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

Matthew P. Heinicke[a,*], Alan R. Lemmon[b], Emily Moriarty Lemmon[b], Kathleen McGrath[c], S. Blair Hedges[c]

[a] Department of Natural Sciences, University of Michigan-Dearborn, 4901 Evergreen Road, Dearborn, MI 48128, USA
[b] Department of Biology, Florida State University, 319 Stadium Drive, P.O. Box 3064295, Tallahassee, FL 32306-4295, USA
[c] Center for Biodiversity, Temple University, 1925 N 12thStreet, Philadelphia, PA 19122, USA

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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;

* Corresponding author.
E-mail addresses: heinicke@umich.edu (M.P. Heinicke), alemmon@fsu.edu (A.R. Lemmon), chorusfrog@bio.fsu.edu (E.M. Lemmon), sbh@temple.edu (S.B. Hedges).

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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

Table 1
Species included in phylogenetic analyses. Specimen codes are as follows: AMNH (American Museum of Natural History), CVULA (Collection of Vertebrates, University of the Andes, Mérida, Venezuela), KU (University of Kansas Natural History Museum), LSUMZ (Louisiana State University Museum of Zoology), MVZ (Museum of Vertebrate Zoology, University of California, Berkeley), QCAZ (Catholic University of Ecuador, Museum of Zoology), ROM (Royal Ontario Museum), SBH (S. Blair Hedges tissue collection), USNM (U.S. National Museum of Natural History, Smithsonian Institution).

Species	Specimen	Locality
Adelophryne patamona	ROM 39578	Guyana: District 7, Mount Ayanganna
Barycholos pulcher	KU 217781	Ecuador: Pichincha, 15 km NE Alluriquin
Brachycephalus ephippium	USNM 207716-22	Brazil: São Paulo, Eugenio Lefevre
Bufotes viridis	N/A	N/A
Ceuthomantis smaragdinus	ROM 40161	Guyana: District 7, Mount Ayanganna
Craugastor (*Craugastor*) *podiciferus*	MVZ 12020	Costa Rica: Heredia, Chompipe, vicinity of Volcán Barba
Craugastor (*Hylactophryne*) *stuarti*	MVZ 143310	Guatemala: Huehuetenango, Finca Chiblac, ca. 12 km N Barillas
Diasporus diastema	MVZ 203844	Costa Rica: Cartago, 1.9 km S Tapanti Bridge over Río Grande de Orosi
Eleutherodactylus (*Eleutherodactylus*) *abbotti*	USNM 564974	Dominican Republic: Pedernales, Alcoa Road
Eleutherodactylus (*Euhyas*) *planirostris*	USNM 565007	Bahamas: New Providence, Nassau
Eleutherodactylus (*Pelorius*) *inoptatus*	SBH 267595	Dominican Republic: Pedernales, Alcoa Road
Eleutherodactylus (*Schwartzius*) *counouspeus*	SBH 269578	Haiti: Massif de la Hotte
Eleutherodactylus (*Syrrhophus*) *marnockii*	SBH 194156	USA: Texas, Austin
Haddadus binotatus	USNM 303077	Brazil: São Paulo, Estação Biológica de Boracéia
Holoaden bradei	USNM-FS 4071	Brazil: Minas Gerais
Hypodactylus dollops	SBH 267862	Colombia
Ischnocnema guentheri	USNM-FS 053312	Brazil: São Paulo, Estação Biológica de Jureia
Lynchius flavomaculatus	KU 218210	Ecuador: Loja, 19.4 km S Yangana
Nanorana parkeri	N/A	N/A
Oreobates saxatilis	KU 212327	Peru: San Martin, Ponga de Shilcayo, ca 4 km NNW Tarapoto
Osteopilus septentrionalis	SBH 267446	Cuba: La Habana
Phrynopus bracki	USNM 286919	Peru: Pasco, 2.9 km N, 5.5 km E Oxapampa
Phyzelaphryne miriame	LSUMZ 16935	Brazil: Amazonas
Pristimantis (*Hypodictyon*) *cremnobates*	KU 177252	Ecuador: Napo, Rio Salado
Pristimantis (*Hypodictyon*) *ridens*	AMNH A124551	Panama: Cocle, El Valle
Pristimantis (*Pristimantis*) *bipunctatus*	KU 291638	Peru: Pasco, 4.5 km E Oxapampa
Pristimantis (*Pristimantis*) *curtipes*	KU 217871	Ecuador: Pichicha, Bosque de Pasocha
Pristimantis (*Pristimantis*) *stictogaster*	KU 291659	Peru: Pasco, 2.9 km N, 5.5 km E Oxapampa
Pristimantis (*Pristimantis*) *thymelensis*	QCAZ 16428	Ecuador: Napo, Paramo de Guamani
Pristimantis (*Pristimantis*) *unistrigatus*	KU 218057	Ecuador: Imbabura, 35 km E Pquela
Pristimantis (*Pristimantis*) *vanadise*	SBH 268288	Venezuela: Mérida, La Manca, Quebrada La Rana
Pseudacris regilla	N/A	N/A
Strabomantis biporcatus	CVULA 7073	Venezuela: Sucre, Parque Nacional de París, Las Melenas, Peninsula de París
Tachiramantis prolixodiscus	SBH 268371	Venezuela
Xenopus tropicalis	N/A	N/A

Pseudacris nigrita and *Rana sphenocephala* probe region sequences as the references for assembly. Assembly clusters of fewer than 175 reads were removed from downstream analyses. Following assembly, orthologous sets with assembled sequences for more than half of the 31 sequenced taxa were retained for analysis. These orthologs were aligned using MAFFT 7 (Katoh and Standley, 2013) using the – genafpair and –maxiterate 1000 settings and the following default parameters: group-to-group gap opening penalty = 1.53, group-to-group offset value = 0.123, local pairwise gap opening penalty = −2, local pairwise offset value = 0.1, local pairwise gap extension penalty = −0.1, gap opening penalty to skip alignment = −6, gap extension penalty to skip alignment = 0. The alignments were then trimmed using the criteria described in Ruane et al. (2015), but with the following exceptions: good alignment sites are defined as any site in which 30% of the sequences have an identical nucleotide; within each sequence, any 20 bp region containing fewer than 10 good alignment sites is masked; and any alignment site with fewer than 15 unmasked sequences is deleted.

