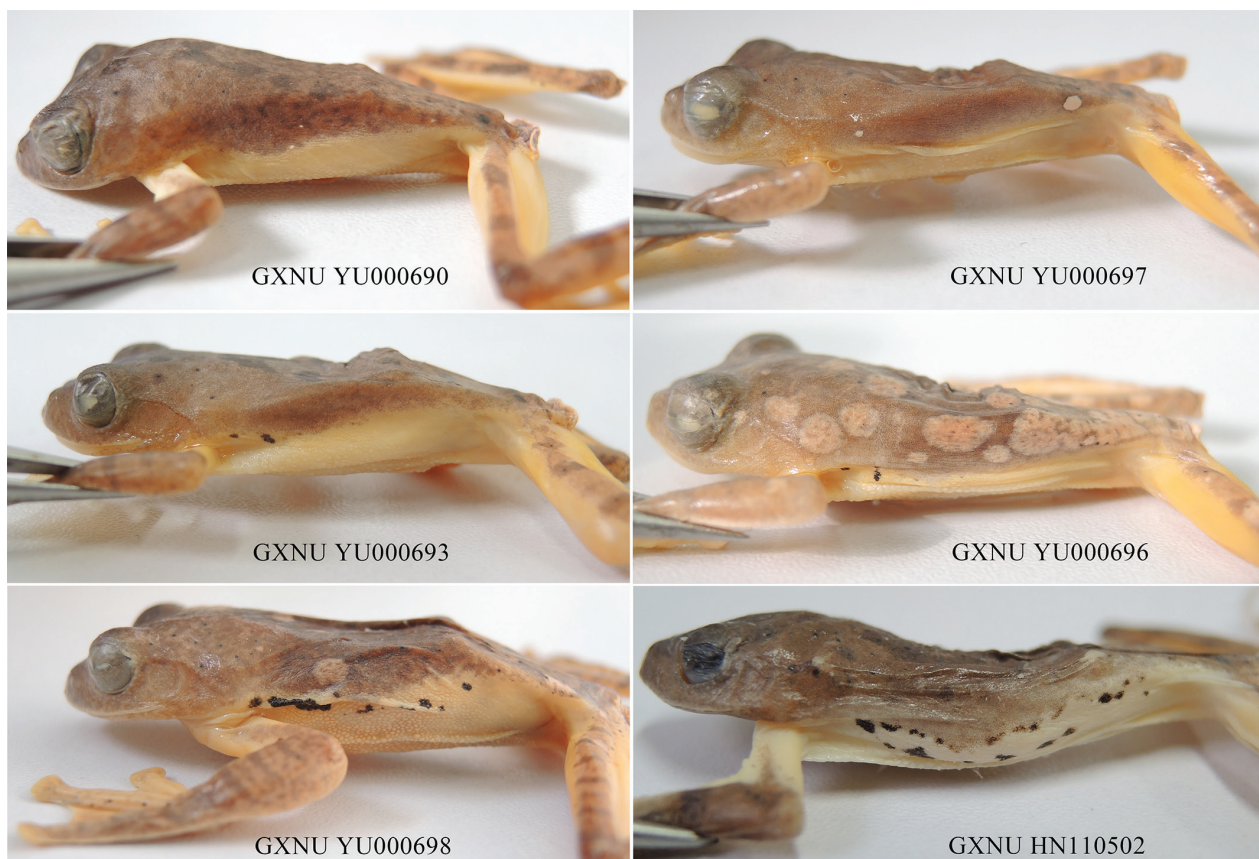


Figure 8. Variation of black spots on flank among paratypes of *R. qiongica* sp. nov. from Hainan, China.



Figure 9. Dorsal view of paratypes GXNU YU000696 (a) and GXNU YU000698 (b) in life.

39.7–44.2 mm [41.3 ± 1.8 , $n = 5$]), snout pointed without dermal appendage on tip (vs. snout pointed with a dermal appendage on tip; Fig. 11), black spots on flanks small (vs. large; Fig. 11), and throat smooth (vs. granular; Fig. 11); and from *R. rhodopus* (Clade C) by black spots on axillar and flanks small (vs. usually large), absence of small black spots on head sides (vs. present; Fig. 12), palm smooth without small tubercles (vs. palm rough with rows of small tubercles; Fig. 12), smaller tympanum, wider upper eyelid, larger distance between nostril

and eye (Table 4), and tibiotarsal articulation reaching beyond eye (vs. tibiotarsal articulation reaching eye).

