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The impact of anchored phylogenomics and taxon sampling on phylogenetic inference in narrow-mouthed frogs (Anura, Microhylidae)

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Abstract

Despite considerable progress in unravelling the phylogenetic relationships of microhylid frogs, relationships among subfamilies remain largely unstable and many genera are not demonstrably monophyletic. Here, we used five alternative combinations of DNA sequence data (ranging from seven loci for 48 taxa to up to 73 loci for as many as 142 taxa) generated using the anchored phylogenomics sequencing method (66 loci, derived from conserved genome regions, for 48 taxa) and Sanger sequencing (seven loci for up to 142 taxa) to tackle this problem. We assess the effects of character sampling, taxon sampling, analytical methods and assumptions in phylogenetic inference of microhylid frogs. The phylogeny of microhylids shows high susceptibility to different analytical methods and datasets used for the analyses. Clades inferred from maximum-likelihood are generally more stable across datasets than those inferred from parsimony. Parsimony trees inferred within a tree-alignment framework are generally better resolved and better supported than those inferred within a similarity-alignment framework, even under the same cost matrix (equally weighted) and same treatment of gaps (as a fifth nucleotide state). We discuss potential causes for these differences in resolution and clade stability among discovery operations. We also highlight the problem that commonly used algorithms for model-based analyses do not explicitly model insertion and deletion events (i.e. gaps are treated as missing data). Our results corroborate the monophyly of Microhylidae and most currently recognized subfamilies but fail to provide support for relationships among subfamilies. Several taxonomic updates are provided, including naming of two new subfamilies, both monotypic.

Amphibian systematics has advanced greatly in the last decade, with many studies attempting to infer relationships in different clades of the amphibian tree. Despite great improvement, some relationships remain obscure and many taxa have not been included in any phylogenetic analyses, their taxonomic status therefore being tentative or arbitrary. An extreme case is the largely unstable relationships among subfamilies of narrow-mouthed frogs, family Microhylidae. Several

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attempts at resolving microhylid relationships have arrived at largely conflicting results (e.g. Frost et al., 2006; Van Bocxlaer et al., 2006; Kurabayashi et al., 2011; Matsui et al., 2011; Pyron and Wiens, 2011; De Sá et al., 2012; Fig. 1). However, the potential causes and sources of such conspicuous topological disagreements have been little discussed.

With 564 named species distributed throughout the World's tropics (with few representatives in temperate zones), Microhylidae accounts for slightly over 8.5% of global frog diversity (Frost, 2015). As currently recognized, Microhylidae is subdivided into 11 subfamilies of largely uncertain relationships (Fig. 1): Asterophryinae (distributed in Southeast Asia and the Australo-Papuan region), Cophylinae (Madagascar), Dyscophinae (Madagascar), Gastrophryninae (New World), Hoplophryninae (Tanzania), Kalophryninae (South and Southeast Asia), Melanobatrachinae (India), Microhylinae (Asia), Otophryninae (New World), Phrynomerinae (sub-Saharan Africa), and Scaphiophryninae (Madagascar). The family, however, remains poorly studied in many aspects of its biology. Around 30% of the species in the family were named since the year 2000, many of which are only known from a handful of specimens. This scenario is further complicated by the fact that many taxa were never included in a complete phylogeny of the group (one including representatives of all subfamilies)—they were tentatively, or arbitrarily, placed in one subfamily or another based on overall similarity or geographical placement, despite some striking disparity in morphological features (e.g. *Adelastes*, *Ctenophryne minor*, *Madecassophryne*, *Parhoplophryne*). Here, we tackle this problem with a combination of a large amount of DNA sequence data. We provide another attempt at inferring microhylid phylogeny using a combination of distinct approaches for taxon and character sampling, including members of all nominal subfamilies and several taxa never sampled before, among which is the enigmatic South American genus *Adelastes*.

Research objectives

Inasmuch as topological shifts observed across different groups of organisms are commonly credited to differences among the datasets (numbers of characters and/or taxa included) or to methodological artefacts, we can expect each non-trivial dataset to respond to an increase in data (i.e. characters and taxa) in a

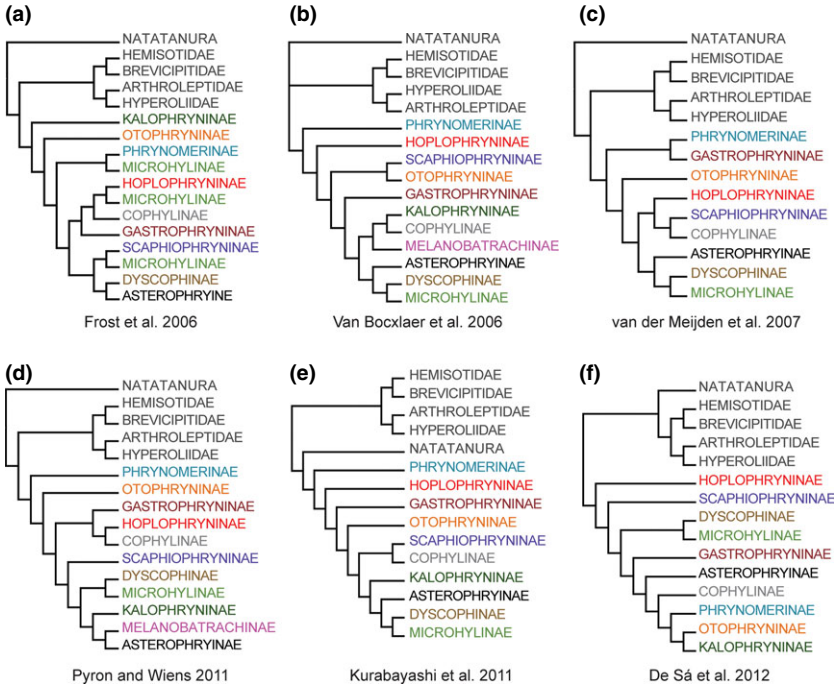

Fig. 1. Alternative hypotheses of relationship among microhylid subfamilies. (a) Frost et al. (2006); 34 spp.; ~4.8 kb; tree-alignment + parsimony; gaps as 5th state. (b) Van Bocxlaer et al. (2006); 28 spp., ~3.1 kb, similarity-alignment + ML; gaps as missing. (c) van der Meijden et al. (2007); 33 spp.; ~4.1 kb, similarity-alignment + ML; gaps as missing. (d) Pyron and Wiens (2011); 136 spp.; ~12.7 kb; similarity-alignment + ML; gaps as missing. (e) Kurabayashi et al. (2011)—tree from supplementary material as it is based on a larger taxon sampling; 53 spp.; ~4.1 kb; similarity-alignment + ML; gaps as missing. (f) De Sá et al. (2012); ~130 spp.—exact number of species is not available, many sister terminals are labelled as "sp" and could pertain to the same taxon; ~2.7 kb; similarity-alignment + ML; gaps as missing. Support values not shown. This figure is available in colour in the online version of the paper.

different way. Beyond changes in the amount of data, other factors must be considered when evaluating conflicting tree topologies—presence and level of missing data (Wilkinson, 1995; Lemmon et al., 2009; Wiens and Morrill, 2011); sequence alignment (Wheeler, 1996; Frost et al., 2001); the treatment of indels (gaps) as informative characters (Giribet and Wheeler, 1999; Simmons and Ochoterena, 2000; Lemmon et al., 2009); and the response of datasets to distinct optimality criteria (e.g. parsimony vs. probabilistic inference). Investigating the potential sources of topological incongruence may help in the design of future experiments and in decision-making regarding, for example, the taxonomy of a given group.

The volatile relationships of microhylid subfamilies and the dataset we have compiled allow us to investigate the effects of several aspects of phylogenetic inference and their underlying assumptions on tree topology. Thus, we evaluate the stability of microhylid relationships to the addition of taxa and characters (i.e. DNA sequence data), and also to the nuances involved in the use of different discovery operations and differential treatment of evidence. The term "discovery operation" refers to the methods that aim to generate and test scientific hypotheses (Grant, 2002). In our case, we are concerned with the methods of analyses—i.e. optimality criteria— used to generate sequence alignments, and implemented for tree search. The term "method" is more general and applied to any operational protocol designed to achieve a particular end. Our approach comprises an exploration of two distinct methods for phylogenetic inference [parsimony and maximum likelihood (ML)] and five different data matrices (varying from 48 taxa and seven loci to 142 taxa for up to 73 loci). The present experiment is therefore a robust empirical evaluation of how optimality criteria, and assumptions regarding character transformations and sequence alignments, may affect tree inference and the hypotheses that are derived from those trees (e.g. taxonomy, biogeography, character evolution).

Material and methods

Taxon sampling

We used a combination of two distinct sequencing techniques. First, we selected 48 taxa for next generation sequencing (NGS) using the anchored phylogenomics sequencing method (Lemmon et al., 2012). The same 48 taxa, plus an additional 94 taxa, were sequenced for seven loci using Sanger sequencing (SGS) techniques. Alternative combinations of the SGS and NGS data resulted in five subsets, which were then used for downstream phylogenetic analyses. Detailed descriptions of these datasets are given below (see Experimental design).

The reduced taxonset (48 taxa) included 44 microhylids, plus four taxa used as outgroups—three Afrobatrachia (representing three of four afrobatrachian families), and one Natatanura (Dicroglossidae: *Limnonectes dabanus*). The extended taxonset (142 taxa) included 129 microhylids, plus 13 taxa used as outgroups—12 Afrobatrachia (representing all four afrobatrachian families) and one Natatanura (*L. dabanus*). *Limnonectes dabanus* was used to root all trees, based on the well-established sister taxon relationship of Natatanura + Allodapanura (Afrobatrachia + Microhylidae) (Frost et al., 2006; Pyron and Wiens, 2011).

For the extended taxonset, we were able to sample all 11 subfamilies currently recognized in Microhylidae (Hoplophryninae and Melanobatrachinae were not included in the 48-taxa dataset). Except for Melanobatrachinae (monotypic) and Hoplophryninae (only *Hoplophryne rogersi* sampled) all subfamilies were sampled from at least two taxa, and thus we were able to test for their monophyly.

A complete list of taxa included is given in Table 1. Metadata for all samples, including locality data, museum collection numbers, and loci included (with GenBank accession numbers) are given in the Supplementary Material (Table S1).

Laboratory protocols

For both NGS and SGS, DNA was extracted and isolated from preserved tissues (liver or muscle) using the Qiagen DNeasy kit following the manufacturer's guidelines.

Sanger sequencing. Fragments targeted for PCR amplification and sequencing were two mitochondrial loci—part of the ribosomal subunit 16S rRNA and part of cytochrome oxidase I (COI)—as well as a segment of the nuclear coding genes brain-derived neurotrophic factor (BDNF), cellular myelocytomatosis oncogene - exon 2 (CMYC), histone H3, seven *in absentia* homolog 1 (SIA1), and tyrosinase. Primers used for PCR amplification and sequencing are drawn from those commonly used in amphibian systematics (Palumbi et al., 1991; Hedges, 1994; Colgan et al., 1999; Bossuyt and Milinkovitch, 2000; Bonacum et al., 2001; Crawford, 2003; Wiens et al., 2005; van der Meijden et al., 2007; Peloso et al., 2014—primer sequences, fragment lengths and annealing temperatures are given in the Supplementary Material: Text S1). PCR and sequencing protocols are detailed in the Supplementary Material.