Additional outgroup sequences were then added to the alignment by performing BLAST searches for each locus against the *Xenopus tropicalis* v9.0 genome assembly (available at www.xenbase.org), the *Nanorana parkeri* v2 genome assembly (available at http://dx.doi.org/10.5524/100132), and transcriptomes of *Bufotes viridis* and *Pseudacris regilla* (NCBI BioProjects PRJNA292872 and PRJNA163143). For each locus, a full length query sequence was chosen at random from the available ingroup samples, and a blastn search was performed under the following parameters: minimum E-value = 10, word size = 11, gap opening penalty = 5, gap extension penalty = 2, match reward = 2, mismatch penalty = 3, and maximum number of target sequences = 1. Homologous sequences of longer than 100 bp were incorporated into the alignment using the –add fragments command in MAFFT. Following this step, the final alignment was quality checked by estimating phylogenies of each locus using RAxML 8.2.8 (Stamatakis, 2014) under a GTR + gamma model of evolution, and inspecting the individual phylogenetic trees by eye for exceptionally long terminal branches (i.e., > 4× as long as any other terminal branch) indicative of low-quality alignment or non-orthology for a particular sequence at a particular locus. All such sequences were removed from the alignment prior to analysis, resulting in removal of 114 outgroup sequences identified in the BLAST searches prior to final analyses.

2.5. Phylogenetic analysis

We employed both concatenated and species-tree approaches in generating phylogenies. The primary concatenated analysis was performed using RAxML 8.2.8, with the full alignment partitioned by gene and the GTR + gamma model employed. One hundred independent analyses were run, and support values for the best-scoring tree of these 100 runs were obtained by performing 1000 rapid bootstrap replicates using the same partitions and model. In addition to the likelihood analysis, a maximum parsimony analysis was performed using MEGA 7 (Kumar et al., 2016), and employed a tree-bisection-reconnection search strategy on 10 random-addition starting trees, with 1000 bootstrap replicates used to assess topological support.

The species-tree analysis was performed using ASTRAL 4.10.12 (Mirarab and Warnow, 2015), which uses individual gene trees as

inputs. Input trees were generated in PhyML 3.1 (Guindon et al., 2010). Best-fitting models of evolution for each individual gene tree were identified using the model-selection tool in the command-line version of MEGA (Kumar et al., 2012), which estimates parameters for 24 common models (Jukes-Cantor, Kimura-2-parameter, Tamura 92, Tamura-Nei, Hasegawa-Kishino-Yano, and General Time Reversible, with all combinations of presence/absence for gamma parameter and invariant sites) and identifies the best fit based on the Bayesian Information Criterion. The best-fitting or most similar available model in PhyML was then employed in order to estimate each gene tree. One hundred bootstrap replicates were obtained for each gene tree using the rapid bootstrapping option in RAxML, under the GTR + gamma model. Once gene trees were estimated, the ASTRAL analysis was performed using the heuristic method, which constrains bipartitions in the output species tree to appear in at least one of the input set of unrooted gene trees (Mirarab et al., 2014), rather than the exact method, which considers all possible bipartitions but is not computationally feasible for a data set with 35 taxa. Support for the tree as a whole was assessed by calculating quartet support (the proportion of gene trees that support a particular node). Branch support was assessed in two ways. First, we performed 100 multi-locus bootstrap replicates. Second, we assessed local quartet support as local posterior probabilities computed as a function of the number of genes and quartet frequency for each branch (relationship described in Sayyari and Mirarab, 2016), which provides a measure of gene tree discordance at particular nodes: nodes with lower values show more discordance in input gene trees.

BEAST 2.4.6 (Bouckaert et al., 2014) was also used to perform both concatenated (standard BEAST) and species tree (*BEAST) analyses in a Bayesian framework. Due to computational limitations, the data matrix for the BEAST analyses was limited to the 50 genes that were complete for all taxa. Each analysis was partitioned by gene, using the best-fitting or most similar available models of evolution previously identified in MEGA. Both analyses used Yule tree priors and uncorrelated lognormal relaxed clocks. The concatenated analysis was run for 575 million generations and the species tree analysis for 1.1 billion generations, with 25 million generations discarded as burn-in. Inspection of effective sample size values of parameters to ensure sample sizes of at least 100 for all parameters showed that the concatenated analysis had adequate sampling for all parameters, while the species tree analysis did not, so the species tree analysis was discontinued after 40 days and not used to infer results.

2.6. Phylogenetic hypothesis testing

We tested three hypotheses of phylogenetic relationships under a ML framework by using the approximately unbiased (AU) test (Shimodaira, 2002). The best-scoring unconstrained tree was tested against the following best-scoring constrained trees: (1) a tree constrained to have *Pristimantis* and *Ceuthomantis* as closest relatives (matching the relationships found in Padial et al., 2014), (2) a tree constrained to have *Strabomantis* as most closely related to *Craugastor*, *Haddadus*, and *Tachiramantis* (matching a relationship found in Pyron and Wiens, 2011), and (3) a tree constrained to have *Haddadus* as the sister taxon to a clade comprising the remaining Craugastoridae + Strabomantidae (matching results we find in one analysis described below). The constrained phylogenies were estimated in RAxML using the same models, settings, and partitions as used in the primary concatenated ML analysis, and once final constrained phylogenies were obtained the log likelihoods were calculated and the AU test was performed in IQ-TREE (Nguyen et al., 2015) using the "-au" option and 10,000 bootstrap replicates.

