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Non-monophyly of *Rhacophorus rhodopus*, *Theloderma* and *Philautus albopunctatus* Inferred from Mitochondrial 16S rRNA Gene Sequences

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Abstract: Mitochondrial gene fragments of 16S rRNA gene of four species (*Rhacophorus rhodopus*, *R. reinwardtii*, *Philautus albopunctatus* and *P. rhododiscus*) from 11 populations were sequenced in this study. Homologous sequences of *R. bipunctatus*, *Theloderma asperum*, *T. corticale* and *Buergeria japonica* were obtained by screening the GenBank database. After excluding all gaps and ambiguous positions, aligned sequences were 500 bp in length with 115 variable sites and 92 parsimony-informative sites. Using *B. japonica* as an outgroup, phylogenetic relationships were analyzed using Bayesian inference, maximum parsimony and maximum likelihood methods. Our results indicated that neither of *R. rhodopus* and *P. albopunctatus* were monophyletic at the species level. The population of *R. rhodopus* from Hainan Island was more close to *R. bipunctatus* than to populations of *R. rhodopus* from Yunnan Province. Furthermore, the populations of *R. rhodopus* from Yunnan Province can be divided into two main lineages. *Theloderma corticale* and *P. rhododiscus* were clustered together and *T. asperum* was nested in *P. albopunctatus*. We considered that *P. albopunctatus* Liu and Hu, 1962, was the synonymy of *T. asperum* Boulenger, 1886, and suggested removing *P. rhododiscus* from *Philautus* into the genus *Theloderma*.

Key words: *Rhacophorus rhodopus*; Phylogenetic relationships; 16S rRNA

基于 16S rRNA 序列推断红蹼树蛙、棱皮树蛙和白斑小树蛙的非单系性

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摘要: 测定了 4 个种(红蹼树蛙、黑蹼树蛙、白斑小树蛙和红吸盘小树蛙)共 11 个种群的 16S rRNA 基因片段。双斑树蛙、马来棱皮树蛙、越南棱皮树蛙以及日本溪树蛙的同源序列通过 GenBank 检索获得。去除所有插入、缺失及模糊位点后, 比对序列长度为 500 bp, 其中变异位点 115 个, 简约信息位点 92 个。以日本溪树蛙为外群, 运用 Bayesian 法、MP 法和 ML 法构建了系统发育树。结果表明红蹼树蛙和白斑小树蛙在种级水平上均不是单系。红蹼树蛙海南种群与双斑树蛙亲缘关系更近, 并且来自云南不同地理种群的红蹼树蛙可以分为两大支系; 越南棱皮树蛙与红吸盘小树蛙聚为一支, 马来棱皮树蛙嵌套在白斑小树蛙不同地理种群中。进而认为白斑小树蛙是马来棱皮树蛙的同物异名, 建议将红吸盘小树蛙并入棱皮树蛙属。

关键词: 红蹼树蛙; 系统发育; 16*S* rRNA
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Rhacophorus reinwardtii, *R. rhodopus*, and *R. bipunctatus* belong to the *R. reinwardtii* species group because of the skin fold on the upside or down side of the anus (Fei, 1999; Fei et al, 2005). Most previous studies about these species were mainly focused on karyotype or Ag-NOR (e.g., Tan et al, 1989; Rao & Yang, 1996; Li

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& Hu, 1996), and the information about the taxonomy and phylogeny of these species is very limited. Wilkinson et al (2002) found that *R. bipunctatus* was more closely related to *R. reinwardtii* than to *R. rhodopus*. However, Frost (2006) placed *R. rhodopus* Liu and Hu, 1959, into the synonymy of *R. bipunctatus* Ahl, 1927, without any discussions.