Despite concentrated efforts at PCR amplification, the SGS matrix is not complete and several sequences are missing for some terminals. Of the seven loci

Table 1
Taxon sampling

Higher taxon	Species	Taxonset
Microhylidae		
Asterophryinae	*Albericus exclamitans* Kraus and Allison, 2005	NGS + SGS
Asterophryinae	*Albericus siegfriedi* Menzies, 1999	SGS
Asterophryinae	*Albericus tuberculus* (Richards, Johnston, and Burton, 1992)	SGS
Asterophryinae	***Asterophrys turpicola* (Schlegel, 1837)**	SGS
Asterophryinae	*Austrochaperina adelphe* (Zweifel, 1985)	SGS
Asterophryinae	*Austrochaperina rivularis* Zweifel, 2000;	SGS
Asterophryinae	*Barygenys flavigularis* Zweifel, 1972	SGS
Asterophryinae	*Barygenys nana* Zweifel, 1972	NGS + SGS
Asterophryinae	*Callulops personatus* (Zweifel, 1972)	NGS + SGS
Asterophryinae	*Callulops robustus* (Boulenger, 1898)	SGS
Asterophryinae	*Choerophryne proboscidea* Van Kampen, 1914	NGS + SGS
Asterophryinae	*Cophixalus balbus* Günther, 2003	NGS + SGS
Asterophryinae	*Cophixalus crepitans* Zweifel, 1985	SGS
Asterophryinae	*Cophixalus hosmeri* Zweifel, 1985	SGS
Asterophryinae	***Copiula oxyrhina* (Boulenger, 1898)**	NGS + SGS
Asterophryinae	*Gastrophrynoides immaculatus* Chan, Grismer, Norhayati, and Daicus, 2009	NGS + SGS
Asterophryinae	***Genyophryne thomsoni* Boulenger, 1890**	NGS + SGS
Asterophryinae	*Hylophorbus rainerguentheri* Richards and Oliver, 2007	NGS + SGS
Asterophryinae	***Hylophorbus rufescens* Macleay, 1878**	SGS
Asterophryinae	***Liophryne rhododactyla* Boulenger, 1897**	SGS
Asterophryinae	*Liophryne schlaginhaufeni* (Wandolleck, 1911)	SGS
Asterophryinae	*Mantophryne infulata* (Zweifel, 1972)	NGS + SGS
Asterophryinae	***Mantophryne lateralis* Boulenger, 1897**	SGS
Asterophryinae	*Metamagnusia slateri* (Loveridge, 1955)	NGS + SGS
Asterophryinae	*Oreophryne brachypus* (Werner, 1898)	SGS
Asterophryinae	*Oreophryne monticola* (Boulenger, 1897)	NGS + SGS
Asterophryinae	*Oreophryne* sp.	SGS
Asterophryinae	*Oxydactyla alpestris* Zweifel, 2000;	NGS + SGS
Asterophryinae	***Sphenophryne cornuta* Peters and Doria, 1878**	SGS
Asterophryinae	*Xenorhina fuscigula* (Blum and Menzies, 1989)	SGS
Asterophryinae	*Xenorhina mehelyi* (Boulenger, 1898)	NGS + SGS
Asterophryinae	*Xenorhina obesa* (Zweifel, 1960)	SGS
Cophylinae	***Anodonthyla boulengerii* Müller, 1892**	SGS
Cophylinae	*Anodonthyla jeanbai* Vences, Glaw, Köhler, and Wollenberg, 2010	SGS
Cophylinae	*Anodonthyla nigrigularis* Glaw and Vences, 1992	NGS + SGS
Cophylinae	*Anodonthyla theoi* Vences, Glaw, Köhler, and Wollenberg, 2010	SGS
Cophylinae	*Anodonthyla vallani* Vences, Glaw, Köhler, and Wollenberg, 2010	SGS
Cophylinae	*Cophyla berara* Vences, Andreone, and Glaw, 2005	SGS
Cophylinae	*Cophyla occultans* (Glaw and Vences, 1992)	NGS + SGS
Cophylinae	*Cophyla* sp1	SGS
Cophylinae	*Platypelis barbouri* Noble, 1940	SGS
Cophylinae	*Platypelis grandis* (Boulenger, 1889)	SGS
Cophylinae	*Platypelis milloti* Guibé, 1950	SGS
Cophylinae	*Platypelis pollicaris* Boulenger, 1888	NGS + SGS
Cophylinae	*Platypelis* sp1	SGS
Cophylinae	*Platypelis tetra* Andreone, Fenolio, and Walvoord, 2003	SGS
Cophylinae	*Plethodontohyla bipunctata* (Guibé, 1974)	SGS
Cophylinae	*Plethodontohyla brevipes* Boulenger, 1882	SGS
Cophylinae	*Plethodontohyla guentheri* Glaw and Vences, 2007	SGS
Cophylinae	*Plethodontohyla mihanika* Vences, Raxworthy, Nussbaum, and Glaw, 2003	SGS
Cophylinae	***Plethodontohyla notosticta* (Günther, 1877)**	SGS
Cophylinae	*Plethodontohyla* sp1	SGS
Cophylinae	*Rhombophryne coronata* (Vences and Glaw, 2003)	SGS
Cophylinae	*Rhombophryne laevipes* (Mocquard, 1895)	SGS
Cophylinae	*Rhombophryne mangabensis* Glaw, Köhler, and Vences, 2010	NGS + SGS
Cophylinae	*Rhombophryne matavy* D'Cruze, Köhler, Vences, and Glaw, 2010	SGS
Cophylinae	*Rhombophryne minuta* (Guibé, 1975)	SGS
Cophylinae	*Stumpffia gimmeli* Glaw and Vences, 1992	SGS
Cophylinae	*Stumpffia hara* Köhler, Vences, D'Cruze, and Glaw, 2010	SGS
Cophylinae	***Stumpffia psologlossa* Boettger, 1881**	SGS
Cophylinae	*Stumpffia pygmaea* Vences and Glaw, 1991	SGS

Table 1
(Continued)

Higher taxon	Species	Taxonset
Cophylinae	*Stumpffia roseifemoralis* Guibé, 1974	NGS + SGS
Cophylinae	*Stumpffia* sp1	SGS
Cophylinae	*Stumpffia* sp2	SGS
Dyscophinae	*Dyscophus guineti* (Grandidier, 1875)	NGS + SGS
Dyscophinae	***Dyscophus insularis* Grandidier, 1872**	SGS
Gastrophryninae	*Adelastes hylonomos* Zweifel, 1986	SGS
Gastrophryninae	*Arcovomer passarellii* Carvalho, 1954	NGS + SGS
Gastrophryninae	***Chiasmocleis albopunctata* (Boettger, 1885)**	SGS
Gastrophryninae	*Chiasmocleis avilapiresae* Peloso and Sturaro, 2008	SGS
Gastrophryninae	*Chiasmocleis bassleri* Dunn, 1949	SGS
Gastrophryninae	*Chiasmocleis capixaba* Cruz, Caramaschi, and Izecksohn, 1997	SGS
Gastrophryninae	*Chiasmocleis carvalhoi* (Nelson, 1975)	NGS + SGS
Gastrophryninae	*Chiasmocleis hudsoni* Parker, 1940	SGS
Gastrophryninae	*Chiasmocleis lacrimae* (Cruz, Caramaschi, and Izecksohn, 1997)	SGS
Gastrophryninae	*Chiasmocleis shudikarensis* Dunn, 1949	SGS
Gastrophryninae	*Ctenophryne aequatorialis* (Peracca, 1904)	SGS
Gastrophryninae	*Ctenophryne aterrima* (Günther, 1901)	SGS
Gastrophryninae	***Ctenophryne geayi* Mocquard, 1904**	NGS + SGS
Gastrophryninae	***Dasypops schirchi* Miranda-Ribeiro, 1924**	NGS + SGS
Gastrophryninae	***Dermatonotus muelleri* (Boettger, 1885)**	NGS + SGS
Gastrophryninae	*Elachistocleis cesarii* (Miranda-Ribeiro, 1920)	SGS
Gastrophryninae	*Elachistocleis helianneae* Caramaschi, 2010	NGS + SGS
Gastrophryninae	*Elachistocleis piauiensis* Caramaschi and Jim, 1983	SGS
Gastrophryninae	*Elachistocleis* sp.	SGS
Gastrophryninae	***Gastrophryne carolinensis* (Holbrook, 1835)**	NGS + SGS
Gastrophryninae	*Gastrophryne mazatlanensis* (Hallowell, 1856)	SGS
Gastrophryninae	***Hamptophryne boliviana* (Parker, 1927)**	NGS + SGS
Gastrophryninae	*Hamptophryne alios* (Wild, 1995)	NGS + SGS
Gastrophryninae	*Hypopachus ustus* (Cope, 1866)	SGS
Gastrophryninae	*Hypopachus variolosus* (Cope, 1866)	NGS + SGS
Gastrophryninae	*Myersiella microps* (Duméril and Bibron, 1841)	NGS + SGS
Gastrophryninae	*Stereocyclops histrio* (Carvalho, 1954)	NGS + SGS
Gastrophryninae	***Stereocyclops incrassatus* Cope, 1870**	NGS + SGS
Hoplophryninae	*Hoplophryne rogersi* Barbour and Loveridge, 1928	SGS
Kalophryninae	*Kalophrynus barioensis* Matsui and Nishikawa 2011	SGS
Kalophryninae	*Kalophrynus interlineatus* (Blyth, 1855)	NGS + SGS
Kalophryninae	*Kalophrynus limbooliati* Matsui, Nishikawa, Belabut, Norhayati & Yong 2011	SGS
Kalophryninae	*Kalophrynus palmatissimus* Kiew, 1984	SGS
Kalophryninae	***Kalophrynus pleurostigma* Tschudi, 1838**	SGS
Kalophryninae	*Kalophrynus stellatus* Tshcudi, 1838	SGS
Kalophryninae	*Kalophrynus subterrestris* Inger, 1966	SGS
Melanobatrachinae	*Melanobatrachus indicus* Beddome, 1878	SGS
Microhylinae	***Calluella guttulata* (Blyth, 1856)**	SGS
Microhylinae	*Calluella yunnanensis* Boulenger, 1919	NGS + SGS
Microhylinae	***Chaperina fusca* Mocquard, 1892;**	SGS
Microhylinae	***Glyphoglossus molossus* Günther, 1869**	NGS + SGS
Microhylinae	*Kaloula baleata* (Müller, 1836)	SGS
Microhylinae	*Kaloula* cf. *taprobanica* Parker, 1934	SGS
Microhylinae	*Kaloula mediolineata* Smith, 1917	NGS + SGS
Microhylinae	*Metaphrynella sundana* (Peters, 1867)	NGS + SGS
Microhylinae	***Microhyla achatina* Tschudi, 1838**	NGS + SGS
Microhylinae	*Microhyla mantheyi* Das, Yaakob, and Sukumaran, 2007	SGS
Microhylinae	*Microhyla nanapollexa* Bain and Nguyen, 2004	SGS
Microhylinae	*Microhyla petrigena* Inger and Frogner, 1979	SGS
Microhylinae	*Microhyla rubra* (Jerdon, 1854)	SGS
Microhylinae	***Micryletta inornata* (Boulenger, 1890)**	NGS + SGS
Microhylinae	*Ramanella* sp.	SGS
Microhylinae	*Ramanella variegata* (Stoliczka, 1872)	SGS
Microhylinae	*Uperodon systoma* (Schneider, 1799)	SGS
Otophryninae	***Otophryne robusta* Boulenger, 1900**	NGS + SGS
Otophryninae	*Otophryne steyermarki* Rivero, 1968	SGS
Otophryninae	*Synapturanus salseri* Pyburn, 1975	NGS + SGS

Table 1
(Continued)

Higher taxon	Species	Taxonset
Phrynomerinae	***Phrynomantis bifasciatus* (Smith, 1847)**	NGS + SGS
Phrynomerinae	*Phrynomantis microps* Peters, 1875	SGS
Scaphiophryninae	*Scaphiophryne brevis* (Boulenger, 1896)	NGS + SGS
Scaphiophryninae	*Scaphiophryne calcarata* (Mocquard, 1895)	SGS
Scaphiophryninae	*Scaphiophryne gottlebei* Busse and Böhme, 1992	SGS
Scaphiophryninae	*Scaphiophryne madagascariensis* (Boulenger, 1882)	SGS
Outgroups		
Arthroleptidae	*Arthroleptis poecilonotus* Peters, 1863	NGS + SGS
Brevicipitidae	*Breviceps gibbosus* (Linnaeus, 1758)	SGS
Brevicipitidae	*Breviceps montanus* Power, 1926	NGS + SGS
Brevicipitidae	*Callulina kreffti* Nieden, 1911	SGS
Brevicipitidae	***Spelaeophryne methneri* Ahl, 1924**	SGS
Hemisotidae	*Hemisus marmoratus* (Peters, 1854)	NGS + SGS
Hyperoliidae	*Afrixalus paradorsalis* Perret, 1960	SGS
Hyperoliidae	*Afrixalus uluguruensis* (Barbour and Loveridge, 1928)	SGS
Hyperoliidae	*Heterixalus betsileo* (Grandidier, 1872)	SGS
Hyperoliidae	*Heterixalus tricolor* (Boettger, 1881)	SGS
Hyperoliidae	*Hyperolius guttulatus* Günther, 1858	SGS
Hyperoliidae	*Tachycnemis seychellensis* (Duméril and Bibron, 1841)	SGS
Dicroglossidae	*Limnonectes dabanus* (Smith, 1922)	NGS + SGS

NGS, taxon was sequenced using the anchored phylogenomics sequencing method. SGS, taxon was sequenced using the Sanger sequencing method. Taxa in bold are the type species of their respective genera.

included, two were sequenced for all 142 samples (16S and BDNF), while the remaining loci have missing sequences for one to ten terminals: COI (141 sequences present), CMYC (132), histone H3 (141), SIA*h*1 (141) and tyrosinase (136). Only four taxa are missing information for more than one locus: *Copiula oxyrhina* (missing COI and CMYC), *Hamptophryne alios* (tyrosinase and CMYC), *Stereocyclops incrassatus* (tyrosinase and CMYC), and *Synapturanus salseri* (SIA and CMYC).