2.7. Phylogenetic comparisons

We used the K tree score computed with the program Ktreedist (Soria-Carrasco et al., 2007) to compare the concatenated ML phylogeny obtained in this study set with the ML phylogeny from Heinicke et al. (2015). This metric allows congruence in both tree topology and in estimates of rate variation among lineages (i.e., relative branch lengths) to be measured, while scaling for differences in global branch lengths. The ML tree from Heinicke et al. (2015) was chosen for comparison because it was estimated using the same set of genes that most previous terraranan phylogenetic studies have employed (*12S*, *16S*, *RAG1*, and *TYR*), and because it shares the most taxa in common with this study. The species tree could not be used in this comparison because ASTRAL does not calculate lengths of terminal branches. Non-overlapping taxa were pruned from the two trees using the R package Ape (Paradis et al., 2004), leaving 28 ingroup taxa for comparison (all ingroup taxa from this study except for *Craugastor stuarti* and *Pristimantis ridens*).

3. Results

3.1. Molecular data characterization

The final data matrix includes 389 loci and 640,022 aligned bases of DNA sequence data and is available in Mendeley Data and in the Dryad repository at http://dx.doi.org/10.5061/dryad.3jg7h; targeted loci not successfully amplified for more than half of the individuals were not included in the final data set. For the individuals sequenced in this study, an average of 8.6 million reads and 1.9 billion bases of sequence data were obtained, with an average of 10% of reads aligning to the target loci. The number of loci per individual for the new sequences in the final data matrix ranges from 288–388 (mean = 364). Homologs were obtained from the genomes of *Xenopus tropicalis* and *Nanorana parkeri* for 383 of 389 included loci, from the transcriptome of *Bufotes viridis* for 262 of 389 loci, and from the transcriptome of *Pseudacris regilla* for 144 of 389 loci. On average, sequence data were available for 32 of 35 taxa per locus, with 50 loci having complete taxon coverage overall and 233 having complete taxon coverage within the ingroup. Taxon sampling, aligned sequence length, and best scoring models of evolution under the BIC for each locus are provided in Appendix S1.

3.2. Phylogenetic relationships

The best-scoring tree found in the concatenated maximum likelihood analysis has a log likelihood of −4,972,902. Nearly all nodes in this phylogeny (Fig. 1a) are well supported, although many of the internodes grouping sets of genera are short. All families as recognized by Heinicke et al. (2009) are recovered as monophyletic. The deepest divergence within Terraranae is between *Ceuthomantis* and all other taxa, with the next-deepest split occurring between Eleutherodactylidae and the remaining genera. The largest genus, *Pristimantis*, is most closely related to the Andean genera *Phrynopus*, *Oreobates*, and *Lynchius*. The recently described genus *Tachiramantis* (Heinicke et al., 2015), is recovered as most closely related to *Craugastor* and *Haddadus*. Within the genus *Pristimantis*, the subgenera *Pristimantis* and *Hypodictyon* are not reciprocally monophyletic, and the subfamily Strabomantinae sensu Hedges et al. (2008a) is also not monophyletic. Only two nodes in the concatenated phylogeny have likelihood bootstrap support values below 94%: a grouping of *Holoaden* and *Barycholos* (= subfamily Holoadeninae) with *Pristimantis* + *Phrynopus* + *Lynchius* + *Oreobates* receives only 89% bootstrap support, and the placement of *Haddadus* as the sister taxon to *Craugastor* receives only 74% support. The parsimony and Bayesian concatenated analyses have nearly identical results and similarly high support values. The only difference for a node affecting intergenric relationships is that both the parsimony and Bayesian analyses recover *Craugastor* and *Tachiramantis* as sister genera rather than *Craugastor* and *Haddadus*. Relationships among included species of *Pristimantis* also differ slightly depending upon analysis, but monophyly of the genus is still strongly supported in all comcatenated analyses.

