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Prevalence of cryptic species in morphologically uniform taxa – Fast speciation and evolutionary radiation in Asian frogs

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ABSTRACT

Diversity and distributions of cryptic species have long been a vexing issue. Identification of species boundaries is made difficult by the lack of obvious morphological differences. Here, we investigate the cryptic diversity and evolutionary history of an underappreciated group of Asian frog species (*Megophrys*) to explore the pattern and dynamic of amphibian cryptic species. We sequenced four mitochondrial genes and five nuclear genes and delineated species using multiple approaches, combining DNA and mating-call data. A Bayesian species tree was generated to estimate divergence times and to reconstruct ancestral ranges. Macroevolutionary analyses and hybridization tests were conducted to explore the evolutionary dynamics of this cryptic group. Our phylogenies support the current subgenera. We revealed 43 cryptic species, 158% higher than previously thought. The species-delimitation results were further confirmed by mating-call data and morphological divergence. We found that these Asian frogs entered China from the Sunda Shelf 48 Mya, followed by an ancient radiation event during middle Miocene. We confirmed the efficiency of the multispecies coalescent model for delimitation of species with low morphological diversity. Species diversity of *Megophrys* is severely underappreciated, and species distributions have been misestimated as a result.

1. Introduction

Cryptic species are morphologically indistinguishable and mistakenly grouped as a single nominal species (Bickford et al., 2007). While they are hard to tell apart visually, these taxa diverge in their mating signals, interrupting gene flow. Acoustic and pheromones signals are typical in insects (Henry, 1994) and vertebrates, including bats (Jones and Barlow, 2004) and frogs (Narins, 1983). Additionally, species inhabiting extreme environments may be under selection for uniform morphology while maintaining ecological adaptations (Schönrogge et al., 2002). Since morphological characters are of no help in species delimitation in such taxa, molecular genetic data have been widely applied to discover cryptic species (Harrington and Near, 2011; Hedin, 2015; Satler et al., 2013). The multispecies coalescent model is the most popular among various approaches that leverage DNA sequence variation to diagnose species (Fujita et al., 2012). However, distinguishing between within-species population structure and among-species divergence remains a challenge (Sukumaran and Knowles, 2017). In addition, most studies delimit species only on the basis of molecular data. As speciation is a continuous process, it is hard to make a cut-off to identify the boundaries, especially when recent introgression is present (Wiens, 2007). Approaches that combine phenotypic and genotypic evaluations are thus potentially more fruitful than either method in isolation.

Here, we sought to explore the species boundaries and the dynamics of *Megophrys*. *Megophrys* species are widely distributed in the eastern and central Chinese mainland, throughout southeastern Asia, and extending to the islands of the Sunda Shelf and the Philippines (Fei et al.,

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2009; Li et al., 2014; Wang et al., 2014). Seven subgenera (Panophrys, Xenophrys, Ophyrophryne, Brachytarsophrys, Atympanophrys, Megophrys, and Pelobatrachus) are currently recognized as members of this genus, comprising approximately 70 species (Mahony et al., 2017; Orlov et al., 2015; Poyarkov Jr et al., 2017; Zhang et al., 2017). The taxonomic status of this group has been controversial due to a lack of molecular phylogenetic and comparative morphological studies (Delorme et al., 2006; Dubois and Ohler, 1998; Fei et al., 2009; Frost et al., 2006; Jiang et al., 2002; Khonsue and Thirakhupt, 2001; Li et al., 2011; Mahony et al., 2011; Ohler, 2003; Pyron and Wiens, 2011; Rao and Yang, 1997; Wang et al., 2012). Only two recent studies used comprehensive data to explore phylogenetic relationships within this group (Chen et al., 2017; Mahony et al., 2017).

Although horned frogs are reportedly widespread in southeastern China, many new species have been published in the past few years (Li et al., 2014; Poyarkov Jr et al., 2017; Wang et al., 2012; Wang et al., 2014; Zhao et al., 2014). Our long-term field investigations and recent publications suggest that the species diversity in this group is greatly underestimated. *Megophyrs* is not prone to dispersal and thrives only in specific habitats (Fei et al., 2009). Morphological similarity often hinders exact species recognition. This leads to misidentification of endemic species as geographic populations of a widely-distributed species, a problem only partially corrected in recent studies. Thus, the real distribution of these species remains to be determined, and it is reasonable to question the correctness of field records. To begin studying the evolutionary history and adaptation within the genus, it is necessary first to understand the phenotypic diversity and geographical distributions of *Megophrys* species.

In the present study, we delimit cryptic species using multiple approaches and confirm the results using morphological characters, revealing striking hidden diversity and distributional heterogeneity. Reconstruction of diversification history reveals an intriguing dispersal pattern of *Megophrys* and an ancient evolutionary radiation within the Panophrys group triggered by drastic climatic change and ancient hybridization. According to records and our results, *Megophrys* appears to be the most diverse amphibian group in China (Fei et al., 2009; Fei et al., 2010). Our results demonstrate the effectiveness of the multispecies coalescent model and emphasize the importance of sexual traits to confirm boundaries of cryptic species. Finally, we discuss the possibility of using cryptic groups such as Panophrys as new models to study patterns and dynamics of speciation and evolutionary radiation.

2. Material and methods

2.1. Sampling and PCR amplification

We sampled 293 individuals in total (Table S1 in Supplementary Material), including 243 from Panophrys, 23 from Xenophrys, 11 from Ophryophryne, six from Atympanophrys, and five from Brachytarsophrys. The extensive sampling of Panophrys was conducted to evaluate its evolutionary history. In addition, six individuals from *Leptolalax* were collected to use as an outgroup. All specimens were collected during field surveys from 2008 to 2016 (Fig. 1). Muscle tissues were collected in 95% ethanol for preservation. DNA was extracted from each muscle tissue sample using a standard extraction kit (Tiangen Biotech, Beijing, China). Four mitochondrial genes (16S, 12S, *CO1* and *CYTB*) and five nuclear genes (*CXCR-4*, *RAG-1*, *RAG-2*, *DISP-2* and *SALL-1*) were amplified in this study. Primers used in this study are listed in Supplementary Material, Table S2. PCR amplifications were performed in a 20 uL reaction volume with the following cycling conditions: an initial denaturing step at 95 °C for 4 min, 35 cycles of denaturation at 94 °C for 40 s, annealing at 45–53 °C for 40 s, extension at 72 °C for 1 min, and a final extension step of 72 °C for 10 min for mitochondrial genes. The *CXCR-4* gene was amplified using the protocol developed by Biju and Bossuyt, (2003). The other nuclear genes were amplified using a nested-PCR protocol (Shen et al., 2013). PCR products were purified using spin columns. The purified products were sequenced with both forward and reverse primers using the BigDye Terminator Cycle Sequencing Kit on an ABI Prism 3730 automated DNA analyzer according to the manufacturer's guidelines. All sequences were deposited in GenBank with accession numbers (Table S1 in Supplementary Material).

2.2. Phylogenetic analysis

Sequences were aligned in MEGA6 using the Clustal W algorithm with default parameters (Tamura et al., 2013). To compare our new sequences with ones downloaded from GenBank, we used seven genes

Fig. 1. Sampling map. Dots represent sampling localities. Numbers in dots correspond to locations listed in the Supplementary Material, Table S1.