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Phylogenomic support for evolutionary relationships of New World direct-developing frogs (Anura: Terraranae)

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ABSTRACT

Phylogenomic approaches have proven able to resolve difficult branches in the tree of life. New World direct-developing frogs (Terraranae) represent a large evolutionary radiation in which interrelationships at key points in the phylogeny have not been adequately determined, affecting evolutionary, biogeographic, and taxonomic interpretations. We employed anchored hybrid enrichment to generate a data set containing 389 loci and > 600,000 nucleotide positions for 30 terraranan and several outgroup frog species encompassing all major lineages in the clade. Concatenated maximum likelihood and coalescent species-tree approaches recover nearly identical topologies with strong support for nearly all relationships in the tree. These results are similar to previous phylogenetic results but provide additional resolution at short internodes. Among taxa whose placement varied in previous analyses, *Ceuthomantis* is shown to be the sister taxon to all other terraranans, rather than deeply embedded within the radiation, and Strabomantidae is monophyletic rather than paraphyletic with respect to Craugastoridae. We present an updated taxonomy to reflect these results, and describe a new subfamily for the genus *Hypodactylus*.

1. Introduction

Phylogenomic approaches reliant on high-throughput sequencing technologies have emerged as a viable approach to infer evolutionary histories of lineages in large radiations (Delsuc et al., 2005; Lemmon et al., 2012; McCormack et al., 2012). Patterns of relationships in some vertebrate mega-radiations that were unsettled using traditional multigene phylogenetic methods have proven resolvable when phylogenomic-scale data sets drawing from hundreds or thousands of loci and millions of nucleotides were employed. Well known longstanding vertebrate phylogenetic problems, including unsettled relationships among avian orders and among spiny-rayed fish families, have been resolved through use of phylogenomic-scale data (Faircloth et al., 2013; Hackett et al., 2008; Jarvis et al., 2014; Li et al., 2007). In addition to these well-known examples, phylogenomic data sets have been used to good effect in inferring phylogenies in many other vertebrate groups as well (e.g. Brandley et al., 2015; Crawford et al., 2015; Shen et al., 2013; Song et al., 2012).

Terraranae is a frog clade of more than 1065 named species, comprising nearly 15% of all amphibians (AmphibiaWeb, 2017; Frost, 2017) and an ideal target for phylogenomics because of controversial high-level relationships (Hedges et al., 2008a; Heinicke et al., 2009; Pyron and Wiens, 2011; Padial et al., 2014). This clade of Western Hemisphere direct-developing frogs (= terraranan frogs) has an unranked name emended from Terrarana (Hedges et al., 2008a) to Terraranae by Duellman et al. (2016). A ranked taxon name (superfamily Brachycephaloidea) could be used instead, but as in previous studies (Hedges et al., 2008a; Duellman et al., 2016) we reject a ranked name because it would unnecessarily constrain taxonomic expansion in this already large clade whose taxonomy continues to grow rapidly.

For many years, most species of terraranans were placed in a single genus, *Eleutherodactylus*, due in part to a lack of easily applied external morphological traits to identify subgroupings (Lynch and Duellman, 1997). Later, multi-gene molecular phylogenetic analyses were used to determine the broad pattern of relationships in Terraranae, to revise the taxonomy of the group, and to identify major macroevolutionary and biogeographic patterns (Amaro et al., 2013; Canedo and Haddad, 2012; Crawford and Smith, 2005; Fouquet et al., 2012; Gonzalez-Voyer et al., 2011; Hedges et al., 2008a; Heinicke et al., 2007, 2009, 2015; Mendoza et al., 2015; Padial et al., 2009, 2014; Pinto-Sánchez et al., 2012, 2014;

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Pyron and Wiens, 2011). Most of these studies used the mitochondrial 12S and 16S genes alone or in combination with a small set of nuclear markers. While all showed similar patterns, key nodes in the tree of terraranans remain unresolved, and there is some conflict in published results. Much of this conflict and lack of resolution involves genera placed in the families Ceuthomantidae, Craugastoridae, and Strabomantidae (recognized as 1–3 families depending on source; AmphibiaWeb, 2017; Frost, 2017; IUCN, 2016), which rapidly diverged from one another in a short timeframe in the early to middle Cenozoic (Heinicke et al., 2007, 2009). This lack of resolution has led not only to differing taxonomic interpretations; it also hinders a full understanding of macroevolutionary and biogeographic patterns of terraranans, as genera placed in each putative family are most diverse in different areas within the Neotropics. A phylogenomic analysis has the potential to provide needed resolution.

Several classes of phylogenomic data have been used to determine relationships among vertebrate lineages. These include RAD-Seq data as well as data obtained via sequence-capture methods such as anchored hybrid enrichment, ultraconserved elements, or transcriptome-based exon capture (Bi et al., 2012; Cruaud et al., 2014; Lemmon and Lemmon, 2013; Lemmon et al., 2012; McCormack et al., 2012, 2013). In general, sequence-capture methods show utility at a variety of phylogenetic depths, and produce data sets easily compared across species. RAD-Seq, on the other hand, produces data sets with potentially low overlap among species, making it better suited for phylogeographic studies (Harvey et al., 2016). Therefore, sequence-capture approaches hold the most promise for determining evolutionary relationships among terraranan frog genera.

