

Molecular Phylogenetic Status of *Rhacophorus laoshan* and *Zhangixalus yinggelingsensis* (Anura: Rhacophoridae) from China

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ABSTRACT

Recently, *Rhacophorus sensu lato* was divided into three genera. In addition, *Rhacophorus laoshan* and *Zhangixalus yinggelingsensis* were placed in the genus *Rhacophorus sensu stricto* and *Zhangixalus*, respectively, only based on morphological characters. The research on the genus *Rhacophorus sensu lato* faces major challenges due to their complex interspecies relationships, and the phylogenetic status of many species are still unclear, which hampered the taxonomy and protection of these species. In this study, we investigated the molecular phylogenetic status of *R. laoshan* and *Z. yinggelingsensis* using mitochondrial DNA (12S rRNA, tRNA^{Val}, 16S rRNA and Cyt *b*) fragments and nuclear DNA (RAG-1, RHOD, TYR) fragments. Our results revealed that *Rhacophorus laoshan* is closely related to *R. verrucopus*, *R. orlovi* and *R. calcaneus*, and *Zhangixalus yinggelingsensis* should indeed be placed under *Zhangixalus*. This research clarified the phylogenetic positions of two species within the genus *Rhacophorus sensu lato*, and it also has an impact on the protection and biogeographic analysis of these species.

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DCJ and KY conceived the study. LMY performed the analysis and prepared the initial manuscript draft. DCJ approved the final version of the manuscript.

Key words

Rhacophorus, *Zhangixalus*, Tree frog

INTRODUCTION

Old world tree frogs of the family Rhacophoridae include 428 species that are widely distributed across Sub-Saharan Africa and southern Asia (from Sri Lanka, Nepal, and India, to Japan, the Philippines and Sulawesi) (Frost, 2020). The genus *Rhacophorus sensu lato* is one of the most diverse genera of the family Rhacophoridae, which contains 94 species and is widely distributed across China, Japan, and India, and from the Philippines to Sulawesi (Frost, 2020).

Many new species of the genus *Rhacophorus sensu lato* have been discovered recently (Dehling, 2015; Hamidy and Kurniati, 2015; Kropachev *et al.*, 2019; Matsui *et al.*, 2013; Nguyen *et al.*, 2017; Pan *et al.*, 2017b; Streicher *et al.*, 2014; Yu *et al.*, 2019), and these studies have made valuable taxonomic contributions of the genus *Rhacophorus sensu lato*. In addition, in the last ten years, there have been many reports that have inferred the phylogenetic

relationships within the genus *Rhacophorus sensu lato* (Chen *et al.*, 2019; Li *et al.*, 2008, 2009, 2012a,b, 2013; O'Connell *et al.*, 2018a,b; Pan *et al.*, 2017a; Yu *et al.*, 2008). However, the molecular data of many species of *Rhacophorus sensu lato* are still poorly known and not publicly available, such as these cryptic species *R. laoshan* and *Z. yinggelingsensis* from southern China. A brown tree frog species, *R. laoshan*, which was first described in Mo *et al.* (2008) based on seven adult male specimens collected from Cenwanglaoshan Nature Reserve, Guangxi, China. This frog can be distinguished from all other Asian *Rhacophorus* Kuhl and van Hasselt, 1822 by the combination of: skin brown and smooth; Y-shaped cartilage visible dorsally on tips of fingers and toes; outer fingers one-third webbed; distinct dermal ridges present on forearms, above vent, and calcars present on heels; anterior and posterior surface of thighs tangerine in color without distinct dark or light spots; tympanum distinct and large, about 6.6% of SVL; dorsum brown with wide dark cross-shaped mark (Mo *et al.*, 2008). To date, *R. laoshan* is still known from type series only, the validity of this species is based on morphological evidence only (Mo *et al.*, 2008). Similarly, another species, *Z. yinggelingsensis*, was first described in Chou *et al.* (2007), and it is known from the type locality in the Yinggelings Mountains of Hainan, China (Frost, 2020). It can be distinguished from other species by the following combination of characteristics: snout rounded, without protruding process; green dorsum with few fine

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white spots, dorsolateral folds absent; skin on dorsum smooth, flanks and venter granulate; metatarsal skinfolds faint; fingers and toes webbed; front of thigh yellow and red-tinged, rear of thigh and inner side of tibia red; white-tipped tuberculous dermal ridge on cloaca present, not well developed; iris silvery, partly light golden in upper half (Chou *et al.*, 2007). In addition, no further specimens or new distribution record has been reported for these two poorly known tree frogs, to the best of our knowledge (Fei *et al.*, 2009, 2012; Frost, 2020).