Next generation sequencing: Anchored Phylogenomics. The discovery of conserved areas across vertebrate genomes (Bejerano et al., 2004) that are flanked by less conserved regions provide a great source of potential data for phylogenetics (Faircloth et al., 2012; Lemmon et al., 2012; McCormack et al., 2012). Lemmon et al. (2012) detected 512 conserved loci present across a range of vertebrate genomes and designed probes for targeted enrichments of these regions generating assemblies of up to 3000 bp per locus.

One caveat of the method of Lemmon et al. (2012) is that there is a considerable decrease in yield with increased genetic distance from the target organism (microhylids in this study) relative to the organism used to design the probes. Probes used here are those employed by Lemmon et al. (2012), whereas the closest organism used for designing the probes is the pipid *Xenopus tropicalis*, with an estimated divergence time of approximately 200 Myr from the taxa included in our experiment (divergence estimate from Hedges et al., 2006). In practice, this leads to less overall coverage, fewer loci sampled across all samples, and an increased number of ambiguities in resulting contigs (Lemmon et al., 2012). Our prediction was to have 127–377 loci with average locus coverage of 1142 bp (A.R.L., personal observation). However, the distance from the model organism and the low quality of the *Xenopus* genome also posed a problem in terms of detection of gene paralogy, further exacerbated by the low quality of many of the recovered reads. Nonetheless, the amount of data that passed our very stringent quality control steps is still large (see below).

Data were collected following the methods of Lemmon et al. (2012) at the Center for Anchored Phylogenomics at Florida State University. Briefly, each genomic DNA sample was sonicated to a fragment size of ~300–700 bp. Subsequently, library preparation and indexing were performed following a protocol modified from that of Meyer and Kircher (2010). Indexed samples were then pooled at equal quantities (eight samples per pool), and enrichments were performed on each multi-sample pool using an Agilent Custom SureSelect kit, which contained probes designed for anchor loci from five vertebrate genomes (described in Lemmon et al., 2012), including *Xenopus tropicalis*. After enrichment, the six reactions were pooled in equal quantities and concentrations for sequencing on one paired-end 100- bp Illumina HiSeq2000 at the HudsonAlpha Institute for Biotechnology, and a second 100- bp single-end sequencing lane at the Florida State University Translational laboratory.

Quality-filtered sequencing reads were processed following the methods described by Lemmon et al. (2012), with some modifications. In short, reads were demultiplexed, with no mismatches to the expected index sequences tolerated. Reads were then scanned for matches to the probe region sequences of *Xenopus tropicalis* using the high-sensitivity approach described by Lemmon et al. (2012), which tolerates up to 45% mismatches to the reference. Reads matching a reference sequence for each individual were then aligned to produce preliminary seed consensus sequences for each locus. The reads were aligned using a script written by A.R.L. that: (i) sorts the reads by number of matches to the reference, (ii) for each read in the sorted list (starting with the best-matched read), notes the position maximizing the match to the previous read in the list, (iii) skips the read if the best matching position does not generate a 90% match (of at least 20 bp), (iv) and repeatedly traverses the entire sorted list until no additional reads have aligned during a traverse. After the preliminary assembly was complete, we performed an additional extension assembly to obtain flanking sequence. This was performed by iteratively matching previously unmapped reads to the current consensus sequence (a new consensus sequence is generated after each traversal through the unmapped reads). Assemblies were complete once no additional reads could be matched. After assemblies were complete, consensus bases were called as follows: (i) all sites with less than three-fold coverage and variant sites with < 10-fold coverage were termed "N", (ii) invariant sites with coverage between three- and ten-fold were called with the observed nucleotide, and (iii) for sites with greater than nine-fold coverage, the most common base was called. After base-calling to obtain a consensus sequence for each locus, mapped reads were removed from the original reads files and the assembly/base-calling process was repeated to obtain additional homologues (if present). Orthology was established by first choosing a reference individual (*Metaphrynella sundana*) for which the number of captured loci was greatest, then performing pairwise alignments for each locus between the longest reference sequence for that reference individual and the sequences obtained for each individual (up to two pairwise alignments performed for each locus × individual combination). The sequence producing the optimal alignment (i.e. minimizing the number of gaps and mismatches) was chosen as the orthologue for each locus. The source codes used for the assembly, base calling and orthology assessment are available at Data Dryad (doi:10.5061/dryad.8112f).

A total of 596 896 432 reads (59 Gb) were obtained, of which 1.8% were mapped. An average of 290 loci were captured per individual (min = 203, max = 350, captured defined as ≥ 10 reads mapped). Length of consensus sequences averaged 579 bp with averages across loci within individuals ranging from 359 to 826 bp. Assemblies of captured loci contained an average coverage of 76-fold.

After assembly, contigs for each locus were aligned using the default parameters in MUSCLE (Edgar, 2004) and these raw alignments were evaluated for the presence of ambiguous characters and gaps (i.e. any base not A, T, C or G). Any site containing more than one ambiguous base was removed from the alignment (a text file containing details of the trimming patterns for each loci is given as Supplementary Material: Text S2). This strict approach is taken to further increase the quality of characters used for downstream analyses by removing ambiguous characters that could have been generated due to sequencing errors and that were not detected during initial quality control steps. Although the vast majority of gaps removed are from the 5′ or 3′ ends of the sequences, this approach also removes a few bases from within the targeted region (most of the removals are the result of low coverage or poor read quality not filtered during quality control steps). We understand that this is a problematic aspect of the method of trimming selected, but it was deemed the best way to deal with the data, given the distance of the target and model organism and the large amount of ambiguities present in the sequences. For future experiments, we expect these problems with read quality to decrease significantly with improvement of the probes (by including neobatrachian genomes when designing the probes—work in progress, A.R.L. and E.M.L., unpublished data). Loci with fewer than 250 bp remaining after trimming were removed from downstream analyses.

Experimental design

After assembly, quality control and trimming, the NGS dataset was reduced to 66 loci larger than 250 bp, which were available for all 48 samples (~25 kb of sequence characters). These 66 loci were combined with the seven loci derived from the SGS method (Table 1) into five distinct datasets for downstream analyses (Fig. 2). Sequences were concatenated and assembled, using Sequence Matrix (Vaidya et al., 2011).

Dataset 1 (SGS48). In total 48 taxa were sequenced for seven SGS loci. The aligned dataset contains 4284 characters (1525 parsimony informative) (Fig. 2a).

Dataset 2 (NGS48). In total 48 taxa were sequenced for 66 NGS loci. These are the same 48 taxa used in dataset 1 (SGS48). The aligned dataset contains 25 301 characters (6422 parsimony informative) (Fig. 2b).

Dataset 3 (CB48). In total this comprises 48 taxa and 73 loci resulting from the combination of datasets

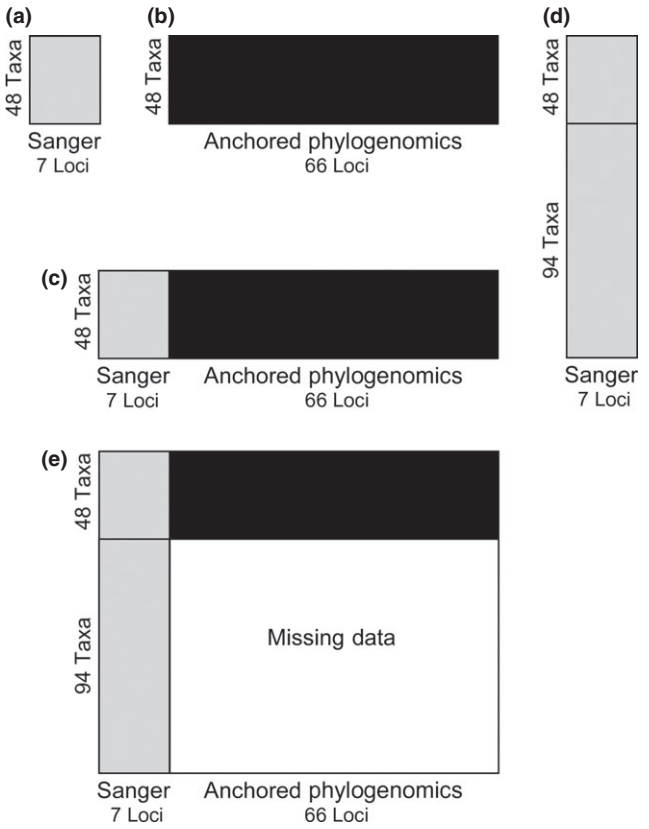

Fig. 2. Alternative datasets derived from the combination of Sanger sequencing (SGS; seven loci) and anchored phylogenomics next generation sequencing (NGS; 66 loci). See text for detailed descriptions of content and abbreviations for the datasets employed in this work. (a) SGS48, (b) NGS48, (c) CB48, (d) SGS142, (e) TE142.

1 (SGS48) and 2 (NG48). The aligned dataset contains 29 585 characters (7947 parsimony informative) (Fig. 2c).

Dataset 4 (SGS142). In total 142 taxa were sequenced for seven SGS loci. The aligned dataset contains 4284 characters (1525 parsimony informative) (Fig. 2d).

Dataset 5 (TE142). In total 142 taxa and 73 loci resulted from the combination of datasets 2 (NGS48) and 4 (SGS142) (Fig. 2e). This dataset includes all characters and taxa, therefore accounting for the best test of character congruence among partitions available to this study (we refer to it as the total evidence dataset: TE). The aligned dataset contains 29 585 characters (7947 parsimony informative).

Phylogenetic analyses: optimality criteria and data exploration

To estimate the response of our dataset to (i) differential taxon and character sampling, (ii) different sets of assumptions (e.g. treatment of indels, use of models in phylogenetic inferences), and (iii) analytical methods, we have applied four distinct discovery operations in search of causal effects of incongruence relating to topological differences in the microhylid phylogeny. We employed four tree search strategies in each of the five datasets described above: (i) maximum likelihood on prealigned sequences treating gaps as missing data—ML; (ii) parsimony on prealigned sequences (similarity-alignment) treating gaps as missing data—TNT4st; (iii) parsimony on prealigned sequences treating gaps as a fifth state—TNT5st; and (iv) direct optimization parsimony—POY (tree-alignment, which by default treats gaps as a fifth state).

Within each of the optimality criteria, we analysed all of the five different taxon/character set combinations described above, thus also evaluating the effects of the addition of sequence data and terminals on phylogenetic inference. Although we evaluated multiple optimality criteria and different datasets to estimate phylogeny of microhylids, we decided *a priori* that all taxonomic and systematic results will be based on the tree-alignment analysis (POY) of the most complete dataset (TE142: Fig. 2e). The justification for this is that this combination of data plus discovery operation makes full use of available data (all taxa and all characters, but moreover because it includes length variation information—i.e. indels), therefore maximizing its descriptive efficiency and explanatory power. The tree-alignment method also minimizes assumptions regarding transformation costs, as the same rate matrix is used for generating implied alignments and calculating tree costs. Additional justifications for the use of tree-alignment parsimony (=dynamic homologies) in phylogenetics are given in Wheeler (1996), Wheeler et al. (2006), Kluge and Grant (2006), Grant and Kluge (2009) and Padial et al. (2014) and in references therein.

Table 2 summarizes the multiple discovery operations applied to the datasets, as well as their significance and contingent explanations that can be derived from each one of them.