The ASTRAL species tree is broadly in agreement with the

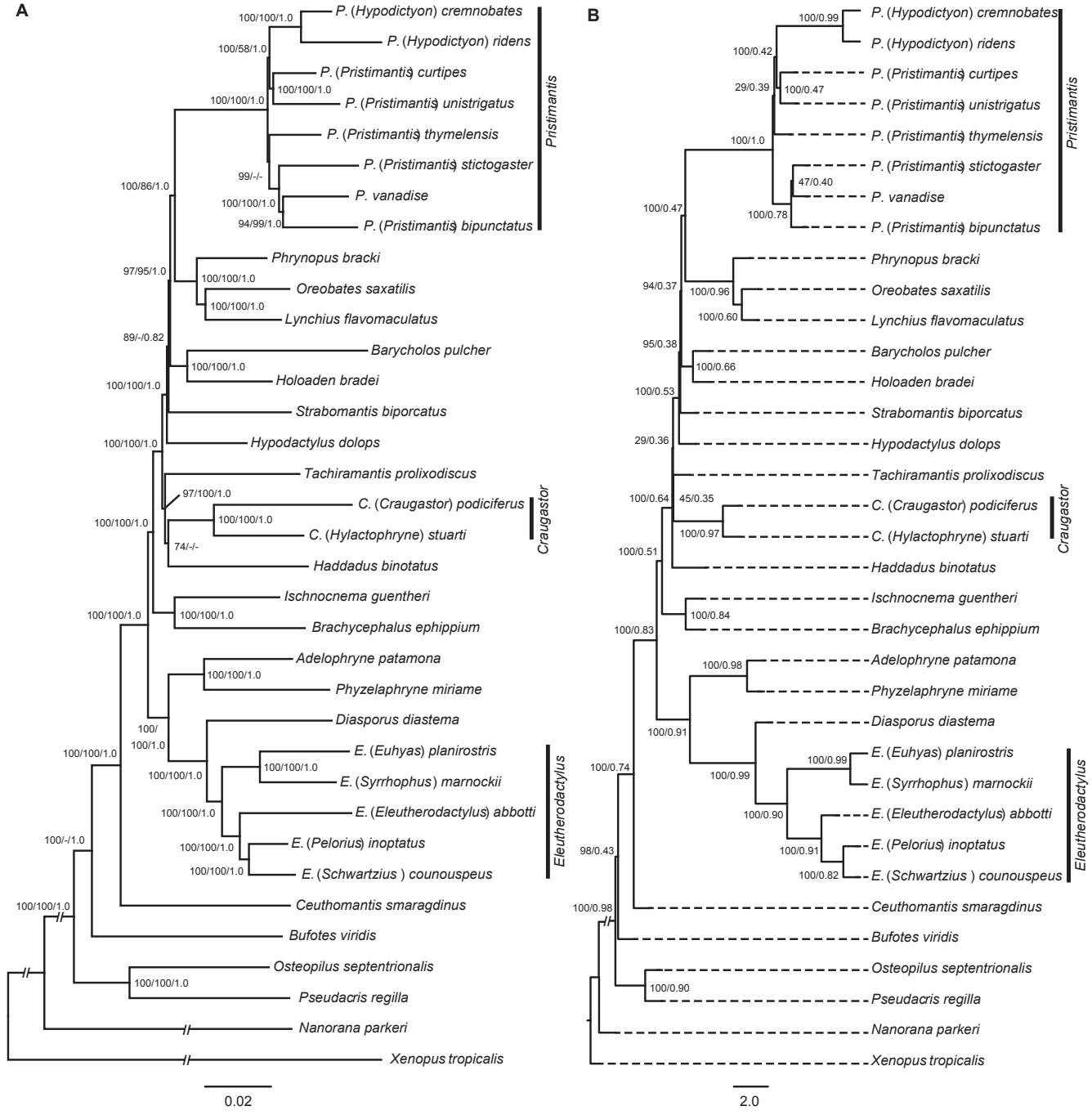

Fig. 1. Phylogenetic relationships among terraranans based on (A) concatenated RAxML and (B) coalescent ASTRAL analyses. Support values are shown at nodes. For (A), these are ML bootstrap/MP bootstrap/Bayesian posterior probabilities from RAxML, MEGA, and BEAST analyses, respectively. For (B), these are multi-locus bootstrap/local posterior probability values from ASTRAL, where local posterior probabilities (= "local quartet support") are calculated as described in Sayyari and Mirarab (2016); these values can be interpreted in an analogous way to typical posterior probabilities.

concatenated maximum likelihood phylogeny in its depiction of evolutionary relationships (Fig. 1b). Nodes that receive strong bootstrap support in the concatenated analysis also receive strong multilocus bootstrap support, although quartet support (local posterior probability) varies. Overall, the normalized quartet score of the tree is 0.818, indicating a high degree of congruence between the species-tree and the input gene trees. Only two nodes differ between the RAxML and ASTRAL analyses. Within *Pristimantis*, the position of *P. thymelensis* changes slightly. More notably, in the ASTRAL analysis *Haddadus* is found to be outside a grouping of all other craugastorid and strabomantid genera, rather than most closely related to *Craugastor*. However, this placement of *Haddadus* receives very low multilocus bootstrap support (29%), and the ASTRAL bootstrap consensus instead places *Haddadus* with *Craugastor* and *Tachiramantis* (multilocus bootstrap support = 45%). The overall normalized quartet score of a tree in which *Craugastor* + *Tachiramantis* + *Haddadus* is monophyletic is also nearly identical to the best scoring tree (0.81811 vs. 0.81848).

3.3. Phylogenetic hypothesis testing and comparison

Two of the three constrained phylogenies were identified as significantly different in likelihood from the unconstrained phylogeny by the AU test (Table 2). The best-scoring tree in which *Ceuthomantis* and *Pristimantis* are constrained to be closest relatives is significantly worse

Table 2
Results of AU test for unconstrained vs. constrained likelihood phylogenies.

Tree	lnL	p-value	Significantly Worse? (α = 0.05)
ML Tree (unconstrained)	−4955121.821	n/a	n/a
Monophyly of *Ceuthomantis* + *Pristimantis*	−4963038.343	0.000	Yes
Monophyly of *Strabomantis* + *Craugastor* + *Haddadus* + *Tachiramantis*	−4956284.349	0.000	Yes
Monophyly of Strabomantidae + Craugastoridae exclusive of *Haddadus*	−4955188.975	0.061	No

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than the best-scoring unconstrained tree (p = 0.000), as is the tree constrained to place *Strabomantis* as most closely related to *Craugastor* + *Haddadus* + *Tachiramantis* (p = 0.000). In contrast, the tree constrained so that *Haddadus* falls outside a grouping of all other craugastorid + strabomantid genera is not significantly worse than the best-scoring unconstrained tree (p = 0.061), at least at the α = 0.05 level. Based on the K tree score, the concatenated ML phylogeny generated in this study is very similar to the ML phylogeny from Heinicke et al. (2015). The calculated K tree score is 0.04254, with a scale factor of 0.15639 (Fig. 2).