Both the present and previous phylogenetic analyses revealed that *R. norhayatae*, *R. reinwardtii*, *R. kio*, *R. borneensis*, and *R. helenae* are imbedded in the *R. rhodopus* and *R. bipunctatus* complexes. The new species can be easily distinguished from these five species by the dorsal surface being red brown (vs. green) and the web between toes being red with no black pigmentation (vs. black pigmentation present).

Table 7. Morphological comparison between the new species and members of *Rhacophorus rhodopus* and *R. bipunctatus* complexes. Characters are: ① dorsal color: 0 = brown, 1 = green; ② black spots on flank: 0 = small, 1 = large; ③ bands on limbs: 0 = distinct, 1 = indistinct; ④ throat: 0 = smooth, 1 = granular; ⑤ snout: 0 = pointed without appendage on tip, 1 = pointed with appendage on tip; ⑥ black spots on head side: 0 = absent, 1 = present; ⑦ palm: 0 = smooth without tubercles, 1 = rough with tubercles; ⑧ tibiotarsal articulation: 0 = reaching beyond eye, 1 = reaching eye. “?” means unknown.

Species	Male SVL	①	②	③	④	⑤	⑥	⑦	⑧
<i>R. qiongica</i> sp. nov.	35.1–38.2 (37.1 ± 1.3, n = 8)	0	0	0	0	0	0	0	0
<i>R. rhodopus</i>	33.1–38.7 (35.3 ± 2.3, n = 6)	0	1	0	0	0	1	1	1
<i>R. bipunctatus</i>	37.8–50.4 (n = 28)	1	1	1	1	0	?	0	0
<i>R. napoensis</i>	39.7–44.2 (41.3 ± 1.8, n = 5)	0	1	0	1	1	1	1	0

Discussion

The taxonomy within the *R. rhodopus* and *R. bipunctatus* complexes is complicated owing to the similar external morphology among members of these two complexes, which has heavily hampered the identification of species and understanding of the species boundary in these two complexes. For example, the Xizang population was once recorded as *R. rhodopus* (e.g., Fei et al. 2010; Li et al. 2012) or *R. bipunctatus* (Che et al. 2020); Nguyen et al. (2008) and Poyarkov et al. (2021) suggested that existing records of these two complexes from Vietnam are actually of *R. rhodopus*, but it has been revealed that Vietnamese *R. rhodopus* may represent a cryptic species (Li et al. 2012), suggesting that a species delimitation within these two complexes based on broad sampling is necessary. In this study, based on newly collected samples and previously published data,



Figure 10. Habitat of *R. qiongica* sp. nov. at the type locality.

our phylogenetic analyses revealed that the *R. rhodopus* and *R. bipunctatus* complexes contain six distinct clades (A–F), and they were assigned to six species by the analysis of species delimitation, indicating the species diversity of these two complexes was underestimated and the distribution range of members of these two complexes needs to be re-defined.

According to previous phylogenetic analyses (Yu et al. 2007; Li et al. 2012), there are two distinct clades (C and D) in Yunnan, China. Clade C contains samples from southern Yunnan, China, northern Laos (Phongsali and Louangphrabang), northern (Phetchabun, Phitsanulok, and Chiang Mai) and central (Chachoengsao) Thailand. Undoubtedly, this clade represents the true *R. rhodopus* because the topotypes of this species were grouped in it. Clade D contains samples from western and southwestern Yunnan, central Myanmar (Mandalay), central Thailand (Prachuap Khiri Khan), and Malaysia. Although it was recovered as the sister taxon to the clade C that represents the true *R. rhodopus*, genetic divergence in 16S sequences between them reaches 5.3%, which is greater than the distance between *R. helenae* and *R. kio* (4.5%), the distance between *R. helenae* and *R. borneensis* (4.9%),