The genus *Philautus*, as currently understood, is clearly heterogeneous in terms of morphology, and possibly also of life-history (Bossuyt & Dubois, 2001). *Philautus albopunctatus* was placed into the *P. albopunctatus* group along with *P. asper* and *P. tuberculatus* by Fei (1999). However, *Philautus asper* has been placed into the genus *Theloderma* as *T. asperum* (e.g., Taylor, 1962; Liem, 1970; Bossuyt & Dubois, 2001; Frost, 2006). According to Channing (1989), Wilkinson et al (2002) and Delorme et al (2005), the genus *Theloderma* was the sister taxa of *Nyctixalus*. However, Frost et al (2006) found that *T. corticale* was more close to *P. rhododiscus* first, and then to *Nyctixalus*. This incongruence is probably due to sampling difference as Wilkinson et al (2002) did not include *P. rhododiscus*, while Frost et al (2006) did not include *T. asperum*.

Thus, the starting points of our work are to re-examine the phylogenetic relationships among *R. reinwardtii*, *R. rhodopus* and *R. bipunctatus*, and to obtain a better understanding of the phylogenetic placement of *P. albopunctatus* and *P. rhododiscus*, although both of the species were still placed in *Philautus* by most herpetologists (Fei, 1999; Bossuyt & Dubois, 2001; Frost, 2006).

1 Materials and Methods

1.1 Samples

Mitochondrial gene fragments of 16*S* rRNA sequences from 14 specimens of *R. rhodopus*, *R. reinwardtii*, *P. rhododiscus* and *P. albopunctatus* were amplified and sequenced. Homologous sequences of *R. bipunctatus*, *T. asperum* and *T. corticale* were obtained by screening the GenBank database. Although the deep relationships among the subfamily Rhacophorinae are not clear, the genus *Buergeria*, distinguished from other Asian/African rhacophorids by the absence of a bony knob on the third metacarpal (Wilkinson et al, 2002), definitely is the sister taxon of other Asian/African rhacophorids based on recent molecular studies (Richards & Moore, 1998; Wilkinson et al, 2002; Frost et al, 2006). Therefore we prudently selected one species of *Buergeria* as an outgroup in the present study. All the retrieved sequences from GenBank were: *Buergeria japonica*, AF458123; *R. bipunctatus*, DQ283050; *T. corticale*, AF458148; *T. asperum*, AF458144. All species used in this study are listed in Tab. 1.

Tab. 1 Species used in this work

Species	Sample locality	GenBank Accession Number
Rhacophorus rhodopus	Simao, Yunnan (SM)	EF646362
	Yongde, Yunnan (YD)	EF646363
	Lvchun, Yunnan (LC)	EF646364
	Jingdong, Yunnan (JD)	EF646365
	Xishuangbanna, Yunnan (XSBN)	EF646366
	Longlin, Yunnan (LL)	EF646367
	Longlin, Yunnan (LL)	EF646368
	Limu Mountains, Hainan (LM)	EF646369
	Limu Mountains, Hainan (LM)	EF646370
R. reinwardtii	Lvchun, Yunnan (LC)	EF646371
	Lvchun, Yunnan (LC)	EF646372
R. bipunctatus	—	AF458144
Philautus rhododiscus	Jinxiu, Guangxi (JX)	EF646373
P. albopunctatus	Jinping, Yunnan (JP)	EF646374
	Jinxiu, Guangxi (JX)	EF646375
Theloderma asperum	—	AF458148
T. corticale	—	DQ283050
Buergeria japonica	—	AF458123

1.2 DNA extraction, PCR amplification and sequencing

All voucher specimens used in this study are deposited at the Kunming Institute of Zoology, the Chinese Academy of Sciences. Total DNA was purified from alcohol-preserved muscle or liver using a standard proteinase K/SDS digest extraction method followed by phenol/chloroform isolation and ethanol precipitation. A total of about 550 base pairs corresponding to part of the 16*S* ribosomal RNA gene were amplified via polymerase chain reaction (PCR) using the primers 16Sar (5′-CGCCTGTTTATCAAAAACAT-3′) and 16*S*br (5′-CCGGTCTGAACTCAGATCACGT-3′) (Palumbi et al, 1991). The lowercase ar and br indicate the amplified directions of light and heavy strand, respectively. PCR amplifications were conducted in a total volume of 50 μL using the following cycling conditions: an initial denaturing step at 95℃ for 3 min; 40 cycles of denaturing at 94℃ for 1 min, annealing at 51℃ for 1 min and

extending at 72℃ for 1 min; and a final extending step at 72℃ for 10 min.