Recently, Jiang *et al.* (2019) divided *Rhacophorus sensu lato* into three genera: genus *Rhacophorus sensu stricto* Kuhl and Van Hassalt, 1822, genus *Leptomantis* Peters, 1867, and a new genus erected, namely *Zhangixalus* Jiang *et al.*, 2019. Furthermore, *R. laoshan* and *Z. yinggelingsensis* were placed in the genus *Rhacophorus sensu stricto* and *Zhangixalus*, respectively, only based on morphological characters (Jiang *et al.*, 2019). Due to the lack of molecular data for the two species, their phylogenetic status remains unclear. Previous studies suggested that it is essential to clarify species' phylogenetic status using molecular data. We thus conducted molecular phylogenetic analyses of the two *Rhacophorus sensu lato* species to clarify their taxonomic status.

MATERIALS AND METHODS

Ethical statement

This study was performed in accordance with the approval of Experimental Animal Ethics Committee of Chengdu Institute of Biology, Chinese Academy of Sciences.

Taxon sampling and data collection

Two specimens were collected in this study, including one specimen of *Z. yinggelingsensis* (HN2018002), which was collected in Hainan, China on 2018 (19°3'3.84N, 109°32'20.60"E) at 1,569 m altitude (Fig. 1), and one specimen of *R. laoshan* (1705014), which was collected from Cenwanglaoshan Nature Reserve (Guangxi, China) (Fig. 1). After euthanization, liver tissues were preserved in 95% ethanol for DNA extraction. Except for the two

individuals, sequences of 39 individuals were collected from GenBank in this study, including sequences for eight species of *Rhacophorus sensu stricto*, one species of *Leptomantis*, and 18 species of *Zhangixalus* as ingroups and two species of *Polypedates* as outgroups according to previous studies (Jiang *et al.*, 2019; Li *et al.*, 2008, 2009). All novel sequences obtained in this study were deposited in GenBank (Accession No. are shown in Supplementary Table S1).

DNA extraction, amplification, and sequencing

Genomic DNA of *R. laoshan* was extracted from ethanol-preserved liver tissue samples using an Ezup Column Animal Genomic DNA Purification Kit (Sangon Biotech, China), according to the protocols of the manufacturer. A total of 1890 bp of the 12S rRNA, tRNA^{Val}, and 16S rRNA was targeted and amplified using the primers that are listed in Table I. Polymerase chain reaction (PCR) were performed at 25 µL volume, including 12.5 µL Taq PCR Master Mix (2 X blue dye), 9.5 µL ddH₂O, 1 µL FS01, 1 µL Rend (C=10 µL/L), and 1 µL DNA template. PCR cycles consisted of initial denaturation at 95°C for 3 min followed by 35 cycles: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min. A final elongation step of 10 min at 72°C was performed.

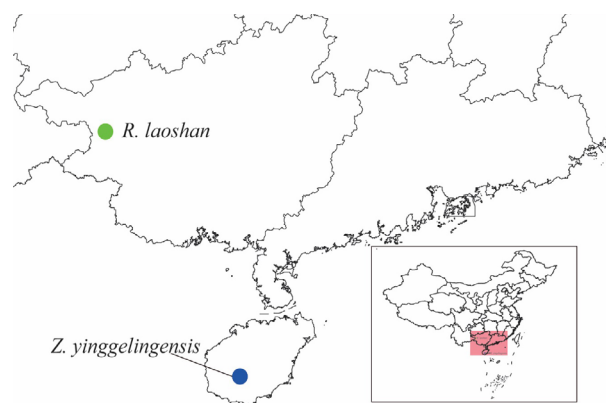


Fig. 1. Localities of *R. laoshan* and *Z. yinggelingsensis* in China.

Table I. Primers used in this study.