We have generated a sequence-capture data set using the anchored hybrid enrichment approach for a set of terraranan frog species representing most genera and all known major lineages in order to clarify evolutionary relationships of New World direct-developing frogs. We employ both concatenated and species-tree approaches in analyzing these data, and employ separate likelihood tests of phylogeny to assess alternative hypotheses for the placement of three genera whose phylogenetic position has important taxonomic implications: *Ceuthomantis*, *Haddadus*, and *Strabomantis* (Heinicke et al., 2009; Padial et al., 2014; Pyron and Wiens, 2011). We also compare anchored hybrid-enrichment results to results from traditional multi-gene phylogenetic data sets in order to assess the reliability of published phylogenies obtained using the most commonly employed mitochondrial and nuclear markers in amphibian phylogenetics.

2. Materials and methods

2.1. Taxon sampling

We included 30 ingroup and five outgroup species in our analyses (Table 1). We chose ingroup specimens for inclusion to maximize taxonomic breadth and to provide exemplars to test alternative hypotheses of relationships suggested in previous molecular phylogenetic studies (Heinicke et al., 2007, 2009; Hedges et al., 2008a; Pyron and Wiens, 2011; Padial et al., 2014). The 30 ingroup taxa are sampled from 18 genera and include multiple representatives of all families and subfamilies recognized in the preceding studies; the most species-rich genera (*Craugastor*, 115 sp.; *Eleutherodactylus*, 191 sp.; *Pristimantis*, 505 sp.) are each represented by multiple samples drawn by different subgenera or clades. Outgroup taxa include four additional neobatrachians (*Bufotes viridis*, *Nanorana parkeri*, *Osteopilus septentrionalis*, *Pseudacris regilla*) with the non-neobatrachian species *Xenopus tropicalis* serving as the most distant outgroup. *Nanorana* and *Xenopus* sequences were derived from published genome assemblies (Sun et al., 2015; Hellsten et al., 2010), and *Bufotes* and *Pseudacris* sequences from published transcriptomes (Gerchen et al., 2016; Robertson and Cornman, 2014). All other sequences were newly generated. Sequence-capture was attempted but yielded poor quality sequences unable to be included in analysis for an additional five ingroup and one outgroup taxa (*Bryophryne cophites*, *Craugastor daryi*, *Eleutherodactylus zeus*, *Noblella lochites*, *Psychrophrynella wettsteini*, *Hemiphractus proboscideus*). Two of the five failed ingroup sequences have congeners represented in the final data set (*Craugastor*, *Eleutherodactylus*), and the other three (*Bryophryne*, *Noblella*, *Psychrophrynella*) belong to an unambiguously monophyletic subfamily with two other genera that were included in the final data set (*Barycholos*, *Holoaden*), suggesting that the exclusion of these samples has little effect on inferences that can be made regarding broad phylogenetic patterns of terraranan frogs.

2.2. Locus selection and probe design

Target loci were derived from the 394 loci of Prum et al. (2015) and Ruane et al. (2015), which were derived from the original 512 vertebrate targets of Lemmon and Lemmon (2013). Amphibian orthologs of these loci were first identified in *Xenopus tropicalis* (Pipidae) using the UCSC Genome Browser (liftover) tool (https://genome.ucsc.edu/cgi-bin/hgLiftOver) to convert genomic coordinates between the human genome (hg19) to the *Xenopus tropicalis* genome (xenTro3). After extracting the corresponding genomic regions in the *Xenopus* genome, orthologous sequences were obtained from ~15x raw genomic reads (Illumina 2500 paired-end 150 bp) of three frog species, *Rana sphenocephala* (Ranidae), *Pseudacris feriarum* (Hylidae), and *Pseudacris nigrita* (Hylidae), following methods described in Ruane et al. (2015), but with the *Xenopus* sequences used as the reference. After aligning the sequences in MAFFT v. 7.023b (Katoh and Standley, 2013, the alignments were inspected in Geneious v. 8.1.8 in order to identify obviously misaligned and/or paralogous sequences. A total of 364 loci were retained after removing loci with poor taxon representation. The average target locus length was 1205 bp. Probes were tiled at 1.2x coverage. Orthologous sequences for 9 additional amphibians were identified by Hime et al. (in preparation) in order to produce an amphibian-wide probe set with increased enrichment efficiency in Ranoidea and Hyloidea; these were used to refine probe design but the sequences themselves are not included in this study.

2.3. Sample preparation and sequencing

New sequence data were generated using the anchored hybrid enrichment method (Lemmon et al., 2012) implemented at the Center for Anchored Phylogenomics (www.anchoredphylogeny.com). DNA extraction was performed using Qiagen DNeasy blood and tissue kits with an additional ethanol purification step to eliminate contaminants. Following extraction, DNA quantity and quality were assessed with a Qu-Bit fluorometer and a 2% TAE agarose gel, respectively. The enrichment and sequencing process followed established protocol (Pyron et al., 2014; Ruane et al., 2015; Tucker et al., 2016; Domingos et al., 2017). Briefly, DNA was fragmented to a size of 150–300 bp using a Covaris E220 sonicator, with indexed library preparation performed using a Beckman-Coulter Biomek FXp liquid-handling robot. Libraries were pooled in two sets for the enrichment step using an Agilent Custom SureSelect kit containing the amphibian-specific probes described above. Following enrichment, the samples were sequenced on one lane using an Illumina HiSeq 2000 PE150 sequencer housed at the Translational Science Laboratory in the Florida State University College of Medicine.

2.4. Sequence assembly and alignment

After sequencing, paired reads were merged using the method described in Rokyta et al. (2012), which merges reads only when the minimum probability of obtaining the observed number of overlapping matches by chance alone is less that 10^{-10} and the next smallest probability is more than 1000 times as large. Merged reads were then assembled using the method described in Ruane et al. (2015), using