Multiple sequence alignment. Padial et al. (2014: see also Wheeler, 1996; Wheeler et al., 2006) provided an extensive discussion on the role of alignment methods and assumptions in assessing homology in DNA sequences. Among the most important observations of Padial et al. (2014) is a clarified distinction of (i) the conventional two-step procedure of multiple sequence alignment + subsequent tree inference (therein referred to as similarity-alignment) from (ii) the iterative improvement of homology assessments based on the co-estimation of trees and novel alignments (therein referred to as tree-alignment, after Sankoff, 1975). In the similarity-alignment, sequences are aligned by the

Table 2
Contingent explanations derived from the evaluation of the response of several datasets to changes in assumptions and treatment of evidence

Aspect under evaluation	Test and justification
Effects of increased taxon sampling	Comparing the datasets SG48 vs. SG142 within the same optimality criteria, same treatment of indels, and transformation matrix. Comparison of the CB48 vs. TE142 would presumably constitute another test of the effects of increasing taxon, but with the peculiarity that the TE142 has 94 taxa that have 66 missing loci.
Effects of increased character sampling	Comparing SGS48 vs. NGS48 vs. CB48 within the same optimality criteria, same treatment of indels, and transformation matrix. Comparing SGS142 vs. TE142 would presumably constitute another test, but TE142 has 94 taxa with 66 missing loci.
Effects of indels (i.e. gaps as a fifth nucleotide state)	Similarity-alignments + Parsimony (MUSCLE + TNT) treating gaps alternatively as missing data (TNT4st), and as a fifth character state (TNT5st). Heuristics aside, the additional evidence provided by gaps must be interpreted as the cause for any differences in tree topology and support values. Only valid if the same dataset is used for comparisons.
Effects of optimality criteria and model assumptions	Similarity-alignments (MUSCLE) + parsimony with gaps as missing data (TNT4st) vs. similarity-alignments + maximum likelihood (GARLI). Caveat: the methods are grounded in much different logical foundations and the model of nucleotide transformations adopted for the ML analyses is not equivalent to the transformation matrix applied to the parsimony analyses. Hidden model assumptions are inserted and should be considered when evaluating the differences between the two discovery operations. It has been shown that under certain models parsimony and ML will yield the same results (Farris, 1973; Tuffley and Steel, 1997), but to use such parsimony-equivalent models (e.g. No Common Mechanism) would be pointless to the present discussion. We use the GTR model instead. This comparison is only valid if the same dataset is used for comparisons.
Effects of alignment	Parsimony + similarity-alignment (MUSCLE + TNT) vs. tree-alignment (POY), with gaps treated as a fifth character state. Only valid if the same dataset is used for comparisons.
The phylogeny of Microhylidae	Tree-alignment parsimony (POY) on the TE142 dataset. This operation makes use of all available evidence (including indels, i.e. gaps as a fifth character state), thus being the most complete possible test of character congruence. This is done without addition of assumptions regarding character transformation (models, character weighting), therefore maximizing descriptive efficiency and explanatory power (see Frost et al., 2001; Kluge and Grant, 2006; Grant and Kluge, 2009; Padial et al., 2014).

For the parsimony analyses all transformations received equal weights (1); for all the ML analyses we used the GTR+I+G model for nucleotide transformations. See text for analytical details.

insertion of gaps and based on some function of similarity (e.g. codon structure, molecular structural architecture, minimum sum of pairs) and this alignment is carried out to all downstream phylogenetic analyses, without any re-evaluation of its content in light of inferred phylogenetic trees. In the tree-alignment, alignments and consequent homology statements are evaluated in reference to phylogenetic trees by optimizing sequences directly onto trees concomitantly with tree search (Sankoff, 1975; Wheeler, 1996).

For all similarity-alignment analyses (both parsimony and ML) each SGS fragment was aligned using the complete taxon set (142 taxa) and only after that were taxa and characters pruned for use in the smaller sub-datasets. For obvious reasons, the NGS loci were aligned using only 48 taxa. All alignments were produced in MUSCLE using default parameters and a maximum of eight improvement iterations per run.

Similarity-alignment + ML. ML analyses were performed with all loci concatenated in a single partition. We used the General Time Reversible model (GTR: Tavaré, 1986) with the distribution of rate variation among sites and the proportion of invariant sites both estimated from the data (GTR+G+I). The model was selected as the best fit for the concatenated dataset (TE142) using PartitionFinder and evaluating all 56 models available therein (Lanfear et al., 2012).

ML analyses were conducted in GARLI 2.01 (Zwickl, 2006). Some search parameters were changed from the default for more thorough searches following the recommendations of Zwickl (2006). Tree searches on all five datasets consisted of 500 replicates with random starting trees (default = stepwise), testing 10 00 different attachments per taxon (default = 50), and a maximum Subtree pruning and regrafting (SPR) distance of 12 branches away from the original location (default = 6). Bootstrap values were calculated from

1000 replicates with the same parameter changes set for tree searched. Only a single tree was saved per bootstrap replicate. ML analyses were conducted on the online server developed by Bazinet and Cummings (2011) with makes use of grid parallel computing through the Lattice Project (Bazinet and Cummings, 2008).

Similarity-alignment + parsimony. Analyses were conducted in TNT 1.1 (Willi Hennig Society version: Goloboff et al., 2008) on an iMac with 3.4-GHz Intel Core i7 processor and 24 GB of RAM.

Tree searches were conducted under the *xmult* command, which randomly implements a variety of tree search algorithms—Random Addition Sequences (RAS), Tree Bisection and Reconnection branch swapping (TBR), Tree Fusing (Goloboff, 1999), Sectorial Searches (Goloboff, 1999), and Tree Drifting (Goloboff, 1999). For each search the best solution must be hit 500 times before the search was stopped (command *hits 500*). Parsimony Jackknife (Farris et al., 1996) was estimated with 1000 replicates with five search iterations using *xmult* per replicate, and a removal rate of e^{-1} (=0.36) following the suggestion of Farris et al. (1996) that this is the value most congruent with bootstrapping.

Tree-alignment + parsimony. One of the caveats with the way we treated the data derived from the anchored phylogenomics loci is that they are unsuitable for analyses using direct optimization (DO). This is true because of the strict trimming we applied (excluding all sites containing more than one ambiguity: see above). Although this procedure usually trimmed the ends of the sequences, it also excluded a few bases in the middle of some loci (see Supplementary Material for trimming pattern). In addition to excluding potentially informative characters (i.e. heterozygous sites and informative gaps), this procedure, in practice, added the assumption that our loci do not correspond to "real sequences," but instead to sets of DNA characters derived from a given loci. This is not problematic for phylogenetic analyses as long as the homology assessments made during multiple sequence alignment (performed prior to trimming) are not further manipulated. Alignment was used as it was inferred from MUSCLE without further manipulation (e.g. by eye, or by hand) and is therefore tied to a well-defined optimality criterion (minimum sum of pairs). Because trimming is done after multiple sequence alignment, it poses no difficulties for similarity-alignment phylogenetic inference as homology statements are already defined prior to site exclusions. It does, however, impede analyses of tree-alignment on loci submitted to such procedure, as the fundamental aspects of the analyses assume that data are sequential and homologies are iteratively re-evaluated concomitantly with tree search. This impediment is theoretical, not practical. POY will analyse any uninterrupted string of characters, but to do this for our dataset (NGS loci) would be logically flawed, as some bases were removed from within the sequences, violating this basic assumption of the method. Given this peculiarity, DO was applied only to the seven Sanger loci, while the NGS loci were treated as pre-aligned. Genes derived from SGS were divided in blocks of putative homologous fragments, defined by variable regions delimited by conserved regions. Blocks were delimited with a hash sign (#) as suggested by Wheeler et al. (2006). By definition, tree-alignment analyses must treat gaps (coded as a dash "–") as a fifth nucleotide state (Wheeler, 1996, 2002; Wheeler et al., 2006) as is required by the logic of the method. Even if for only a subset of characters, this analysis provides evidence for the effects of treating sequences as similarity-aligned versus tree-alignment insofar as they test homology patterns during tree search.

Tree-alignment analyses were conducted in POY, versions 4.1.2 or 5.1.1 (Varón et al., 2010; Wheeler et al., 2014). Initial tree searches were conducted with the command *search*, which implements a time-constrained search with as many replicates of RAS + TBR as possible, followed by Parsimony Ratchet (Nixon, 1999), and Tree Fusing. Several runs can be initiated in parallel whereas the command (*search*) stores the shortest trees of each independent run and implements a final round of Tree Fusing using the pooled trees as a source of topological diversity. For each dataset, we performed ten consecutive 12-h searches in 16 parallel Intel Xeon 3.0-GHz processors in one of the American Museum of Natural History cluster computers (*Enyo*). For a more thorough search, the best trees from the initial searches were submitted to additional rounds of TBR under an iterative pass (IP) optimization (Wheeler, 2003b) and the implied alignments (Wheeler, 2003a) from optimal trees recovered from the IP search were used for additional searches in TNT.

Results

The number of equally parsimonious trees and respective tree lengths (for parsimony trees), as well as –log likelihood scores (for ML trees) for all of the analyses performed are given in Table 3.

The monophyly of the outgroup Afrobatrachia (Arthroleptidae, Brevicipitidae, Hemisotidae and Hyperoliidae) is not recovered in all analyses (Figs 3–6). The ML analyses, including those with the reduced character sets, recovered a monophyletic Afrobatrachia. On

Table 3
General results from all analyses performed for this study (SGS48, NGS48, CB48, SGS142, TE142; for detailed description of datasets see Material and methods)

Optimality criteria	Dataset	No. of trees	Score
Similarity-alignment + maximum-likelihood (Gaps as missing)	SGS48	1	–logL 55418.96
	NGS48	1	–logL 191832.90
	CB48	1	–logL 250499.70
	SGS142	1	–logL 124935.50
	TE142	1	–logL 321657.60
Similarity-alignment + parsimony (Gaps as missing)	SGS48	5	12 623
	NGS48	3	30 356
	CB48	3	43 040
	SGS142	6	29 788
	TE142	4	60 205
Similarity-alignment + parsimony (Gaps as a 5th state)	SGS48	1	13 253
	NGS48	1	31 188
	CB48	1	44 503
	SGS142	28	31 358
	TE142	18	62 605
Tree-alignment + parsimony (Gaps as a 5th state)	SGS48	1	12 705
	NGS48	1	31 188
	CB48	1	43 980
	SGS142	1	29 966
	TE142	1	61 216

the other hand, in the parsimony analyses (TNT4st, TNT5st and POY) with the reduced character sets (SGS48 and SGS142) Afrobatrachia is not unambiguously monophyletic (some trees are unresolved and monophyly cannot be supported).

With respect to the ingroup (Microhylidae), the analyses show a large amount of incongruence. Microhylidae is monophyletic in all of the analyses, whereas several subfamilies are not consistently and unambiguously recovered as monophyletic. Relationships among subfamilies varied widely with respect to the dataset and discovery operation applied. The trees shown in Figs 3–6 are condensed for clarity (i.e. to highlight subfamily relationships). Complete trees are available at Data Dryad (doi:10.5061/dryad.8112f). A summary of the major agreements and disagreements among the numerous different analyses employed to the five different datasets is given in Table 4.

Within-subfamily relationships

To assess generic relationships, we only take into consideration the TE142 dataset (Figs 7–8) —justification for this approach is given above. Our results generally corroborate the current arrangement of 11 subfamilies in Microhylidae. Two exceptions pertain to the positions of *Adelastes hylonomos* and *Chaperina fusca*. The position of *Adelastes* varies with the analytical methods employed—the sister of Otophryninae in the three parsimony analyses, and the sister of Gastrophryninae in the ML analysis. The position of *Chaperina* in the parsimony analyses renders Microhylinae paraphyletic. In the POY analysis, *Chaperina fusca* is the sister taxon of all Microhylidae, whereas in the ML analysis it is the sister of a clade containing *Calluella*, *Glyphoglossus*, and *Microhyla*. The ML analysis recovered a monophyletic Microhylinae, albeit with low support values. Both TNT analyses (gaps as missing and gaps as fifth state) resulted in identical poorly resolved strict consensuses, with Microhylinae divided into four clades of uncertain relationships.

Several genera were not recovered as monophyletic (Figs 7–8), among which are several that had been already known or suspected to be para- or polyphyletic (e.g. *Austrochaperina*, *Calluella*, *Kaloula*, *Oreophryne*). Of all cophyline genera sampled, only *Anodonthyla* and *Plethodontohyla* are monophyletic. On the other hand, *Microhyla*, which was recovered as para- or polyphyletic in the past (Matsui et al., 2011; De Sá et al., 2012), is monophyletic in all of our analyses. Our results strongly corroborate the long recognized monophyly of Asterophryinae, which was recovered by all analyses. On the other hand, of the nine genera of asterophryines sampled for more than a single species, four are not monophyletic in the POY analysis (i.e. *Albericus*, *Austrochaperina*, *Cophixalus*, *Oreophryne*—*Austrochaperina* is monophyletic in the ML analysis: see Supplementary Material). A brief taxonomic revision, based on our results, is given in Appendices I and II.