4. Discussion

4.1. Phylogenetic relationships

Evolutionary relationships found in our analyses are in broad agreement with relationships found using multi-gene phylogenetic data sets (Hedges et al., 2008a; Heinicke et al., 2007, 2009, 2015; Padial et al., 2014; Pyron and Wiens, 2011). The most jarring discordance among previous analyses was the placement of *Ceuthomantis*, which in most studies (e.g. Heinicke et al., 2009; Pyron and Wiens, 2011; Heinicke et al., 2015) was found to be the sister taxon to all other Terraranae, but was deeply embedded as the sister group to *Pristimantis* + *Yunganastes* in Padial et al. (2014), even though all of these studies used overlapping data sets including mitochondrial 12S and 16S ribosomal RNA sequences along with a small number of nuclear loci. The apparent explanation for this is the use of a direct optimization tree alignment method (Varón et al., 2010) in Padial et al. (2014) rather than similarity- or structure-based alignment methods (Edgar, 2004; Stocsits et al., 2009; Thompson et al., 1994) used in the other studies. Similarly conflicting results among these methods when using 12S and 16S ribosomal RNA sequences have also been demonstrated for bufonid frogs (Pauly et al., 2009). Some studies have shown that direct optimization methods may fare poorly when dealing with variable-length ribosomal RNA sequences such as 12S and 16S (Ogden and Rosenberg, 2007; Kjer et al., 2007). Both our concatenated and coalescent analyses, and our SH test results, strongly reject a clade comprising *Ceuthomantis* and *Pristimantis*, and demonstrate that the earliest divergence in Terraranae separates the ancestral lineage of *Ceuthomantis* from that of all other terraranans.

No other deep relationship within Terraranae (i.e., among families, genera, or subgenera) showed such deep discordance as the phylogenetic position of *Ceuthomantis* in previously published analyses, but a number of branches that had remained unresolved are resolved here. In *Eleutherodactylus*, the subgenera *Pelorius* and *Schwartzius*, both endemic to Hispaniola, are resolved as closest relatives. Previously, the relationships among the subgenera *Eleutherodactylus*, *Pelorius*, and *Schwartzius* were not resolved (Hedges et al., 2008a). Within the genus *Pristimantis*, the subgenus *Pristimantis* is shown to be non-monophyletic, in agreement with Padial et al. (2014). The phylogenetic positions of

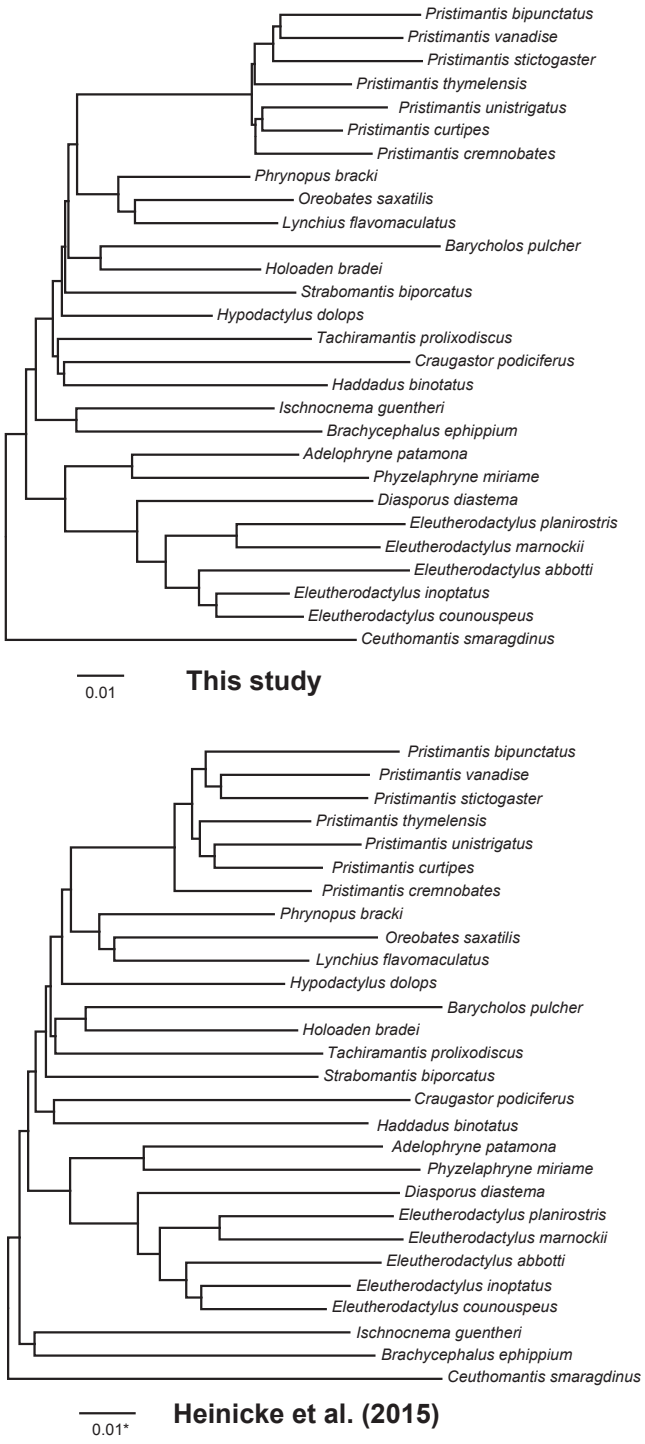

Fig. 2. Pruned 389-gene ML phylogeny (this study) compared with pruned 12S/16S/RAG1/TYR ML phylogeny from Heinicke et al. (2015), following branch length normalization in Ktreedist using a scale factor of 0.15639. The scale with an asterisk depicts normalized, rather than raw, branch lengths. The K tree score is 0.04254, indicating high congruence in tree topology and relative branch lengths between the two trees.