PCR products were purified using the "High Pure PCR Product Purification Kit" (Bioteke) and sequenced in an automated DNA sequencer (ABI PRISM 3730) using the BigDye terminator v3.1 by Shanghai Sangon Biological Engineering Technology & Services CO., Ltd. Both sense and antisense strands were sequenced using the corresponding PCR primers. All sequences were deposited in GenBank (Accession Nos. EF646362-EF646375).

1.3 Molecular and phylogenetic analyses

Sequences were aligned using CLUSTAL X version 1.83 (Thompson et al, 1997) with default gap costs and revised by eye in order to maximize homology of position. After cutting off both ragged sides, we obtained sequences of 540 bp. Considering the uncertainties of alignment of hypervariabel regions, we preferred using a strongly conservative approach, which excluded all regions of the gene fragments that could not be clearly aligned among all taxa, as recommended by Swofford et al (1996). Therefore all ambiguous alignments and gaps were removed from the alignment using GBLOCKS 0.91b (Castresana, 2000) with default parameters. The revised alignment was 500 bp in length.

Observed proportional sequence divergence (p-distance) and the number of transitions and transversions in pairwise comparisons were obtained using the computer program MEGA (version 3.1; Kumar et al, 2004) and PAUP* (version 4.0b10; Swofford, 2002), respectively. To test for the saturation in base substitutions, we plotted p-distances against pairwise transition and transversion differences. The amount of sequence saturation is inferred from the shape of the trend line, with a linear relationship indicating that the sequence is unsaturated and an asymptotic relationship indicating the presence of saturation (Guo et al, 2005). The saturation plot was showed in Fig. 1, which confirmed that 16S fragments did not reach saturation.

Prior to phylogenetic analyses, a g1 test was conducted to assess the amount of phylogenetic signal (Hillis and Huelsenbeck, 1992). We generated 10 000 random trees and calculated the skewness (g1) of the resulting tree length distribution with PAUP* 4.0b10. The data was subjected to three different phylogenetic analyses using: maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). MP and ML analyses were carried out with PAUP*. The MP method was performed using heuristic searches with 1 000 random addition sequence replicates, tree bisection reconnection (TBR), branch swapping and transitions and transversions given equal weight. Maximum likelihood (ML) analysis was based on the best substitution model and phylogenetic parameters, which was selected using the Akaike Information Criterion (AIC: Akaike, 1973) as implemented in the computer program Modeltest version 3.7 (Posada & Crandall, 1998). For the ML analysis, a heuristic search with the TBR branch swapping algorithm and 10 random addition replicates was employed. The robustness of the resulting MP and ML tree topologies was tested using bootstrap analyses (Felsenstein, 1985), with 1 000 replicates for MP and 100 for ML. Finally, BI was carried out using MrBayes version 3.1.2 (Huelsenbeck & Ronquist, 2001). To ensure that the Bayesian analyses were not trapped in local optima (Huelsenbeck & Bollback, 2001; Leaché & Reeder, 2002), two runs were performed simultaneously with four Markov chains (three heated and one cold) starting from random trees. The Markov chains were run for 1 000 000 generations, sampling every 100 generations thinned the data to 10 000 sample points. The program Tracer 1.3 (Rambaut & Drummond, 2003) was used to determine when the log likelihood (ln L) of sampled trees reached a stationary distribution. Generations sampled before the chain reached stationarity were discarded as burn-in, and the remaining trees were used to estimate Bayesian posterior probabilities (BPPs).

