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Molecular phylogenetics and dating of the problematic New Guinea microhylid frogs (Amphibia: Anura) reveals elevated speciation rates and need for taxonomic reclassification

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ABSTRACT

Asterophryinae is a large monophyletic subfamily of Anurans containing over 300 species distributed across one of the world's most geologically active areas – New Guinea and its satellite islands, Australia and the Philippines. The tremendous ecological and morphological diversity of this clade, with apparent specializations for burrowing, terrestrial, semi-aquatic, and arboreal lifestyle, suggests an evolutionary process of adaptive radiation. Despite this spectacular diversity, this and many other questions of evolutionary processes have received little formal study because until now the phylogeny of this spececies-rich clade has remained uncertain. Here we reconstruct a phylogeny for Asterophryinae with greatly increased taxon and genetic sampling relative to prior studies. We use Maximum Likelihood and Bayesian Inference methods to produce the most robust and comprehensive phylogeny to date containing 155 species using 3 nuclear and 2 mitochondrial loci. We also perform a time calibration analysis to estimate the age of the clade. We find support for the monophyly of Asterophryinae as well as need for taxonomic reclassification of several genera. Furthermore, we find increased rates of speciation across the clade supporting the hypothesis of rapid radiation. Lastly, we found that adding taxa to the analysis produced more robust phylogenetic results over adding loci.

1. Introduction

The subfamily Asterophryinae is the largest part of one of the largest amphibian families in the world with nearly 300 described species of frogs (AmphibiaWeb; http://www.amphibiaweb.org/, accessed February 2017). The majority of this diversity is centered in the Papuan region (comprising New Guinea and its satellite islands, the Admiralty and Bismark Archipelagos, and the Solomon Islands) and extends into northeastern Australia, the southern Philippines, and the eastern islands of the Sunda Shelf. Peloso et al. (2015) found asterophryine to be monophyletic and has most recently been organized into 21 genera. However, intergeneric relationships remain uncertain despite decades of study, hampering further evolutionary studies of this group.

In addition to its prolific numbers of species, Asterophryinae frogs are most notable for their high degrees of endemism and ecological diversity relative to the other four anuran families in the region. Taxonomists have identified six types of burrowing (fossorial), terrestrial, semi-aquatic, scansorial, and arboreal forms (Zweifel and Tyler, 1982) or putative "ecomorphs," indicating exceptional morphological disparity. While many questions remain unsettled in the Asterophryinae phylogeny, some of the ecomorphs appear in seemingly unrelated lineages (Köhler and Günther, 2008), suggesting repeated, independent evolution to novel lifestyles. Indeed, Asteroprhyinae is an excellent candidate adaptive radiation - a rapidly radiating lineage coupled with ecological diversification (Schluter, 2000).

The great ecological and species diversity may be linked in part to the geological history of New Guinea. The Papuan region lies at the junction of three active plates: the northward-moving Australian Plate, the west-northward-moving Pacific Plate the stable Eurasian Plate (Kroenke, 1996, 1984; Hall, 1998; Klootwijk et al., 2003), which have combined to move entire island-arc systems as well as shearing off and rearranging sections of continental land masses. These tectonic activities have resulted in a great expansion of land area as both island arc systems and offshore terranes have

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sequentially collided to form the large present-day New Guinea island and its smaller offshore islands (Pigram and Davies, 1987; Klootwijk et al., 2003). In addition, these large-scale geological events, such as the docking of the Fold Belt, have given rise to the high mountains traversing central New Guinea creating topographical complexity and further expanding ecological opportunity. It seems likely that Asterophryinae was the first anuran lineage to colonize New Guinea as the island is estimated to be approximately 30 MY old (Davies et al., 1996, 1997), consistent with the lower end of the 30–60 MY range estimated by other molecular phylogenetic studies on Asterophryinae, and it is the most widespread and diverse anuran lineage (Roelants et al., 2007; van Bocxlaer et al., 2006; van der Meijden et al., 2007).

1.1. Taxonomic problems

Many previous studies have used morphological characters to infer the Asteroprhyinae phylogeny, however, some of the character data contain insufficient information to resolve the phylogeny. For example, the clavicles and procoracoids have been lost several times independently and are thought to be homoplastic traits (Burton, 1986; Duellman and Trueb, 1986) and therefore not informative regarding evolutionary relationship. There is also great variability in the cranial elements (Burton, 1986), which may potentially lead to erroneous conclusions about species relationships. It is therefore not entirely surprising that homoplasy is common, yielding conflicting phylogenetic conclusions based on morphological data (Burton, 1986; Menzies, 2006; Zweifel, 1972, 2000). As a result, taxonomy of the Asterophryinae has been fluid throughout the 20th Century, increasing from nine genera (Parker, 1934) to 21 in the span of 70 years, with several genera variously synonymized, resurrected, or redefined (Burton, 1986; Burton and Zweifel, 1995; Frost et al., 2006; Günther, 2009; Kraus, 2013; Menzies and Tyler, 1977; Peloso et al., 2015; Zweifel, 1956, 2000; Zweifel and Allison, 1982; Zweifel and Parker, 1989).