Strabomantis and *Hypodactylus* also receive strong support, and a clade comprising *Strabomantis* and *Craugastor* (sensu Pyron and Wiens, 2011; Padial et al., 2014) is rejected. At the family level, the relationships among Brachycephalidae, Eleutherodactylidae, and Craugastoridae + Strabomantidae were previously not resolved (Heinicke et al., 2009). Our analyses show that Eleutherodactylidae, which includes most West Indian species of terraranans as well as a few from Central America and northern South America, is the sister taxon to the remaining families,

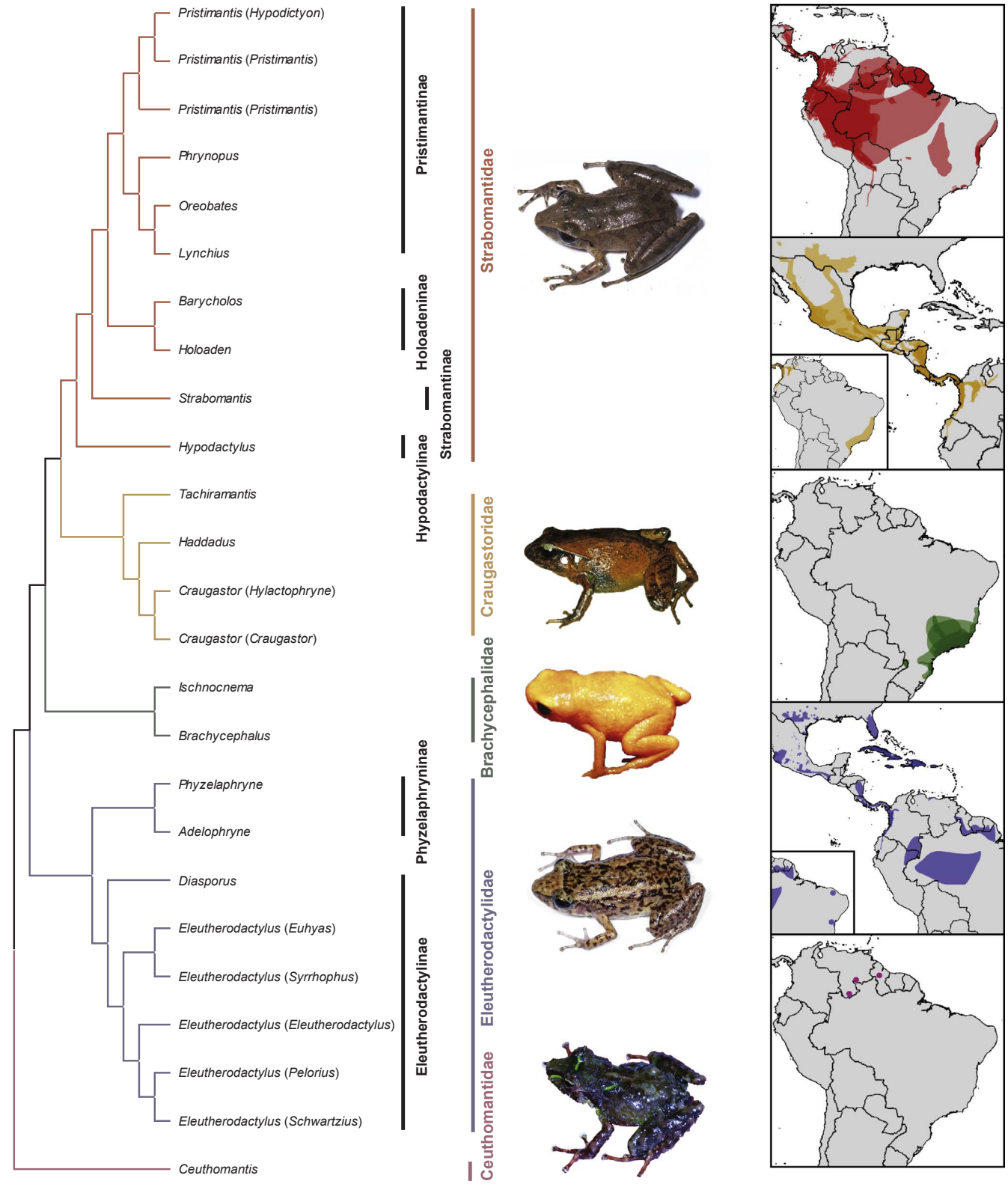

Fig. 3. Higher classification of New World direct developing frog genera and subgenera included in this study. Phylogenetic relationships and geographic ranges of families and subfamilies are indicated. Representative species from each family (*Pristimantis bipunctatus*, *Craugastor podiciferus*, *Brachycephalus ephippium*, *Eleutherodactylus planirostris*, *Ceuthomantis smaragdinus*) are shown.

which are most diverse in Central America, Andean and Amazonian South America, and southeastern Brazil (Fig. 3).

We were not able to include sequence data for all genera in our analyses. Of the excluded genera, published molecular phylogenetic analyses unambiguously place *Bryophryne*, *Euparkerella*, *Microkayla*, *Noblella*, *Psychrophrynella*, and *Yunganastes*, and there is no reason to believe that the phylogenetic positions of these genera would change. *Yunganastes* is most closely related to *Pristimantis* (Padial et al., 2014; Heinicke et al., 2015). The other five unsampled genera form a well-supported clade with the included genera *Barycholos* and *Holoaden*

(Canedo and Haddad, 2012; Padial et al., 2014; Heinicke et al., 2015; De la Riva et al., 2017). Four other genera have never been included in a molecular phylogenetic study: *Atopophrynus*, *Dischidodactylus*, *Geobatrachus*, and *Niceforonia*. Of these genera, species of both *Geobatrachus* and *Niceforonia* have been observed and collected in recent years (e.g., Pacheco-Florez and Ramírez-Pinilla, 2014; Romero-Garcia et al., 2015; Buitrago-González et al., 2016), providing hope that sequence data may be obtainable in the near future. In contrast, no *Atopophrynus* or *Dischidodactylus* have been collected in recent decades, so phylogenetic placement of these genera may ultimately rely on other lines of evidence, such as morphology.