Discussion

The next sections individually discuss the effects of particular aspects of phylogenetic inference, i.e. use of

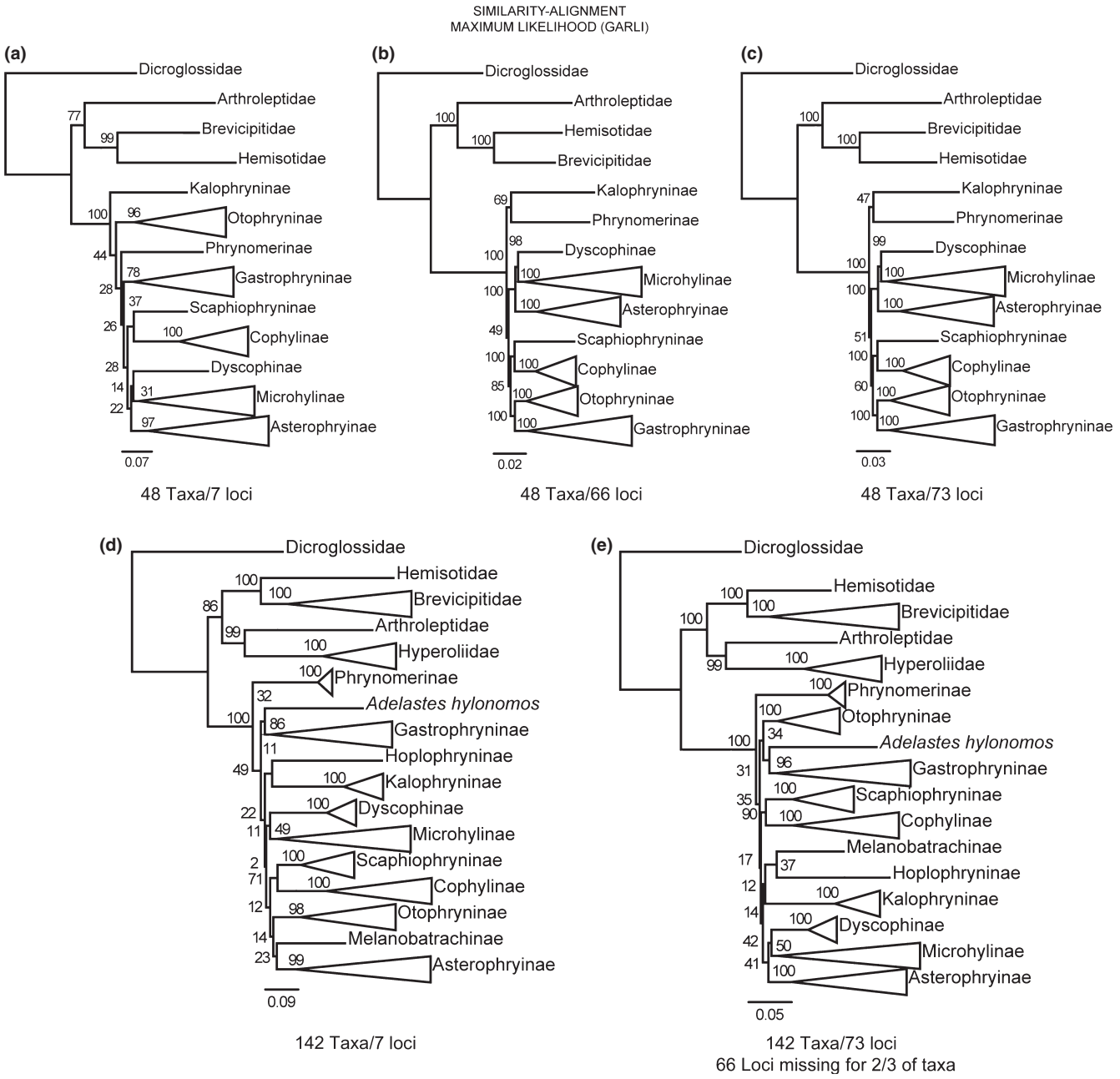

Fig. 3. Maximum-likelihood trees inferred from the similarity-alignments in GARLI (ML) and treating gaps as missing data (major clades are collapsed into a single terminal). (a) SGS48, (b) NGS48, (c) CB48, (d) SGS142, (e) TE142—details about the datasets are described in the text and Fig. 2. Numbers on nodes are bootstrap values.

data, incorporation of indels, optimality criteria and so on. Contingent explanations that can be derived from the many distinct combinations of datasets and discovery operations are given in Table 2.

More characters, more taxa, more disagreement

From the parsimony analyses, it seems that an increase in taxon sampling does not help much in resolving microhylid subfamily relationships if characters are not increased as well. In both TNT analyses in which only the seven SGS loci are included (SGS48 and SGS142), the strict consensus of the most-parsimonious trees is largely unresolved and several well-corroborated clades are not recovered as monophyletic (e.g. Afrobatrachia, Gastrophryninae, and Otophryninae). The addition of characters, both to the reduced (48 taxa) and to the larger (142 taxa) datasets, generally resulted in increased tree resolution and rescued Afrobatrachia, Gastrophryninae, and Otophryninae

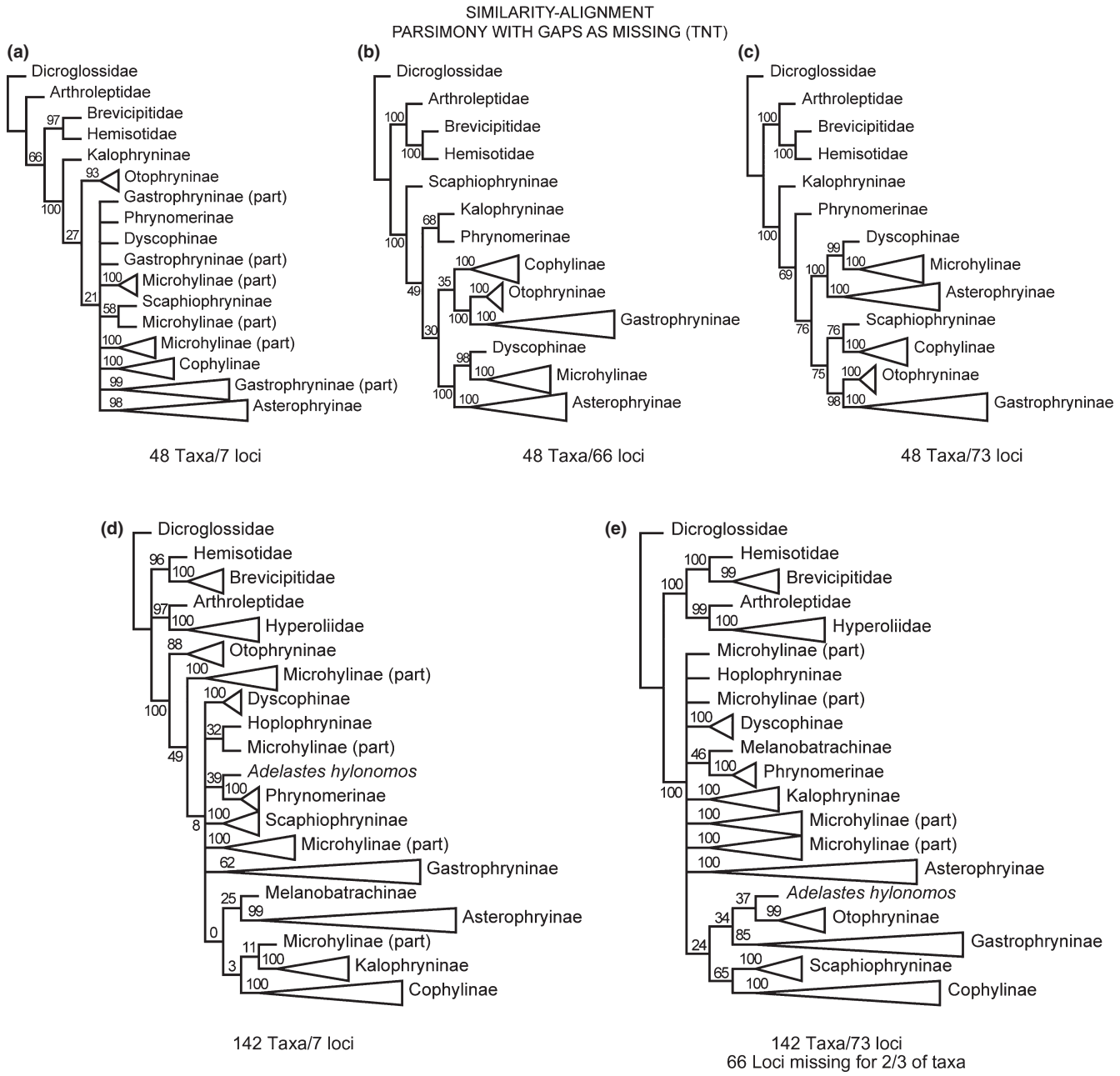

Fig. 4. Strict consensus of most-parsimonious trees inferred from the similarity-alignments on TNT and treating gaps as missing data (major clades are collapsed into a single terminal). (a) SGS48, (b) NGS48, (c) CB48, (d) SGS142, (e) TE142—details about the datasets are described in the text and Fig. 2. Numbers on nodes are parsimony jackknife values.

from non-monophyly (although not in all of the analyses). In the POY analyses, both trees are fully resolved but Afrobatrachia and Gastrophryninae are still not monophyletic in the SG142 analysis. In the ML analyses, the addition of characters predominantly affects nodal support values, which increased for most nodes of the tree (although it also caused minor shifts in topology).

Many of the taxa added to the 142-taxa datasets that were not sampled in the reduced 48 taxa (94 taxa; Fig. 2) and sequenced only for up to seven loci are members of genera sampled in the reduced datasets. Whereas most of these additional taxa fall in expected places in the tree (e.g. assumed correct subfamilies and genera, based on previous knowledge of their phylogenetic position) some of the relationships were not inferred with much certainty (e.g. for taxa never sampled before, or for those that conflict with previous work). The inclusion of taxa increased the support values in many internal nodes whereas in

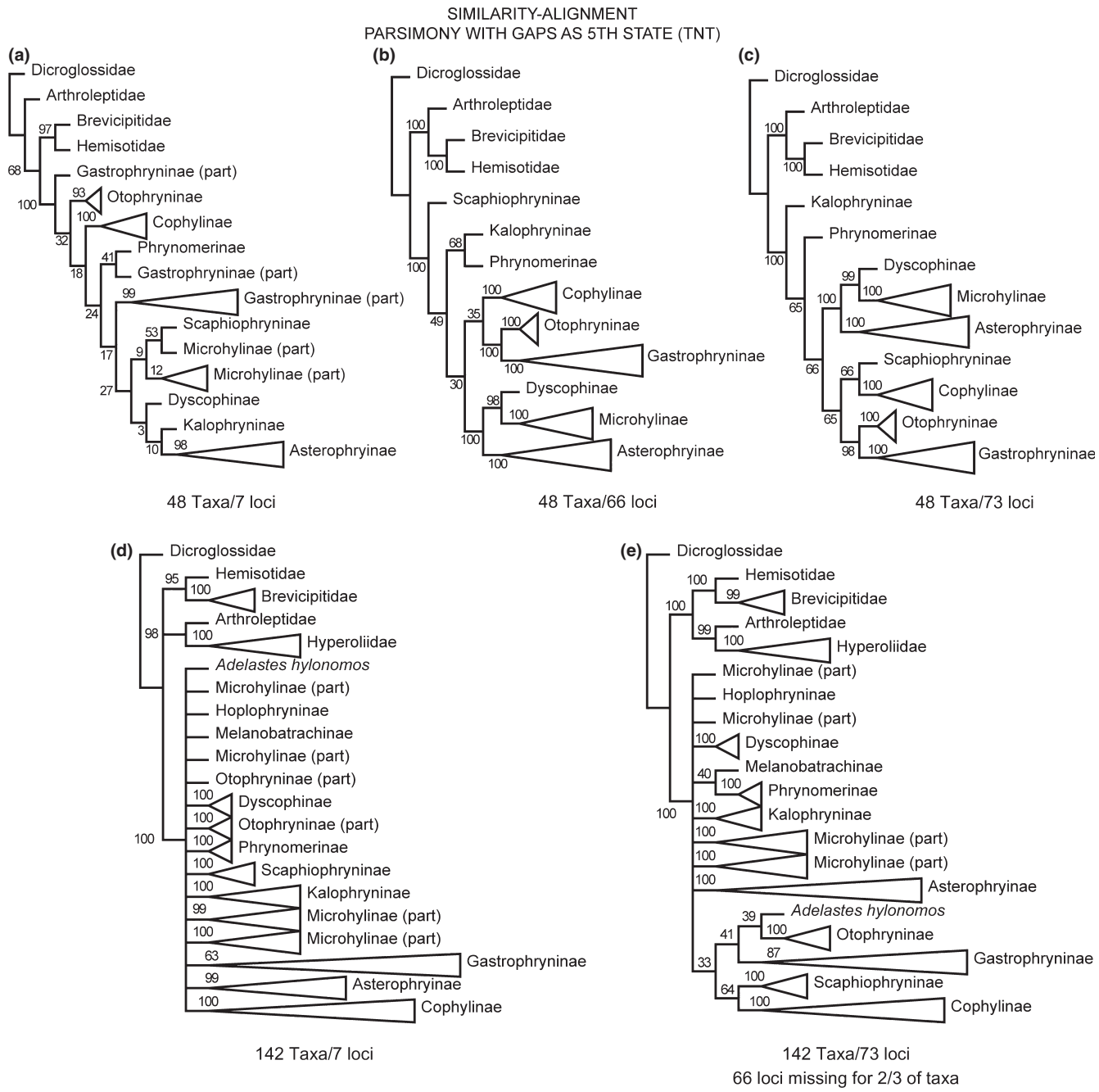

Fig. 5. Strict consensus of most-parsimonious trees inferred from the similarity-alignments on TNT and treating gaps as a fifth character state (major clades are collapsed into a single terminal). (a) SGS48, (b) NGS48, (c) CB48, (d) SGS142, (e) TE142—details about the datasets are described in the text and Fig. 2. Numbers on nodes are parsimony jackknife values.

general it did not improve support values at basal nodes (i.e. subfamilies). By contrast, in many cases support was diminished and caused the collapse of nodes supported in the reduced taxon sets (e.g. in the TNT5st analysis) with the same character sets. This may be caused by the addition of a few taxa with hard-to-infer relationships that were not sampled in the reduced datasets (i.e. *Adelastes hylonomos*, *Chaperina fusca*, *Hoplophryne rogersi*, *Melanobatrachus indicus*).