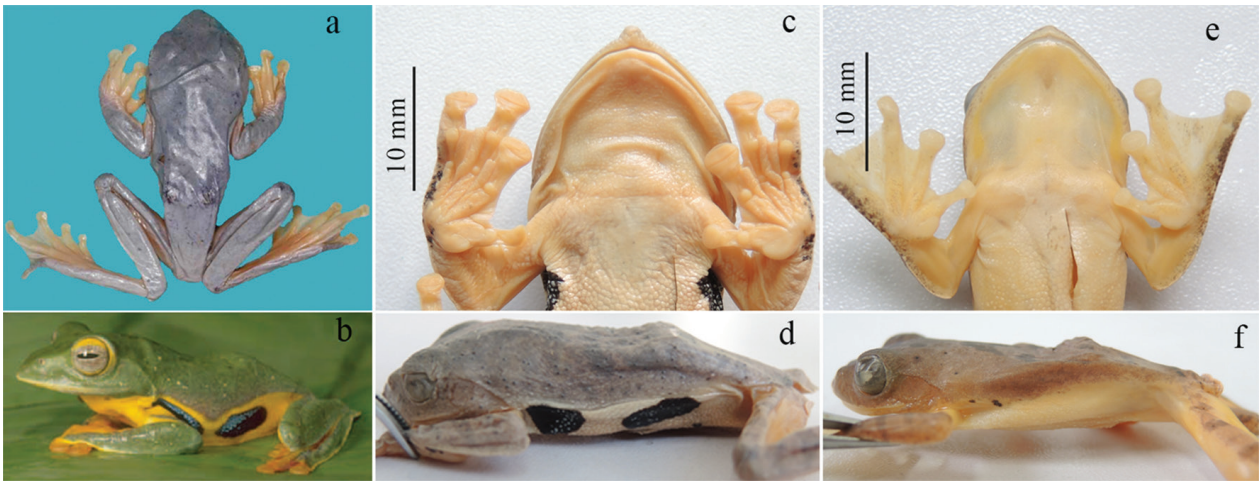


Figure 11. Dorsal (a. BMNH 1872.4.17.127, lectophoront from Khasi Hills, India) and lateral (b. CAS 229893, collected from Putao, Kachin State, Myanmar) views of *R. bipunctatus*, throat (c) and lateral view (d) of *R. napoensis* (GXNU YU000170), and throat (e) and lateral view (f) of *R. qiongica* sp. nov. (GXNU YU000693). The images of a and b were reproduced from Bordoloi et al. (2007) and Wilkinson et al. (2005), respectively.