4.2. Anchored phylogenomics vs. multi-gene phylogenetics

The low K tree score illustrates the broad agreement between the phylogeny generated here using 389 Anchored Phylogenomics loci with one based on the *12S*, *16S*, *RAG1*, and *TYR* genes (Fig. 2). This is true even though raw untransformed branch lengths are about six times longer (based on scale factor) in the four-gene tree than the 389-gene tree, as would be expected because the four-gene data set includes a large proportion of fast-evolving mitochondrial data. An important implication of this result is that, at least in amphibians, published phylogenetic results based on the *12S*, *16S*, *RAG1*, and *TYR* genes should be considered generally reliable, at least for branches receiving significant bootstrap values or posterior probabilities. These genes have been the most widely applied markers in amphibian phylogenetics for several decades (e.g., Bossuyt et al., 2006; Frost et al., 2006; Hay et al., 1995; Roelants et al., 2007; Vieites et al., 2009), with thousands to tens of thousands of sequences of each gene deposited in GenBank for amphibians (as of January 2017). Because the similarity measured by the K tree score incorporates both topology and branch-length variation, this conclusion can be extended to results and interpretations that rely on branch lengths, such as biogeographic interpretations based on time-trees. Thus, we expect that continued application of phylogenomic approaches will refine multi-gene phylogenetic results, as in this study, rather than replace them wholesale.

4.3. Taxonomic considerations

One significant role of taxonomic categorization is to provide information about evolutionary relationships (De Queiroz and Gauthier, 1990). Although not a requirement of the Code of Zoological Nomenclature, we take the view that taxonomic entities should be monophyletic. Currently, major databases utilize conflicting taxonomies for terraranan frogs (AmphibiaWeb, 2017; Frost, 2017; IUCN, 2016), in part because different studies with conflicting results have been used to generate these taxonomic arrangements. Our results provide a robust framework for revising and standardizing the family- and subfamily-level taxonomy of terraranan frogs based almost entirely on names and entities previously recognized (Table 3) (Hedges et al., 2008a; Heinicke et al., 2009, 2015; Pyron and Wiens, 2011; Padial et al., 2014). We recognize five families of terraranan frogs, an arrangement originally proposed in Hedges et al. (2008a) and Heinicke et al. (2009): Brachycephalidae, Ceuthomantidae, Craugastoridae, Eleutherodactylidae, and Strabomantidae (Fig. 3). The content of Brachycephalidae and Eleutherodactylidae is identical to all recent usage, with Brachycephalidae containing no subfamilies and Eleutherodactylidae containing the subfamilies Eleutherodactylinae and Phyzelaphryninae. Ceuthomantidae is restricted to the genus *Ceuthomantis*.

Craugastoridae and Strabomantidae have been merged in a number of recent taxonomic treatments (e.g. Pyron and Wiens, 2011; Padial et al., 2014). We recognize these as distinct families because they are reciprocally monophyletic in our concatenated ML analysis, there is not strong evidence of non-monophyly in our species-tree analysis, each contains 100+ species with centers of diversity on different landmasses (Central America for Craugastoridae, South America for Strabomantidae; Fig. 3), and there is precedent for use of this arrangement in the literature (e.g. Kaiser et al., 2015; Lehr and Duellman, 2009; Padial et al., 2012). The recently described genus *Tachiramantis* is placed in Craugastoridae. We use four subfamilies within Strabomantidae: the previously recognized subfamilies Holoadeninae, Pristimantinae, Strabomantinae, and a new subfamily for *Hypodactylus*, which our phylogenetic analyses show not to form a monophyletic grouping with any one of the three previously recognized subfamilies.

Hypodactylinae subfam. nov.

Type Genus: *Hypodactylus* Hedges et al. (2008b)

Content: *Hypodactylus* Hedges et al. (2008b)

Definition: As for the genus (Hedges et al., 2008a, 2008b): Small to midsized strabomantid frogs (adult snout-vent length 19–49 mm) with head narrower than body, and tympanic membrane differentiated (only tympanic annulus visible under skin in some species). Cranial crests are absent, but vomers have prominent tooth-bearing processes. Trigeminal nerve passes lateral to the adductor mandibulae posterior subexternus muscle (="S" condition of Starrett, 1968). Terminal discs on digits not expanded, but do have weak circumferential grooves. Terminal phalanges are narrowly T-shaped. Finger I is as long as or longer than Finger II; toes III and V are equal in length. Subarticular tubercles are not prominent. The dorsum is smooth or weakly tuberculate, while the venter is smooth.

Below the genus level, our results show that the two recognized subgenera within the genus *Pristimantis* are not reciprocally monophyletic. Recognizing only monophyletic groups would thus require either abandoning use of subgenera or else naming additional subgenera. Since *Pristimantis* is the largest vertebrate genus, with over 500 species, recognition of additional subgenera would be of broad utility in communicating evolutionary relationships within the genus, and we in general favor that approach. However, adequate revision of the genus would require analyses of phylogenetic relationships, along with incorporation of morphological, ecological, and geographic data for far more than the 1.5% of the *Pristimantis* species diversity included in this study. We make no attempt to revise the genus here, and plan to revisit the issue in a focused future study.