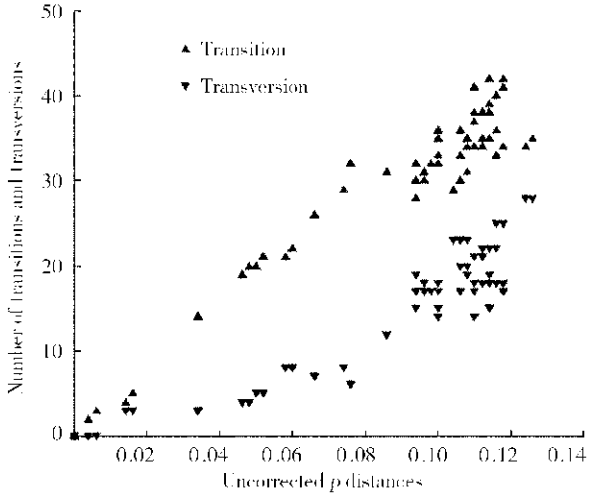

Fig. 1 The saturation plot of pairwise transitions and transversions against uncorrected p sequence divergences

2 Results

2.1 Sequence and tree statistics

From 10 000 random trees, the g1 statistic significantly supported that our data contained sufficient phylogenetic signals: g1 = −0.96; $P < 0.01$; mean ± *SD* tree length = 412.43 ± 20.78. Of 500 bp in the alignment, 115 were variable and 92 positions were phylogenetically informative (23.0% and 18.4%, respectively). Pairwise uncorrected *p* distances among taxa are shown in Tab. 2. Levels of sequence divergence between the outgroup and ingroup lineages ranged from 9.4% (between *B. japonica* and *R. reinwardtii*) to 12.6% (between *B. japonica* and *P. albopunctatus*). Sequence divergence within the ingroup taxa was as high as 11.8% (*R. reinwardtii* compared to *P. albopunctatus*, and *Theloderma* compared to *R. rhodopus*). The smallest divergence between two species was *R. bipunctatus* and *R. rhodopus* from Hainan Island (0.4%). The average transition/transversion (Ti/Tv) ratio was 3.0 among ingroups.

The GTR + I + G model was selected as the best-fit model of nucleotide substitution for the ML analyses using Akaike Information Criterion (AIC) as implemented in the Modeltest 3.7. Settings for this model were as follows: *R*-matrix = (14.5704, 27.8375, 14.2252, 0.6341, 110.7702 and 1.0000); base frequencies = (A = 0.3062, C = 0.2562, G = 0.2196 and T = 0.2180); proportion of invariant sites = 0.3694; and the shape-parameter of the gamma distribution = 0.3840. This model was also used to calculate the phylogenetic parameters when BI analysis was performed.

We obtained two most parsimonious trees (MPT) with 194 evolutionary steps, a consistency index (CI) of 0.768 and a retention index (RI) of 0.855. The likelihood values of the ML tree was ln *L* = −1596.63, and the likelihood values of the consensus tree in the Bayesian approach were ln *L* = −1609.76 and −1612.12 for cold chain of run 1 and run 2, respectively. The burn-in in the BI occurred before 250 000 generations, so the first 2 500 samples were discarded and the remaining 7 500 sampling trees (whose log-likelihoods converged to stable values) were used to construct a 50% consensus tree using the command "sumt".