Primer	Primer sequence	Resource
FS01	5'-AAC GCT AAG ATG AAC CCT AAA AAG TTC T-3'	Wilkinson <i>et al.</i> , 2002
Rend	5'-GAC CTG GAT TAC TCC GGT CTG A-3'	Wilkinson <i>et al.</i> , 2002
F0483	5'-GAA GAG GCA AGT CGT AAC ATG G -3'	Wilkinson <i>et al.</i> , 2002
F1209	5'-CTC GTA CCT TTT GCA TCA TG-3'	Yang, 2018
R1209	5'-CAT GAT GCA AAA GGT ACG AG-3'	Yang, 2018

In order to conduct further research, we used high-throughput sequencing technology to extract genes of *Z. yinggelingensis*. A total of 0.88 µg DNA extracted from *Z. yinggelingensis* muscle tissue was sheared into short fragments (<500bp) using the Covaris system to construct pair-end DNA libraries. DNA libraries were constructed and sequenced using Illumina-Hi-Seq 2500. The sample was sequenced to a target 10×depth (30Gbp) of its genome. Reads containing more low-quality bases (phred score <30) than 20% were filtered out. Gene fragments of other species in this study were aligned using MEGA 7.0 (Kumar *et al.*, 2016) and adjusted manually. Then, we used the MEANGS (<https://github.com/YanCCscu/MEANGS>) to get the purpose genes. The process are as follows: The alignments of genes that aligned using MEGA 7.0 were used in construction profile HMM(s) with hmmer-build in HMMER V3.16 (Eddy, 2008). Hereafter, nhmmer were used to search reads that fitting the hmm profiler for each gene from high-throughout sequencing data. Finally, we assembled these matched reads with the de novo assembler SSAKE v2.2.1 (Warren *et al.*, 2007), and the assembled scaffolds were identified by blasting to the purpose DNA fragments, including four mitochondrial fragments, 12S rRNA, tRNA^{Val}, 16S rRNA, Cytochrome b (Cyt b), and three nuclear genes, Recombination activating gene 1 (RAG-1), Exon 1 of tyrosinase (RHOD) and exon 1 of tyrosinase (TYR). In order to evaluate the accuracy of the assembled genes of *Z. yinggelingensis*, we mapped all reads back to the assembled genes, and statistic the mapping quality and average depth of these genes.

Phylogenetic analyses

Phylogenetic trees were reconstructed by only mitochondrial fragments (12S rRNA, tRNA^{Val}, 16S rRNA) and combined mitochondrial and nuclear fragments (12S rRNA, tRNA^{Val}, 16S rRNA, Cyt b, RAG-1, RHOD, TYR), respectively. Sequences were aligned using MEGA 7.0 (Kumar *et al.*, 2016) with default settings and rechecked in MAFFT version 7 (Katoh and Standley, 2013), then adjusted manually. Different gene fragments of each individual were concatenated by using Phylosuite (Zhang *et al.*, 2020), and positions of indels were treated as missing data for all datasets. The most optimum substitution model of evolution (GTR+I+G for mitochondrial fragments, HKY+I for RAG-1 gene and RHOD gene, and SYM+G for TYR gene) was calculated by Partition Finder v2.1.1 (Lanfear *et al.*, 2017) under Akaike Information Criterion (AIC) (lnL= -18812.57, AICc= 37858.23). We then reconstructed phylogenetic trees using the Bayesian inference (BI) and maximum likelihood (ML) methods, respectively.

The Bayesian phylogenetic tree was constructed using

MrBayes 3.2.0 (Ronquist *et al.*, 2012). Runs of Markov chains for 5,000,000 generations were summarized and sampled every 100 generations. The first 25% of samples as burn-in, and convergence was investigated using the parameter of average standard deviation of split frequencies ≤ 0.01 (Ronquist *et al.*, 2012). Nodes were considered strongly supported when BPP ≥ 0.95 . Software RAXML v8.2.10 (Stamatakis, 2014) was used to implement Maximum Likelihood (ML) analysis under the best-fit model of evolution (GTRGAMMA) based on the AIC criterion. And bootstrap proportions (BSP) were evaluated with 1000 nonparametric bootstrap pseudoreplications, and it were considered significantly supported when the node's BSP ≥ 70 . In addition, uncorrected pairwise distance (*p*-distances) of the four mitochondrial fragments (12S rRNA, tRNA^{Val}, 16S rRNA and Cyt b) and three nuclear genes (RAG-1, RHOD and TYR) dataset among species in *Rhacophorus sensu lato* were calculated using MEGA 7 (Kumar *et al.*, 2016), with the “*P*-distance” model and “Transitions + Transversions” substitutions to include.