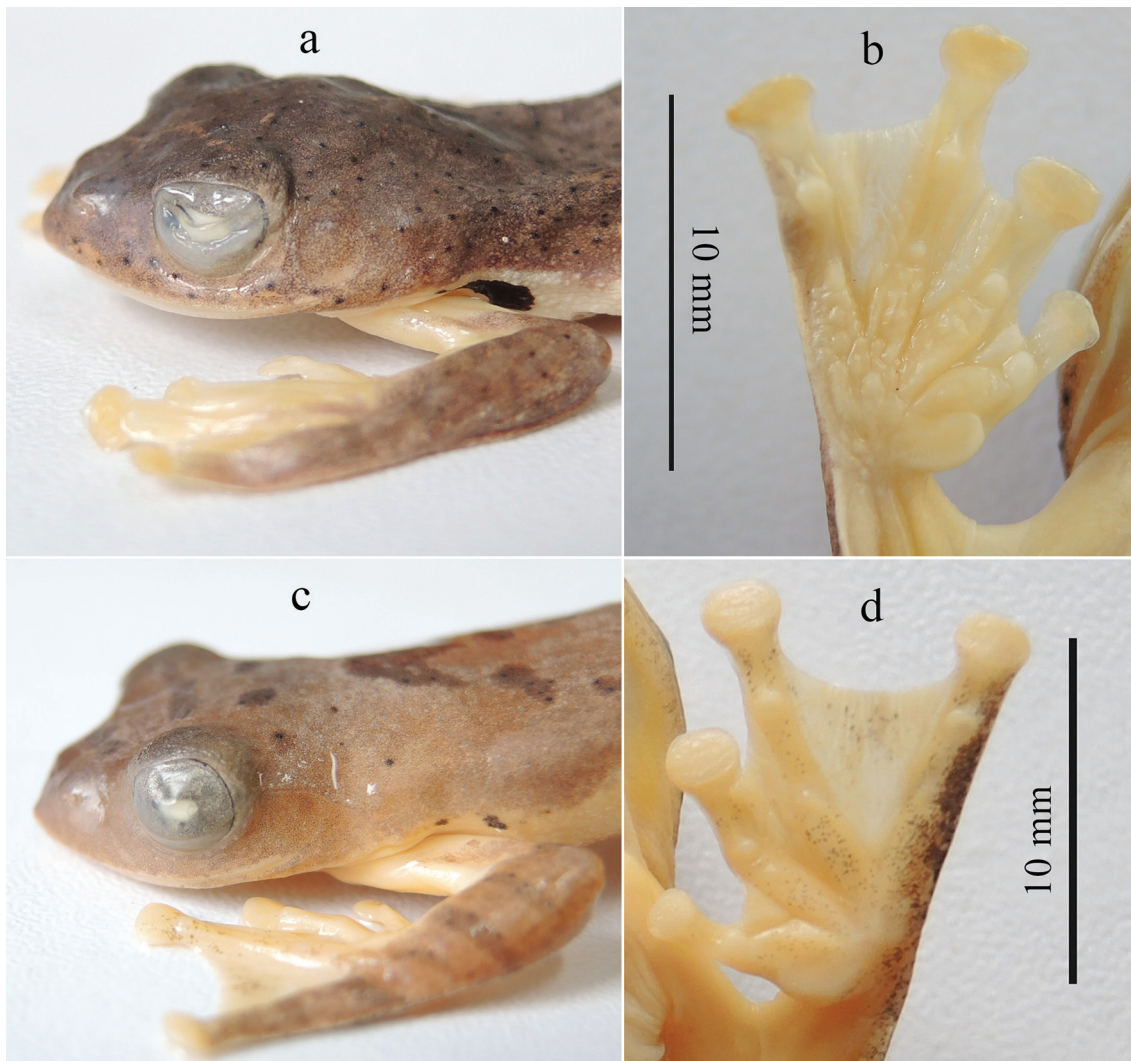


Figure 12. Head side and ventral surface of the hand of *R. rhodopus* (a, b, GXNU YU090186) and *R. qiongica* sp. nov. (c, d, GXNU YU000693).

and the distance between *R. norhayatiae*, *R. borneensis*, and *R. reinwardtii* (4.0%–4.8%; Table 2), and they were assigned into two different species by the analyses of species delimitation. Therefore, we consider that clade D probably represents a cryptic species pending additional morphological studies.

Rhacophorus bipunctatus was originally described from Khasi Hills, Northeast India. Previous phylogenetic analyses (Li et al. 2012; Che et al. 2020) revealed that samples of *R. bipunctatus* from Myanmar and southern Xizang, China form two distinct clades. Owing to the absence of *R. bipunctatus* samples from Northeast India in these two previous studies, it is confusing which clade represents the true *R. bipunctatus*. In this study, we found that *R. bipunctatus* from Northeast India (Mizoram), north Myanmar (Kachin), and west Myanmar (Chin) form a clade (Clade F), whereas samples from southeastern Xizang form an independent clade (Clade E). Considering that Mizoram is close to Khasi Hills, the type locality of *R. bipunctatus*, and records from there have been confirmed based on morphology (Decemson et al.

2020), we consider that clade F is true *R. bipunctatus* and the Xizang population (Clade E) probably represents a cryptic species. Morphologically, the Xizang population differs from *R. rhodopus* and *R. qiongica* sp. nov. by throat granular (Che et al. 2020) since the latter two are smooth (Liu and Hu 1960; this study), and from *R. napoensis* by smaller body size (male SVL 31.6–38.7 mm, mean = 34.9 mm, n = 10), head slightly longer than wide, and tibiotarsal articulation reaching eyes (Che et al. 2020) (vs. SVL 39.7–44.2 mm [mean = 41.3, n = 5] in males, head wider than long, and tibiotarsal articulation reaching snout; Li et al. 2022). Additionally, bands on limbs in the Xizang population are distinct (Che et al. 2020), but bands on limbs in specimens of *R. bipunctatus* from northeastern India and Myanmar are indistinct (Bordoloi et al. 2007; Fig. 11).

Records in Vietnam were once placed in *R. bipunctatus* or *R. rhodopus*, and recently, Poyarkov et al. (2021) conjectured that all existing records of *R. rhodopus* and *R. bipunctatus* complexes in Vietnam are actually of *R. rhodopus*. In this study, we found that there are two

distinct clades in Vietnam (Clades A and B). The samples from northern Vietnam formed Clade B with *R. napoensis*, indicating that they actually refer to *R. napoensis*, while the samples from central Vietnam (Gia Lai) and Hainan, China formed Clade A, which is morphologically distinguishable from other known species of *R. rhodopus* and *R. bipunctatus* complexes and is described as *R. qiongica* sp. nov. herein. The amphibian and reptile flora of Hainan Island is clearly dominated by Oriental species (Shi 2002; Wang et al. 2004, 2009), and the species composition seems to be closely related to the southern part of the Chinese mainland and Vietnam. Although Hainan is separated from the Asian mainland by the Beibu Gulf and Qiongzhou Strait, they have been joined frequently over the past million years (Ali 2018). *Rhacophorus qiongica* sp. nov. likely colonized Hainan via landbridge dispersal during Pleistocene climatic oscillations, which has been suggested for another tree frog in Hainan, *K. hainanus*, a species widely distributed in Vietnam and southern China (Yu et al. 2020).