2.2 Phylogenetic relationships

All four analyses gave similar results, differing only where bootstrap support was weak, thus only one phylogeny is presented (Fig. 2). In all analyses, the exclusive monophyly of *Philautus* and *Theloderma* was not supported. *Philautus rhododiscus* was more closely related to *T. corticale* than to *P. albopunctatus* with 100, 78 and 97 for BI, MP and ML support values respectively, while *T. asperum* was nested in *P. albopunctatus* (100, 79 and 91 for BI, MP and ML support values respectively). Within *R. rhodopus* the population from Hainan Island was sister to *R. bipunctatus* with strong support (100, 100 and 98 for BI, MP and ML, respectively). Populations from Yunnan Province of mainland China can be divided into two clades: one was com-

Tab. 2 Pairwise uncorrected *p* distances based on partial 16S rRNA gene sequences from eight species used in present study

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Rhacophorus reinwardtii																	
R. reinwardtii	0.000																
R. rhodopus (LM)	0.060	0.060															
R. rhodopus (LM)	0.060	0.060	0.000														
R. bipunctatus	0.058	0.058	0.004	0.004													
R. rhodopus (YD)	0.066	0.066	0.052	0.052	0.050												
R. rhodopus (JD)	0.066	0.066	0.052	0.052	0.050	0.000											
R. rhodopus (LL)	0.066	0.066	0.052	0.052	0.050	0.000	0.000										
R. rhodopus (LL)	0.066	0.066	0.052	0.052	0.050	0.000	0.000	0.000									
R. rhodopus (LC)	0.076	0.076	0.046	0.046	0.048	0.034	0.034	0.034	0.034								
R. rhodopus (SM)	0.076	0.076	0.046	0.046	0.048	0.034	0.034	0.034	0.034	0.000							
R. rhodopus (XSBN)	0.076	0.076	0.046	0.046	0.048	0.034	0.034	0.034	0.034	0.000	0.000						
Buergeria japonica	0.094	0.094	0.096	0.096	0.098	0.100	0.100	0.100	0.100	0.106	0.106	0.106					
Philautus rhododiscus	0.094	0.094	0.094	0.094	0.096	0.086	0.086	0.086	0.086	0.100	0.100	0.100	0.116				
Theloderma corticale	0.114	0.114	0.108	0.108	0.108	0.110	0.110	0.110	0.110	0.118	0.118	0.118	0.114	0.074			
P. albopunctatus (JX)	0.118	0.118	0.104	0.104	0.106	0.116	0.116	0.116	0.116	0.110	0.110	0.110	0.126	0.096	0.110		
T. asperum	0.116	0.116	0.106	0.106	0.108	0.118	0.118	0.118	0.118	0.112	0.112	0.112	0.124	0.100	0.114	0.006	
P. albopunctatus (JP)	0.112	0.112	0.106	0.106	0.108	0.114	0.114	0.114	0.114	0.112	0.112	0.112	0.118	0.100	0.110	0.016	0.014

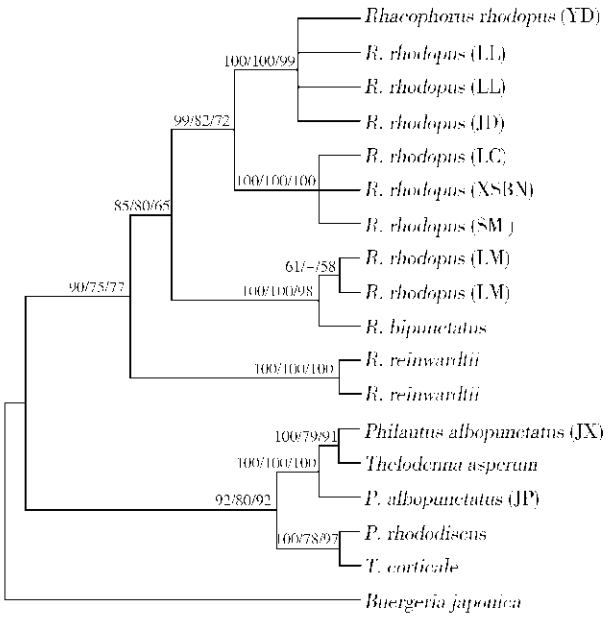

Fig. 2 Bayesian tree based on 500 bp of 16S rRNA with *B. japonica* defined as the outgroup; BI/MP/ML support values (%) are given for nodes that occurred in all applied methods of tree reconstruction.

posed of populations from Southern Yunnan Province including Lvchun, Simao and Xishuangbanna County with robust support (100 for BI, MP and ML), while another one consisted of populations from Southwestern Yunnan Province including Yongde, Longlin and Jingdong County with strong support (100/100/99 BI, MP and ML support values respectively). The average *p* distances within these two clades were 0, and the average distance between these two clades was 3.4%.