RESULTS AND DISCUSSION

The mapping average depth of 12S rRNA, tRNA^{Val}, and 16S rRNA, Cyt-b, RAG-1, RHOD, and TYR for the *Z. yinggelingensis* is 3331, 2315, 13.95, 5.847, 9.681, respectively. Simultaneously, the mitochondrial fragments consisted of 1890 bp, and the combined mitochondrial and nuclear fragments consisted of 3746 bp, including 1890 bp for 12S rRNA, tRNA^{Val}, and 16S rRNA, 546 bp for Cyt-b, 507 bp for RAG-1, 279 bp for RHOD, 524 bp for TYR. The present molecular phylogeny is concordant with previous molecular studies regarding the monophyly of *Rhacophorus sensu lato* (Chan *et al.*, 2018, 2019; Li *et al.*, 2008, 2009, 2012a, 2013; O'Connell *et al.*, 2018b; Pan *et al.*, 2017a). Both mitochondrial fragments (Supplementary Fig. S1) and combined mitochondrial and nuclear fragments (Fig. 2 and Supplementary Fig. S2) resulted in virtually identical topology. Although interspecific relationships were not fully resolved, the phylogenetic trees from both the BI and ML methods showed that all species contained in *Rhacophorus sensu lato* were clustered to three clades as monophyletic groups and each clade consisted of the same species. According to phylogenetic trees from combined mitochondrial and nuclear fragments, clade A was congruent with genus *Zhangixalus* and was strongly supported by a bootstrap proportion of 83 and a posterior probability of 0.96. Clade B was congruent with genus *Leptomantis* and it was sister group to *Zhangixalus* that supported by a bootstrap proportion of 80 and a posterior probability of 0.96, which was consistent with Jiang *et al.* (2019), but was different from Chen *et al.* (2019). Chen *et al.* (2019) indicated that *Leptomantis* was sister group

fall within the known distribution sites of *R. calcaneus*. On the other hand, some species are poorly diagnosed yet and characters highly convergent in previous studies (David *et al.*, 2007, 2015a; Guo *et al.*, 2014). Consequently, we suspect that the two specimens (FMNH 256465, AMNH A163749) of *R. calcaneus* were misidentifications. Due to we do not have the two specimens, it is need to confirm whether the two specimens were misidentified in future study. Moreover, it may be that some sequences of the species *R. calcaneus* from the NCBI have problems, because taxonomic identification problems of existing Genbank sequences are very common (Guo *et al.*, 2014; Ren *et al.*, 2018).

Phylogenetic tree showing the relationships between various species, primarily focusing on the genus *Zhangixalus*. The tree is rooted at the bottom left with *Polypedates leucomystax* and *Polypedates megacephalus*. The main clade of *Zhangixalus* is highlighted in blue, with *Z. yinglingensis* and *Z. laoshan* highlighted in green. Other clades include *Leptomantis* (red), *Rhacophorus* (green), and *Leptomantis* (red). Bootstrap values are provided at the nodes. Scale bar = 0.04.

Clade A: *Zhangixalus*

- Zhangixalus duboisi*
- Zhangixalus omeimontis*
- Zhangixalus dorsoviridis*
- Zhangixalus lishuiensis*
- Zhangixalus zhokuiyue*
- Zhangixalus zhokuiyue*
- Zhangixalus hongchiensis*
- Zhangixalus hongchiensis*
- Zhangixalus hongchiensis*
- Zhangixalus wui*
- Zhangixalus wui*
- Zhangixalus hui*
- Zhangixalus puerensis*
- Zhangixalus puerensis*
- Zhangixalus yinglingensis*
- Zhangixalus molitrecti*
- Zhangixalus arboreus*
- Zhangixalus nigropunctatus*
- Zhangixalus nigropunctatus*
- Zhangixalus chenfei*
- Zhangixalus chenfei*
- Zhangixalus fecae*
- Zhangixalus fecae*
- Zhangixalus achantharhena*
- Zhangixalus dultensis*