Few genera have been explicitly defined on the basis of synapomorphies; it is possible that some groups are paraphyletic or polyphyletic (Köhler and Günther, 2008). Köhler and Günther (2008) provided the most complete asterophryine phylogeny to date but were only able to sample one-fifth of the total current species, lacked some genera and ecomorphs, and lacked high support values for some newly proposed relationships. Consequently, relationships among many asterophryine taxa remain uncertain.

All currently recognized asterophryine genera merit explicit testing for monophyly given the uncertain evidence often available from morphology. Most hypotheses of monophyly to have been implicit in current or prior taxonomies of asterophryines. These include testing the monophyly of each of the 21 recognized genera. Furthermore, the synonymization or division of taxa proposed by previous studies should be formally tested, including the following hypotheses: (a) the synonymy of *Albericus* + *Choerophryne* (Kraus, 2013; Peloso et al., 2015); (b) the synonymy of *Albericus* + *Aphantophryne* + *Cophixalus* + *Copiula* + *Choerophryne*; (c) the division of *Asterophrys* into *Asterophrys* and *Hylophorbus* (Zweifel, 1972); (d) the division of Sphenophryne into *Sphenophryne*, *Austrochaperina, Oxydactyla*, *Liophryne* (Zweifel, 2000); and (e) the synonymy of *Xenorhina* + *Xenobatrachus* (Frost et al., 2006). Köhler and Günther (2008) suggested that several of these hypotheses are probably false, but they lacked the degree of resolution and taxonomic sampling required to make firm conclusions. Because taxonomic revision should be based on robust resolution of phylogenetic relationships, more comprehensive testing of each group's monophyly is required to achieve a reliable taxonomy and to promote further evolutionary studies.

Here, we construct the most comprehensive and well-supported molecular phylogeny to date for the Asterophryinae clade in order to clarify higher taxonomic units and intergeneric relationships. We will explicitly test the hypothesis of monophyly of the asterophryine lineage and hypotheses (a)–(e) above. We use this phylogeny to infer the number of independent origins of each of the several ecomorphs. Furthermore, we perform a time-calibration analysis to estimate the age of the clade and explore whether major geological events may have facilitated diversification within this clade. We also perform an ancestral-reconstruction analysis to test whether lineages with the same ecology are closely related or have arisen independently. Lastly, we comment on challenges encountered during our reconstruction of the phylogeny, our solutions to them, and suggestions for further research to resolve uncertainties.

2. Material and methods

2.1. Specimens and genetic sequencing

Our study included 155 species of Asterophryinae representing 21 proposed genera. We obtained liver samples from specimens housed at the Bishop Museum, Honolulu, Hawaii (BPBM), Museum of Vertebrate Zoology at Berkeley (UMZ), University of Michigan Museum of Zoology (UMMZ), Zoologisches Museum Berlin (ZMB), and University of Kansas Biodiversity Institute and Natural History Museum (KU; see appendix for specimen list), as well as liver tissue collected in the field by the authors. We rooted the phylogeny using 3 outgroup taxa that are thought to include the sister taxon and more distantly related lineages – *Dyscophus antongilii*, *Scaphiophryne marmorata*, and *Platypelis grandis* (van der Meijden et al., 2007). We sequenced three unlinked nuclear loci and two mitochondrial loci: Seventh in Absentia (*SIA*), Brain Derived Neurotrophic Factor (*BDNF*), Sodium Calcium Exchange subunit-1 (*NXC-1*), Cytochrome oxidase *b* (*Cyt b*), and NADH dehydrogenase subunit 4 (*ND4*), resulting in a total of ~2800 base pairs of sequence data (primer details in Table 1). Originally, we amplified three additional loci (*Rag-1*, *Rag-2*, and *BMP2)* but we were not confident in these data because they had high discordance among sequences. To eliminate the possibility of including non-homologous non-coding duplications, these loci were not used in the analysis.

DNA sequencing followed standard protocols. Total DNA was extracted from liver tissue using a Qiagen DNA extraction kit. We performed bidirectional PCR amplification using 25 µl reactions containing 0.25 units of GOTaq DNA polymerase (Promega), <50 ng/µl genomic DNA, 0.5 µmol of each primer, 15 nmol of dNTP, and PCR buffer. We used an initial denaturing period of 95 °C for 5 min, followed by 35 cycles of 95 °C for 45 s, annealing temperatures for 30 s, and an extension period of 72 °C for 60 s followed by a final extension period of 5 min. Annealing temperature is as follows for each locus: 62 °C for *SIA*, 56 °C for *BDNF*, 55 °C for *NXC-1*, 57 °C for *Cyt b* and 51.4 °C for *ND4*. We cleaned the reactions with Exo-SAP and sequenced using Applied Biosystems BigDye terminator chemistry on an ABI 3730XL sequencer following standard protocols. Sequencing was conducted at the University of Hawaii at Manoa's Advanced Studies of Genomics, Proteomics and Bioinformatics facility.

2.2. Sequence alignment and phylogenetic analyses

We prepared the sequence data for analysis as a concatenated dataset as well as a locus-by-locus partitioned dataset. We read and manually edited each sequence using Sequencher v5.2 (Sequencher v5.2). We aligned the sequences using ClustalX using