3 Discussions

Wilkinson et al (2002) found that *R. reinwardtii* was the sister taxon of *R. bipunctatus*. However, our results strongly support that *R. reinwardtii* is close to the clade consisting of *R. rhodopus* and *R. bipunctatus*. This maybe due to the difference in sequence length. Frost (2006) united *R. rhodopus* into *R. bipunctatus*, but no discussions were given. According to Fei (1999), *R. bipunctatus* has a green dorsal and two black spots on its axilla while the dorsal of *R. rhodopus* is red-brown and has only one black spot on its axilla. Furthermore, in China, *R. bipunctatus* is only distributed in Xizang while *R. rhodopus* has a wide distribution including Xizang, Yunnan, Guangxi and Hainan Province. In our study, the monophyly of *R. rhodopus* is not supported. The population from Hainan Island is the sister-group of *R. bipunctatus*, and the populations from Yunnan Province form a clade. Within the Yunnan group, the populations from Southern Yunnan with a low elevation including counties of Lvchun, Simao, and Xishuangbanna form a clade, while the populations from Southwestern Yunnan with a high elevation including counties of Longlin, Yongde, and Jingdong form another clade. Furthermore, the average *p* distance between these two clades is significantly higher than the distances within them. These findings indicate that the genetic structure of *R. rhodopus* is complicated. We do not agree with Frost (2006) on placing *R. rhodopus* Liu and Hu, 1959, into the synonym of *R. bipunctatus* Ahl, 1927, because the distinctive lineages within *R. rhodopus* may represent some cryptic species and just one of these lineages such as the population from Hainan Island is close to *R. bipunctatus*. Unfortunately, we have no samples from Guangxi Province and adjacent countries such as Vietnam and Burma. So more studies should be carried to unveil the general phylogenetic structure within *R. rhodopus*.

The genus *Philautus* is characterized by the aerial direct development of eggs into froglets, without going through an aquatic tadpole stage. Although Bossuyt and Dubois (2001) considered that some of the nominal species included in *Philautus* by Fei (1999) do not belong to this genus and the whole taxonomy of this group proposed by Fei (1999) needs re-evaluation, up to now there have no demurrers about the taxonomic positions of *P. albopunctatus* and *P. rhododiscus*. Frost et al (2006) restored *P. rhododiscus* as the sister taxa of *T. corticale*, but the taxonomy of *P. rhododiscus* was not changed because, in their study, *T. corticale* and *P. rhododiscus* were the single representative of *Theloderma* and *Philautus*, respectively, and the sister relationship between these two species only can be treated as an indication of a close relationship between *Philautus* and *Theloderma*. However, in this study, the monophyly of *Theloderma* as restored by Wilkinson et al (2002) is not supported. Furthermore, *P. albopunctatus* and *P. rhododiscus* are not sister taxon to each other. As showed in Fig. 2, all analyses supports that *T. corticale* is more closely related to *P. rhododiscus* with high supporting values, and *T. asperum* is nested in *P. albopunctatus*. Moreover, the genetic distance (uncorrected *p* distance) between *P. rhododiscus* and *T. corticale* is as high as 7.4%, and the distance between *T. asperum* and *P. albopunctatus* is only 0.6%–1.4%. Therefore, to correct the paraphyly situation, we suggest

that *P. albopunctatus* Liu and Hu, 1962 should be placed into the synonymy species of *T. asperum* Boulenger, 1886, and *P. rhododiscus* be removed from *Philautus* and put into the genus *Theloderma*.

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