The addition of characters proved to be beneficial in terms of increased support values and tree resolution. In general, when compared with the datasets that only have the seven SGS loci, the datasets with 66 loci (NG48) and those with 73 loci (CB48 and TE142) are usually better supported and better resolved. The

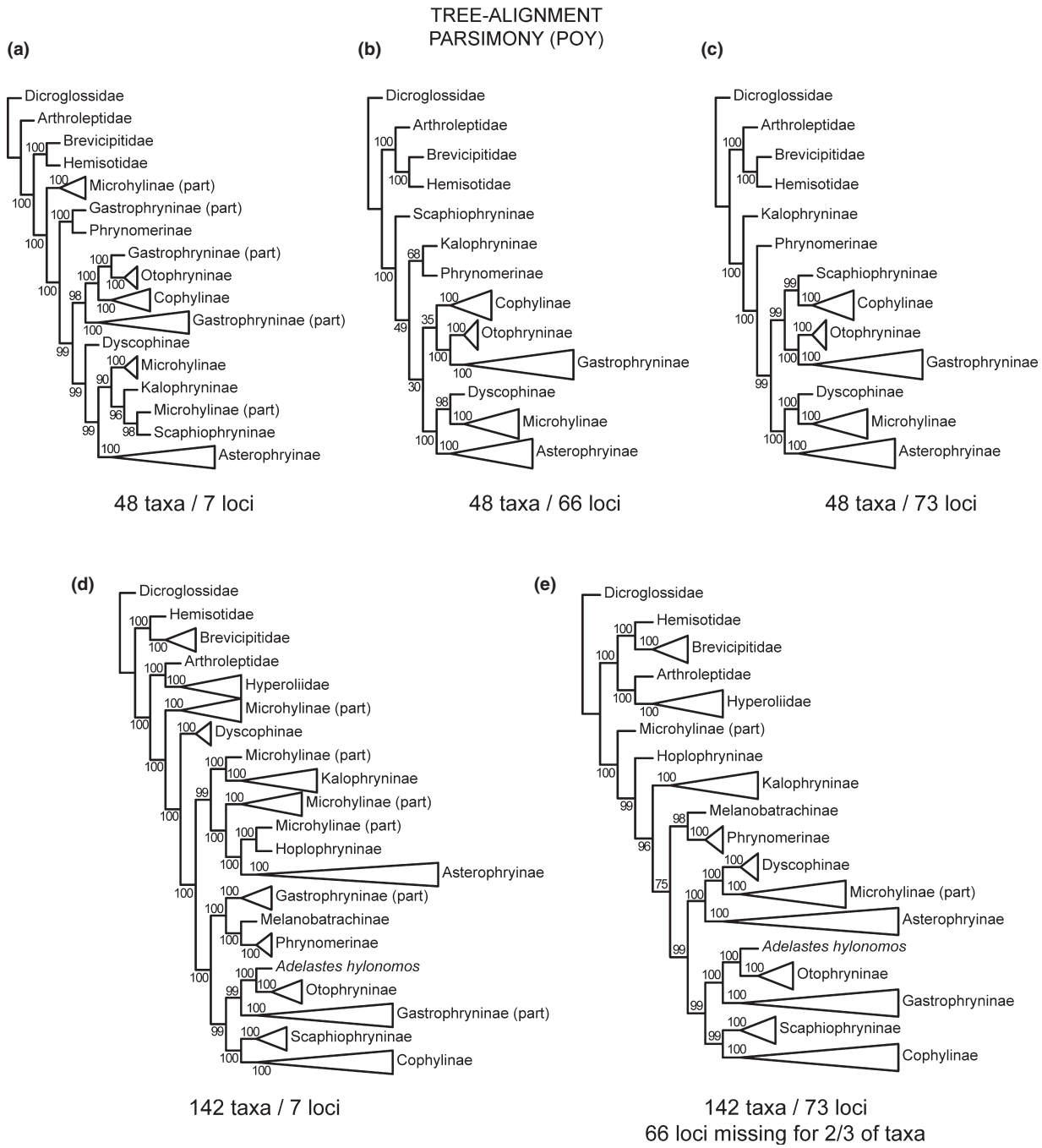

Fig. 6. Strict consensus of most-parsimonious trees inferred from using a tree-alignment method in POY (major clades are collapsed into a single terminal). (a) SGS48, (b) NGS48, (c) CB48, (d) SGS142, (e) TE142—details about the datasets are described in the text and Fig. 2. Numbers on nodes are parsimony jackknife values.

addition of characters also rescues some taxa (e.g. Afrobatrachia, Gastrophryninae and Microhylinae) from para- or polyphyly, an effect easily noticeable in the parsimony analyses.

Effects of indel information. As expected (because transformations to and from gaps count as additional steps in the optimization process), trees inferred with gaps as a fifth state (TNT5st, Fig. 4) are longer (3.2–5.0%) than those with gaps as missing data (TNT4st, Fig. 5).

The topologies obtained from the NGS48, CB48 and TE142 datasets are exactly the same regardless of gap treatment. Topologies for the NGS48 and CB48

Table 4
Recovery of clades in trees inferred from alternativee optimality criteria applied to five distinct datasets (SGS48, NGS48, CB48, SGS142, TE142; for detailed description of datasets see Material and methods)

Optimality Criteria	Dataset	Afrobatrachia monophyletic	Microhylidae monophyletic	Gastrophryninae monophyletic (A)	Microhylinae monophyletic	Cophylinae + Scaphiophryninae	Gastrophryninae + Otophryninae (single New World lineage)
Similarity-alignment + maximum likelihood (gaps as missing)	SGS48	+	+	+	+	+	–
	NGS48	+	+	+	+	+	+
	CBS48	+	+	+	+	+	+
	SGS142	+	+	+	+	+	–
	TE142	+	+	+	+	+	+ (B)
Similarity-alignment + parsimony (gaps as missing)	SGS48	–	+	–	–	–	–
	NGS48	+	+	+	+	–	+
	CB48	+	+	+	+	+	+
	SGS142	–	+	+	–	–	–
	TE142	+	+	+	–	+	+ (B)
Similarity-alignment + Parsimony (gaps as a 5th state)	SGS48	–	+	–	–	–	–
	NGS48	+	+	+	+	–	+
	CB48	+	+	+	+	+	+
	SGS142	Unresolved	+	+	–	–	Unresolved
	TE142	+	+	+	–	Unresolved	+ (B)
Tree-alignment + parsimony (gaps as a 5th state)	SGS48	–	+	–	–	–	–
	NGS48	+	+	+	+	–	+
	CB48	+	+	+	+	+	+
	SGS142	–	+	–	–	+	–
	TE142	+	+	+	–	+	+ (B)

*(A) Adelastes is not considered a priori to be part of Gastrophryninae. We followed Frost et al. (2006) and considered this taxon as incertae sedis. Pyron and Wiens (2011) did not sample *Adelastes* but arbitrarily included it in the subfamily, without any recourse to evidence.

†(B) The relationship of Gastrophryninae and Otophryninae is corroborated, but *Adelastes hylonomos* is nested within the clade, as sister of either one or the other subfamily.

differ, between datasets, in the position of Scaphiophryninae: sister of all Microhylidae in NGS48 and nested within the family, versus sister of Cophylinae in the CB48. The TE142 trees are identical and support values are also very similar.

In the SGS48 dataset, the inclusion of indel information increased tree resolution, as five equally parsimonious trees were recovered in the TNT4st analysis (resulting in an almost complete polytomy involving all subfamilies) versus a single tree in the TNT5st. Interestingly, the contrary is true for the SG142 dataset analyses, where the TNT5st is almost entirely collapsed (28 equally optimal trees) whereas the TNT4st is slightly better resolved (six trees). Therefore, although not conspicuous, some effects of including indel information are easily noticeable. There is a significant change in the number of trees recovered (Table 3) as well as changes in support values for several nodes. Often, the addition of indel information slightly improved support in some nodes that are shared among the analyses, consistent with the general pattern of increased observations enhancing resolution and support.

In practice, our results reinforce that at least some of the phylogenetic information contained in analyses of nucleotide sequences lies in the insertion and deletion transformation events. We did not, however, rediscover the wheel, as serious discussion about importance and usefulness of insertion/deletion information is at least 15 years old (Giribet and Wheeler, 1999; Simmons and Ochoterena, 2000; McGuire et al., 2001; Yang, 2006) but the vast majority of authors still do not incorporate gap information in their analyses, probably for normative rather than scientific reasons. The incorporation of gaps in phylogenetic discovery operations has long been the standard in parsimony analyses (the use of gaps as a fifth nucleotide state is the default in both POY and TNT, and it can be easily implemented in PAUP*), but this is not usually the case in model-based inferential methods. Whereas positive aspects of incorporating indel information in phylogenetic inference have long been stressed (Giribet and Wheeler, 1999; Simmons and Ochoterena, 2000; Lemmon et al., 2009), the negative effects of the omission of indels have been little discussed, especially in model-based methods. Lemmon et al. (2009) and Denton and Wheeler (2012) stressed some of these negative effects of gaps in phylogenetic estimates when they are not explicitly modelled (i.e. when they are treated as ambiguities or missing data), but also alerted to the fact that "most software treat gaps as ambiguous characters because explicit models of indels are rarely implemented" (Lemmon et al., 2009; p. 141). It is, to a certain point, disappointing that the most popular implementations of model-based methods (e.g. Beast, MrBayes, GARLI, and RAxML) are lagging in the inclusion of indels in the pool models they can deal with. The use of models in phylogenetics has been largely justified on the premise that models "have been carefully crafted to account for new biological phenomena as they were discovered" (Huelsenbeck et al., 2011, p. 226), when obviously they have not. Insertions and deletions, even within coding sequences, are well-known real processes in nature (Soding and Lupas, 2003; Taylor et al., 2004), and the reasons why they are not more often incorporated in models completely evade us. Several models that explicitly incorporate indels have been proposed and vary from the simple variant of a Neyman model where all transformations, including indels, are equally probable to a more parameterized GTR-like model (Neyman, 1971; Tavaré, 1986; McGuire et al., 2001; Wheeler, 2006). We feel the development of additional models and their full implementation should be more thoroughly explored in ML- and Bayesian inference-based methods.

Effects of optimality criteria. When considering the same character and taxon sets, trees inferred from parsimony (TNT4st) and ML differ in several aspects. The datasets with only the seven SGS loci (SGS48 and SGS142) resulted in polytomies in the TNT4st and in poorly supported trees in the ML analyses. The topologies for the NGS48 dataset differ only in the position of Scaphiophryninae, whereas the topologies for CB48 differ only in the position of Phrynomerinae. The TE142 again resulted in a polytomy in the parsimony analysis and in a poorly supported tree in the ML analyses.