5. Conclusions

This study, in combination with other recently published studies, further demonstrates the utility of anchored hybrid enrichment and other phylogenomics approaches in determining relationships in anurans (Alexander et al., 2016; Peloso et al., 2016; Portik et al., 2016). Although many terraranan genera, especially within Craugastoridae and Strabomantidae, diverged in a short time frame, as shown by short internodes in the phylogeny, many previously ambiguous relationships were successfully resolved and are in agreement whether using concatenated or coalescent approaches. Even with nearly 400 loci, however, a few relationships do remain equivocal in parts of the tree where rapid divergences occurred, most notably the relationships of *Haddadus* and *Tachiramantis*, and relationships among the sampled *Pristimantis* species. Denser taxon sampling and additional sequence data hold promise for at least some additional resolution.

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Table 3
Comparison of taxonomic arrangements of New World direct-developing frog genera in recent comprehensive studies. Genera marked with an asterisk were not included in the indicated studies, with placement based on other data (genetic, morphological, or geographic).

Genus	Hedges et al. (2008a)/Heinicke et al. (2009)	Pyron and Wiens (2011)	Padial et al. (2014)	This study
Ceuthomantis	Ceuthomantidae	Ceuthomantidae	Craugastoridae: Ceuthomantinae	Ceuthomantidae
Brachycephalus	Brachycephalidae	Brachycephalidae	Brachycephalidae	Brachycephalidae
Ischnocnema	Brachycephalidae	Brachycephalidae	Brachycephalidae	Brachycephalidae
Diasporus	Eleutherodactylidae: Eleutherodactylinae	Eleutherodactylidae: Eleutherodactylinae	Eleutherodactylidae: Eleutherodactylinae	Eleutherodactylidae: Eleutherodactylinae
Eleutherodactylus	Eleutherodactylidae: Eleutherodactylinae	Eleutherodactylidae: Eleutherodactylinae	Eleutherodactylidae: Eleutherodactylinae	Eleutherodactylidae: Eleutherodactylinae
Adelophryne	Eleutherodactylidae: Phyzelaphryninae	Eleutherodactylidae: Phyzelaphryninae	Eleutherodactylidae: Phyzelaphryninae	Eleutherodactylidae: Phyzelaphryninae
Phyzelaphryne	Eleutherodactylidae: Phyzelaphryninae	Eleutherodactylidae: Phyzelaphryninae	Eleutherodactylidae: Phyzelaphryninae	Eleutherodactylidae: Phyzelaphryninae
Craugastor	Craugastoridae	Craugastoridae: Craugastorinae	Craugastoridae: Craugastorinae	Craugastoridae
Haddadus	Craugastoridae	Craugastoridae: Craugastorinae	Craugastoridae: Craugastorinae	Craugastoridae
Tachiramantis	N/A	N/A	N/A	Craugastoridae
Strabomantis	Strabomantidae: Strabomantinae	Craugastoridae: Strabomantinae	Craugastoridae: Craugastorinae	Strabomantidae: Strabomantinae
Hypodactylus	Strabomantidae: Strabomantinae	Craugastoridae (s. f. *incertae sedis*)	Craugastoridae: Holoadeninae	Strabomantidae: Hypodactylinae
Barycholos	Strabomantidae: Holoadeninae	Craugastoridae: Holoadeninae	Craugastoridae: Holoadeninae	Strabomantidae: Holoadeninae
Bryophryne	Strabomantidae: Holoadeninae	Craugastoridae: Holoadeninae	Craugastoridae: Holoadeninae	Strabomantidae: Holoadeninae
*Euparkerella**	Strabomantidae: Holoadeninae	Craugastoridae: Holoadeninae	Craugastoridae: Holoadeninae	Strabomantidae: Holoadeninae
Holoaden	Strabomantidae: Holoadeninae	Craugastoridae: Holoadeninae	Craugastoridae: Holoadeninae	Strabomantidae: Holoadeninae
Noblella	Strabomantidae: Holoadeninae	Craugastoridae: Holoadeninae	Craugastoridae: Holoadeninae	Strabomantidae: Holoadeninae
Psychrophrynella	Strabomantidae: Holoadeninae	Craugastoridae: Holoadeninae	Craugastoridae: Holoadeninae	Strabomantidae: Holoadeninae
*Microkayla**	N/A	N/A	N/A	Strabomantidae: Holoadeninae
*Niceforonia**	Strabomantidae: Strabomantinae	Craugastoridae (s. f. *incertae sedis*)	Craugastoridae: Holoadeninae	Strabomantidae: Pristimantinae
Lynchius	Strabomantidae: Strabomantinae	Craugastoridae: Pristimantinae	Craugastoridae: Holoadeninae	Strabomantidae: Pristimantinae
Oreobates	Strabomantidae: Strabomantinae	Craugastoridae: Pristimantinae	Craugastoridae: Holoadeninae	Strabomantidae: Pristimantinae
Phrynopus	Strabomantidae: Strabomantinae	Craugastoridae: Pristimantinae	Craugastoridae: Holoadeninae	Strabomantidae: Pristimantinae
Pristimantis	Strabomantidae: Strabomantinae	Craugastoridae: Pristimantinae	Craugastoridae: Ceuthomantinae	Strabomantidae: Pristimantinae
Yunganastes	Strabomantidae: Strabomantinae	Craugastoridae: Pristimantinae	Craugastoridae: Ceuthomantinae	Strabomantidae: Pristimantinae
*Dischidodactylus**	Strabomantidae: Strabomantinae	Craugastoridae (s. f. *incertae sedis*)	Craugastoridae: Ceuthomantinae	Strabomantidae: Pristimantinae
*Atopophrynus**	Strabomantidae: Strabomantinae	Craugastoridae (s. f. *incertae sedis*)	*incertae sedis*	Strabomantidae (s. f. *incertae sedis*)
*Geobatrachus**	Strabomantidae: Strabomantinae	Craugastoridae (s. f. *incertae sedis*)	*incertae sedis*	Strabomantidae (s. f. *incertae sedis*)

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2017.09.021.

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