Clade B: *Leptomantis*

- Leptomantis monticola*
- Leptomantis*

Clade C: *Rhacophorus*

- Rhacophorus calcaeus*
- Rhacophorus orlovi*
- Rhacophorus verrucosus*
- Rhacophorus laoshan*
- Rhacophorus calcaeus*
- Rhacophorus roberteri*
- Rhacophorus bipunctatus*
- Rhacophorus bipunctatus*
- Rhacophorus bipunctatus*
- Rhacophorus rhodopus*
- Rhacophorus rhodopus*
- Rhacophorus kio*
- Rhacophorus reinwardtii*

Other species:

- Polypedates leucomystax*
- Polypedates megacephalus*

Fig. 2. Phylogenetic relationships of species of *Zhangixalus*, *Leptomantis* and *Rhacophorus* based on four mitochondrial genes (12S rRNA, tRNA^{Val}, 16S rRNA and Cyt b) and three nuclear DNA genes (RAG-1, RHOD, TYR). Different colors correspond to three clades: Clade A (blue), Clade B (red), Clade C (green). Numbers beside the nodes are given as Bayesian posterior probabilities (BPP) and bootstrap proportions (BSP) for maximum likelihood analyses. Photographs of *R. laoshan* and *Z. yinggelingensis* are used from [Mo et al. \(2008\)](#) and [Chou et al. \(2007\)](#), respectively.

For *Z. yinggelingensis*, the *p*-distances varied from 3.0% to 7.0% between it and other species of *Zhangixalus* that collected in this study [minimum distance to *Z. duboisi*; maximum distance to *Z. achantharhena* (Supplementary Table S2)]. Additionally, Chou *et al.* (2007) suggested that *Z. yinggelingensis* seems to be closely related to *Z. arvalis*,

Z. aurantiventris, *Z. chenfui*, *Z. dorsovirens*, *Z. hungfuensis*, *Z. taipeianus*, and *Z. yaoshanensis*, *Z. moltrechti*, *Z. nigropunctatus* on the basis of morphological evidences. However, there are many morphologically similar and conservative species in *Zhangixalus* (Li *et al.*, 2012), and we can't distinguish them well. Thus, we suggest that it not only use morphological and molecular datasets, but also their distributions when we identified the species, which can reduce the probability of misidentifications.

Although phylogenetic status of *Z. yinggelingsensis* is not resolved well, we recognize the validity of it using the molecular data for the first time. However, why phylogenetic status of *Z. yinggelingsensis* is not resolved well? The possible reasons are as follows: (1) The sequence of *Z. yinggelingsensis* in this study is short; (2) There are only some species of *Zhangixalus* in this study; (3) the genus *Zhangixalus* evolution history is complex. As genomic data have become more common in phylogenetic studies, many studies have been shown that it can use genomic data resolving both shallow and deep-scale evolutionary relationships (Brandrud *et al.*, 2019; Burbrink and Gehara, 2018; Chen *et al.*, 2019; Godefroid *et al.*, 2019; Kuntner *et al.*, 2019; Spriggs *et al.*, 2019). Thus, it seems possible that we can use genomic data to resolve the phylogenetic status of *Z. yinggelingsensis* in future study. In addition, the research on amphibians in Southern China was scarce, and there have been some cryptic species were discovered in the past three years (Liu *et al.*, 2017; Pan *et al.*, 2017b; Yu *et al.*, 2019). Finally, we suggest that we can strengthen the investigation of amphibian diversity in southern China, which lay a foundation for the study of biogeography in southern China.

CONCLUSIONS

In this study, we have clarified the phylogenetic status of *R. laoshan* and *Z. yinggelingsensis* using mitochondrial fragments (12S rRNA, tRNA^{Val}, 16S rRNA and Cyt *b*) and nuclear DNA (RAG-1, RHOD, TYR) fragments. Our results suggest that *R. laoshan* and *Z. yinggelingsensis* belongs to *Rhacophorus sensu stricto* and *Zhangixalus*, respectively. Finally, this research play an important role in the further studies, such as protection, biogeographical patterns and the evolution of life history strategies of these species.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: <https://dx.doi.org/10.17582/journal.pjz/20200611050629>

Statement of conflict of interest

The authors have declared no conflict of interest.

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