Although sometimes receiving low support, Afrobatrachia, Gastrophryninae, and Microhylinae are always monophyletic in the ML analyses, whereas this is not true in the TNT4st analyses. This observation can be extended to the other implementations of parsimony (TNT5st and POY). Heuristics aside—and controlling for the fact that treatment of gaps is the same, i.e. as missing data—we are to credit this pattern of increased resolution, support and clade stability in the ML analyses solely to the optimality criteria (and its embedded assumptions: e.g. model of nucleotide transformation). It appears the parsimony analyses could not identify sufficient characters supporting those clades supported by the ML analyses but, clearly, the method and model assumptions could. The one case that attracted our attention the most is the monophyly of Microhylinae, which shows opposite trends—monophyletic in ML and not monophyletic in parsimony. To what level this increase in resolution and stability in the ML analyses should be viewed as a reason for preference of ML over alternative methods remains to be seen. Padial et al. (2014) have shown an example where ML makes a decision on the placement for a

taxon that completely lacks evidence for its final position, whereas parsimony collapses the entire branch where that taxon is ambiguously placed given the evidence. Simmons (2012a,b) had also shown cases in which ML can favour spurious topologies even in the complete absence of parsimony-informative characters, both in the presence of missing data and in complete matrices. Consilience of the examples shown by Padial et al. (2014) and Simmons (2012a,b) suggests that the increased resolution in ML analyses in comparison with parsimony should be carefully evaluated. In the absence of sufficient evidence—or in the presence of too much ambiguous evidence, i.e. homoplasy—parsimony will tend to collapse branches due to topological conflicts and ambiguous placement of rogue taxa, whereas ML will often return fully resolved trees. As noted by Siddall (2010) and Simmons (2012b: quoted here) "relying upon increased resolution in parametric analyses relative to parsimony to conclude superior performance of the former is unwise". This is particularly problematic in implementations of parametric analyses—both for tree search and for bootstrapping—that hold a single optimal tree that is usually fully resolved (Simmons, 2012b; Simmons and Goloboff, 2013), as is the case for GARLI (used here) and other commonly used ML programs. Wolsan and Sato (2010, p. 181), for example, compared the relative performance of a parametric optimality criterion (Bayesian) versus parsimony and concluded "BI [Bayesian] analysis on the all-species supermatrix outperformed the MP [Maximum Parsimony] analysis in that the former yielded a completely resolved consensus topology with a larger share of strongly supported relationships". It is precisely this type of conclusion that we do not want to jump to with our results—inasmuch as we could, given that POY resulted in a single fully resolved and highly supported tree. We are, however, aware that jackknife values calculated over the implied alignment derived from optimal POY trees tend to be high. We highlight here the importance of investigating the response of these datasets to additional partition schemes and to alternative models of nucleotide evolution.

Effects of alignment assumptions (similarity-alignment vs. tree-alignment). Although only seven of the 73 loci were subjected to DO, the effects of it are immediately clear. For equivalent datasets POY optimal trees are always shorter (1.2–4.6% shorter) than those of TNT5st. In practice, this means that POY found better alignment/tree combinations than those generated from the two-step, similarity-alignment procedure required by TNT. The NGS48 dataset resulted in the same topology and scores—this is, however, expected as these datasets derive entirely from the anchored phylogenomics loci, which were treated as prealigned for the POY searches.

Also expected is the that the implied alignments of optimal trees are longer (more columns in the matrix). This is because some (or many) of the estimated homologies found by the similarity-alignment have been tested in light of a tree topology found to be false. In practice, this means that the optimal tree-alignment requires more gaps than the alignment generated by MUSCLE (POY implied alignments are 1.6–22.2% longer). This derives from the fact that POY is truly dealing with the tree alignment problem (*sensu* Sankoff, 1975; Sankoff and Cedergren, 1983; Felsenstein, 1988)—in which we want to determine median sequences assignments given a distance function such that the overall tree cost is minimal—and is evaluating sequence homology in light of cladograms (Wheeler, 2001, 2003a), and not based on overall sequence similarity (as inferred by multiple sequence alignment software). As a consequence, in many cases where the same base is shared by distant taxa, the similarity-alignment method will infer homoplasy, whereas the tree-alignment method, as implemented in POY, will commonly infer these two characters to be part of independent transformation series.

Data exploration

We have shown that differential taxon and character sampling, as well as the choice of discovery operations, may result in largely incongruent results. Therefore, for any experiment, taxon and character sampling should be carefully planned prior to phylogenetic analyses. It would be simple to find a large quorum of investigators to agree that, when inferring relationships in a complex group, the more taxa and more characters the better. Yet, there are always tradeoffs between increasing one versus the other (e.g. financial costs, time, addition of missing data, computational burden). Our results support the notion that background knowledge of the phylogenetic tree can be used to identify those taxa that should be more thoroughly sampled (e.g. by using NGS techniques) versus those for which limited character sampling suffices for unambiguous placement. It is expected that just a few characters (e.g. a single or few loci) may be enough to resolve most recent splits (closely related species) as long as there is enough phylogenetic information to unambiguously resolve relationships. Therefore it may be informative to compare the results of two types of analyses: one with maximal taxon sampling, and a second with minimal missing data. This strategy will also identify, after tree inference, taxa for which focus should be placed on gathering additional characters for improving subsequent estimates. Subsequent sampling should be focused on clearly identified rogue taxa, on those clades that show unstable or poorly supported topologies, or even in those that are firmly

placed in alternative places depending on dataset analysed or method used, as they are more likely to be suffering from lack of, or ambiguous, phylogenetic signal in available characters.

We also reinforce that the choice of discovery operation is hugely influential on the final topologies and all subsequent inferences we may make based on the trees (e.g. classification, character optimizations for studies in evolution and biogeography). The parsimony analyses were shown to be more prone to instability with changes in the dataset (e.g. conspicuous topological shifts, low jackknife supports). On the other hand, topologies inferred from ML analyses are a great deal more "stable" to addition of taxa and characters. It is unclear, however, how much of this stability comes from the method over-fitting uncertainty (i.e. ambiguous data) into predictable transformation events generated by model assumptions. This is an important and under-appreciated area of investigation in phylogenetic inference that, once thoroughly explored, will invariably have to include investigations of the effects of incorporating indel events (gaps) as part of the models (Denton and Wheeler, 2012). Although already largely incorporated in parsimony analyses and proven to influence phylogenetic inference, the incorporation of indel information in model-based approaches is still in its infancy. Models are available and so is software capable of dealing with them—apparently, the real problem is the reluctance by the scientific community to evolve from its state of inertia and actually incorporate these models into the analyses.

Concluding remarks

As with any historical science, phylogenetics can prove no theory correct any more than it can prove any wrong—only differentially falsified (Popper, 1968; Cracraft, 1978; Kluge, 1997). Yet, we still seek to infer the relationships among taxa (i.e. phylogenetic relationships) and do so by means of discovery operations designed to reconcile available evidence (i.e. characters) into a hypothetical and concise explanation (i.e. trees). The choice of discovery operation should therefore be thoroughly justified, but this is rarely the case. Often several methods are applied indiscriminately in search of common patterns that are, by induction, then taken to be accurate or precise, whereas conflicts are commonly explained *post hoc*. By doing this, one can adapt any preconceived beliefs of how a given group's phylogeny has unfolded into an argument in favour of one preferred solution against all of the conflicting ones. This sort of data exploration was attacked by Grant and Kluge (2003) on the grounds that pluralistic approaches lack scientific rigour and logical justification. On the other hand, we see the application of contrasting methods to the same sets of evidence as the best way to evaluate the effects of the choice of discovery operation in the interpretation of the data (see also Giribet et al., 2002). This approach allows us to identify specific areas of a given methodology where conflicting results may be linked to a causal explanation (methodological artefacts, differential treatment of data, model assumptions, heuristics, etc.). Our experiment is therefore part of a heuristic approach to evaluate the effects of taxon and character sampling, as well as two of the commonly used optimality criteria in modern-day phylogenetics (parsimony and ML), in the phylogenetic inference of microhylids. When employing these tests, however, we are not implying that concordance between methods and datasets should be viewed as support for a given topology. The contrary is also true—discordance does not mean conclusive falsification of any given relationship.

From our analyses, it seems that a denser character sampling is needed within a few microhylid taxa. Special attention is warranted to *Micryletta* and *Chaperina*—these two taxa seem to be the most sensitive to changes in the amount of available evidence, parameterization and optimality criteria (Frost et al., 2006; Pyron and Wiens, 2011; Matsui et al., 2011; our own results). Other taxa that we easily point as targets for increased character sampling are the Hoplophryninae (*Hoplophryne rogersi*, *H. uluguruensis*, and *Parhoplophryne usambarica*), *Melanobatrachus indicus* (Melanobatrachinae) and *Adelastes* (Adelastinae subfam. nov.). Some of these taxa that we identify for potential character augmentation were probably predictable even before we conducted our experiment and analyses, and we regret not being able to include them in the anchored phylogenomics dataset for technical reasons.

In conclusion, our results show, again, that attempting to solve relationships of microhylids is a phylogeneticist's nightmare. But there is light at the end of the tunnel: we have identified a number of clades that seem to be stable regardless of the addition of data, taxa and assumptions (i.e. monophyly of Asterophryinae, Cophylinae, Otophryninae, and several relationships within subfamilies).

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Note added in proof

After this paper was accepted for publication (04 February 2015), a new species of Stumpffia was named in Glaw et al. (2015, publication date of 26 February 2015): Stumpffia kibomena. By implication of our results, this species should be treated as a member of Rhombophryne Boettger, 1880, as Rhombophryne kibomenus (Glaw, Vallan, Andreone, Edmonds, Dolch, and Vences, 2015) new combination.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Metadata for samples included in phylogenetic analyses (including Genbank accession numbers).

Text S1. Material and Methods.

Text S2. Trimming pattern of the Anchored Phylogenomics loci.

Text S3. Hennig 86 file with the Implied Alignment for the otimal tree inferred in POY (tree-alignment) from the TE142 dataset.

Appendix 1

Taxonomy of Microhylidae

All of our taxonomic decisions are based on the topology from the total evidence (TE142) tree-alignment parsimony analyses in POY (Figs 7 and 8; see justification for this approach in the section *Phylogenetic analyses: optimality criteria and data exploration*).

Adelastes hylonomos Zweifel, 1986

The monotypic genus *Adelastes* was named based on a series of male specimens from Departamento Rio Negro, Venezuela (Zweifel, 1986). In the original description Zweifel (1986) provided a phylogenetic analysis of the species, which was found in an unresolved polytomy with the gastrophrynines *Arcovomer*, *Chiasmocleis*, *Hamptophryne* and "*Syncope*" (the last now considered a synonym of *Chiasmocleis*: Peloso et al., 2014). Wild (1995), using a slightly larger character set, found *Adelastes* to be the sister species of a clade including (*Chiasmocleis* ("*Syncope*" + *Arcovomer*)). We sampled *Adelastes* from a single female specimen (ROM 38258) from Guyana. Despite the fact that all of the types of *Adelastes* are males (Zweifel, 1986), based on external morphology and osteological features we are confident that the specimen sampled here is an *Adelastes*. We are tentative about its specific status but assign this specimen to *A. hylonomos*, pending further investigation of the taxonomic status of the Guyana population. *Adelastes* was only sequenced for the SGS loci and therefore was only included in the more taxonomically inclusive datasets (SGS142 and TE142). In the ML analyses *Adelastes* is sister to Gastrophryninae, whereas in the parsimony analysis, it is the sister to Otophryninae. Given the instability of the position and the presence of at least one autapomoprhic trait (morphology of the parahyoid) we place *Adelastes* in its own subfamily.

Adelastinae subfam. nov.

Type genus. Adelastes Zweifel, 1986, by monotypy.

Content. Monotypic.