Like Li et al. (2012) and Chan et al. (2018), phylogenetic relationships within *R. rhodopus* and *R. bipunctatus* complexes were also not resolved well in the present study. Our results strongly supported that the clade containing topotypes of *R. rhodopus* (Clades C) is sister to the clade containing samples from western Yunnan (Clade D), and the two together are sister to the clade composed of samples from Xizang, China (Clade E), which is consistent with Li et al. (2012). Chan et al. (2018) also recovered the clade containing samples from Laos (labeled as Clade 2 in Fig. 4 of Chan et al. (2018) and corresponds to Clade C of this study) as sister to the clade containing samples from Malaysia (labeled as Clade 3 in Fig. 4 of Chan et al. (2018) and corresponds to Clade D of this study) with strong support, but they recovered these two clades together as sister to the clade containing samples from Hainan, China and Gia Lai, Vietnam (labeled as Clade 1 in Fig. 4 of Chan et al. (2018) and corresponds to Clade A of this study). This difference may be caused by the absence of samples from Xizang, China in Chan et al. (2018). In addition, Chan et al. (2018) did not resolve the phylogenetic placement of the clade of the *R. reinwardtii* group, but both the present study and Li et al. (2012) recovered that the clade consisted of the *R. reinwardtii* species group as sister to the group formed by clades C–E with strong support. Neither this nor the two previous studies (Li et al. 2012; Chan et al. 2018) resolved the phylogenetic placement of the three clades corresponding to *R. bipunctatus*, *R. napoensis*, and *R. qiongica* sp. nov., respectively. Therefore, more studies are needed to resolve the phylogenetic relationship among the *R. rhodopus* and *R. bipunctatus* complexes.

Additionally, it is worth noting that some old GenBank sequences of *R. reinwardtii* need to be updated with the correct species name. *Rhacophorus norhayatae* and *R. borneensis* were described as distinct species by Chan and Grismer (2010) and Matsui et al.

(2013), respectively, and both of them were confused with *R. reinwardtii* prior to their naming (Chan and Grismer 2010; Matsui et al. 2013). The type locality of *R. reinwardtii* is Mount Pangerango, Java, Indonesia. In this study, we found that the two samples under the name of *R. reinwardtii* from Malaysia (Rao081205 and NMBE 1056517) were not grouped together with the lineage of *R. reinwardtii* from Indonesia (Java and Sumatra), but clustered together with *R. norhayatae* and *R. borneensis*, respectively. The sequence from the specimen Rao081205 (JX219443) was submitted to GenBank in June 2012, posterior to the taxonomic revision of the *R. reinwardtii*/*R. norhayatae* group (Chan and Grismer 2010), and the sequence from the specimen NMBE 1056517 (JN377366) was submitted to GenBank in July 2011, prior to the taxonomic revision of the *R. reinwardtii*/*R. borneensis* group (Matsui et al. 2013). These evidences suggest that the specimen Rao081205 is a misidentification of *R. norhayatae*, and the name of the specimen NMBE 1056517 is not yet updated in GenBank. This result is partially consistent with the viewpoint of Chan and Grismer (2010) that records of *R. reinwardtii* from Malaya are referable to *R. norhayatae*.

In summary, based on newly collected samples and previously published data, we obtained a clearer delineation of species boundaries within the *R. bipunctatus* and *R. rhodopus* complexes. We recovered six distinct clades (A–F) in these two complexes. *Rhacophorus rhodopus* (Clade C) is distributed in southern Yunnan, China, northern Laos, and northern and central Thailand; *R. bipunctatus* (Clade F) is only distributed in northeast India and western and northern Myanmar; *R. napoensis* (Clade B) is distributed in Guangxi, China and northern Vietnam; and *R. qiongica* sp. nov. (Clade A) is distributed in Hainan, China and central Vietnam. There are two cryptic species requiring additional morphological studies: one only contains samples from Motuo, Xizang, China (Clade E), and the other is distributed in western Yunnan, China, central Myanmar, central Thailand, and Malaysia (Clade D). More studies are needed to resolve the phylogenetic relationship among the *R. rhodopus* and *R. bipunctatus* complexes.

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