Definition. This taxon is characterized by a series of transformations in the ribosomal gene 16S and in the protein coding genes BDNF, COI, CMYC, histone H3, SIA*h*1, and tyrosinase. Transformations can be visualized in the implied alignment (Wheeler, 2003a) of the

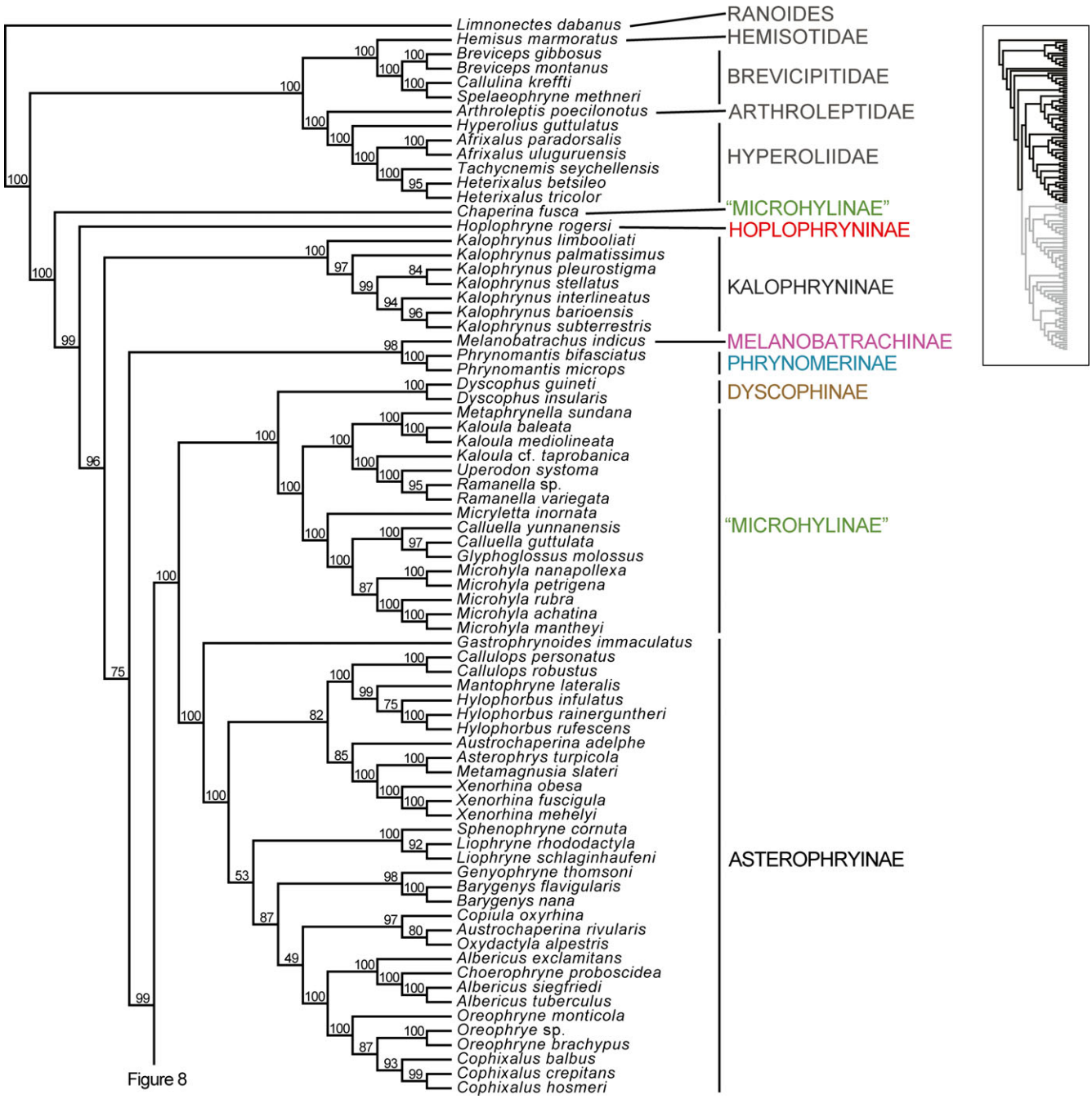

Fig. 7. Single most-parsimonious (part 1) tree inferred from the total evidence dataset (TE142) in POY using the tree-alignment method (61 216 equally weighted steps). Numbers on nodes are jackknife values inferred from the implied alignment. For the rest of the tree, see Fig. 8. This figure is available in colour in the online version of the paper.

TE142 tree-alignment topology, which is given as Supplementary Material (Text S3).

A diagnosis for *Adelastes* is available in Zweifel (1986). Because *Adelastes* is the sole genus in Adelastinae, the same diagnosis can be extended to characterize the new subfamily. The large ossified parahyoid with a free border on the hyoglossal sinus is apparently unique among Microhylidae (Zweifel, 1986) and may be a morphological autapomorphy of Adelastinae. We are unaware of any additional morphological autapomorphies.

Chaperina fusca Mocquard, 1892

In the POY-TE142 (Fig. 7), *Chaperina fusca* (currently assigned to Microhylinae) was found as the sister to all remaining microhylids, rendering Microhylinae paraphyletic. Based on that, we remove *Chaperina fusca* from Microhylinae and place it in its own subfamily.

Chaperininae subfam. nov.

Type genus. Chaperina Mocquard, 1892, by monotypy.

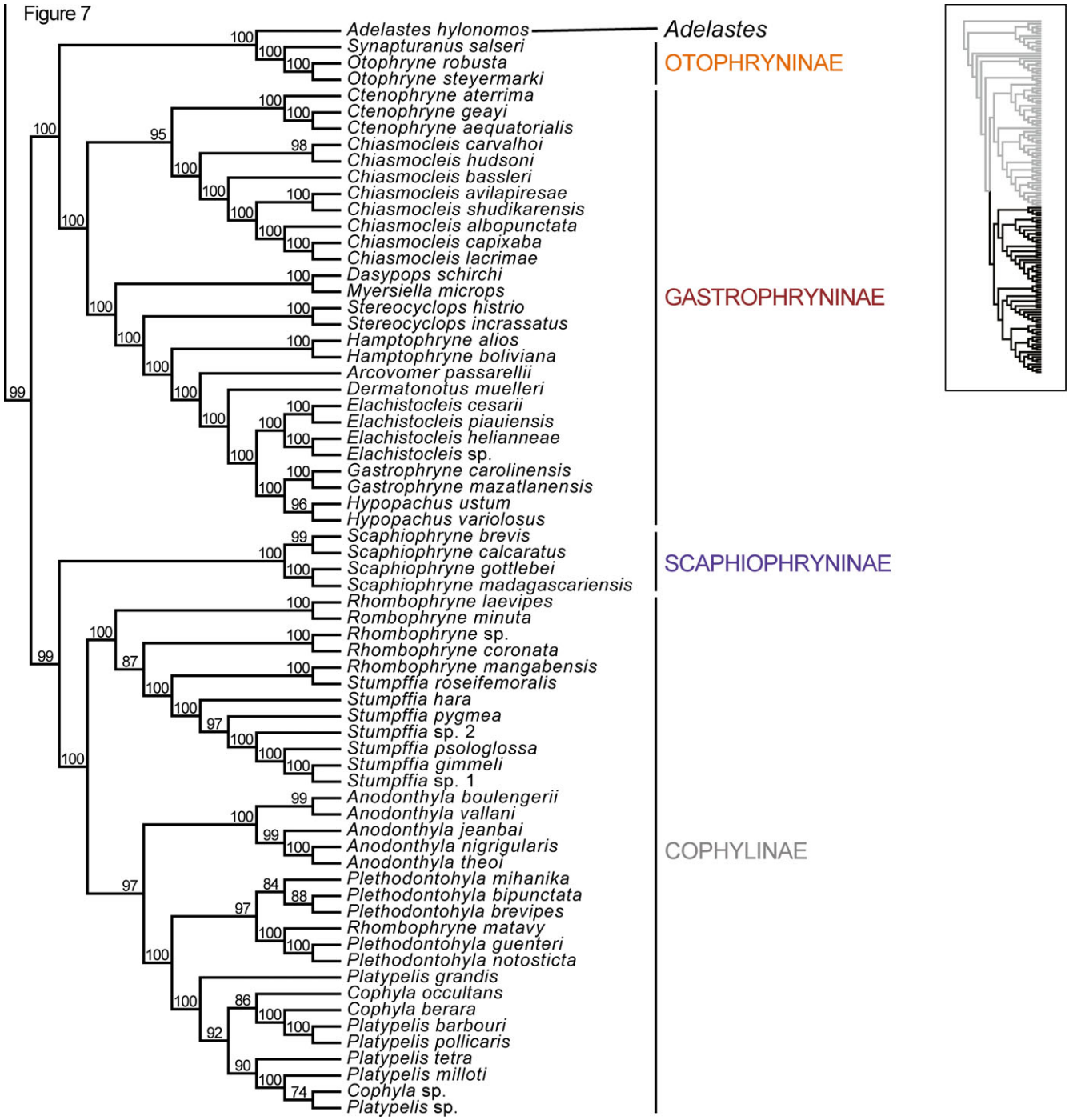

Fig. 8. Single most-parsimonious tree (part 2) inferred from the total evidence dataset (TE142) in POY using the tree-alignment method (61 216 equally weighted steps). Numbers on nodes are jackknife values inferred from the implied alignment. For the remainder of the tree, see Fig. 7.

Content. Monotypic.

Definition. This taxon is characterized by a series of transformations in the ribosomal gene 16S and in the protein coding genes BDNF, COI, CMYC, histone H3, SIA*h*1, and tyrosinase. Transformations can be visualized in the implied alignment (Wheeler, 2003a) of the TE142 tree-alignment topology, which is given as Supplementary Material (Text S3).

Diagnoses for *Chaperina* are available in Mocquard (1892) and in Parker (1934). Because *Chaperina* is the sole genus in Chaperininae, the same diagnoses can be extended to characterize the new subfamily. A long and narrow dermal spine is present in each elbow and calcaneus of *Chaperina* and may be a morphological autapomorphy of Chaperininae. We are unaware of any additional morphological apomorphies for Chaperininae.

Subfamily Asterophryinae

The coupling of our results with previous ones (most notably, Köhler and Günther, 2008; Pyron and Wiens, 2011; Rittmeyer et al., 2012) points to an urgent need for a major revision in the taxonomy of asterophryines. We are, however, hesitant in proceeding with this major revision as our taxon sampling is limited when compared with some previous work and considering the size of the clade (298 species as of 1 October 2014: Frost, 2014). Nonetheless, we feel obliged to take this first step to pave the way for a more thorough review of classification of the subfamily, one that we hope will be available soon. Limited by our taxon sampling of 32 asterophryines (11% of the subfamily's content) and the lack of several genera (and many type species), but impelled by our desire to make progress, on a few occasions we refer to previously published phylogenetic evidence in a in an attempt to promote progress in the taxonomy of the group.

Albericus Burton and Zweifel, 1995, is paraphyletic, with respect to *Choerophryne proboscidea* which is nested within it. Kraus (2013) suggested that the only morphological character differentiating *Albericus* from *Choerophryne* Van Kampen, 1914—the angle of the allary process of the premaxillae—is not consistent and observed an intermediate state in *C. bryonopsis*. However, Kraus (2013) did not formalize the synonymy until phylogenetic evidence is available. Such evidence is provided here, and the two genera are combined under a single name, for which *Choerophryne* Van Kampen, 1914, is the senior name.

The two species of *Austrochaperina* sampled herein render the genus polyphyletic. *Austrochaperina adelphe* is the sister of a clade containing *Asterophrys*, *Metamagnusia* and *Xenorhina*, whereas *Austrochaperina rivularis* is sister of *Oxydactyla crassa*, with the two forming the sister clade of *Copiula oxyrhina*. Köhler and Günther (2008) found a non-monophyletic *Austrochaperina* as two unnamed species were found as the sister of *Copiula major*, with that clade being the sister of another formed by (*A. derongo* (*Copiula obsti* + *Copiula pipiens*)). In the meantime, *Oxydactyla crassa* was found in a clade with a paraphyletic *Liophryne* plus *Sphenophryne*. Pyron and Wiens (2011) found similar results, with *Copiula* being paraphyletic in relation to *Austrochaperina—Copiula major* being sister to (*Austrochaperina derongo* (*C. obsti* + *C. pipiens*)). Rittmeyer et al. (2012) found both *Copiula* and *Austrochaperina* to be polyphyletic. In their analysis, *C. major* is the sister of *Liophryne schlaginheufeni*, while the remaining *Copiula* are nested in clade containing most species of *Austrochaperina*—only *A. palmipes* is not found in that clade; instead it is sister of a major clade containing several other genera. It is clear that at least some of species from *Austrochaperina* and *Copiula* cannot be treated as separate entities. In case of synonymy, *Copiula* Méhely, 1901, has priority over *Austrochaperina* Fry, 1912. We, however take a different approach to this problem, as we have not sampled the *Austrochaperina robusta* (the type species of the genus) and at present do not know where its relationships lie. *Austrochaperina robusta* was sampled by Hoskin (2004) but the fact that his analysis is restricted to four Australian species of *Austrochaperina* (none of them sampled by Köhler and Günther, 2008; or by Rittmeyer et al., 2012) makes it of little help to the nomenclature problem we are trying to solve. Because we sampled the type species of *Copiula* (*Phrynixalus oxyrhinus* Boulenger, 1898 = *C. oxyrhina*), the clade where it was recovered will maintain the name *Copiula* and we only transfer species of *Austrochaperina* that were shown to be related to *Copiula* to the latter genus (i.e. *A. derongo*, *A. guttata*, *A. rivularis*). In the meantime, we maintain *Austrochaperina* as a valid genus name, and maintain *A. adelphe* in that genus. We expect future work will show more species of *Austrochaperina* to be nested within—or perhaps, the sister taxa of—*Copiula*, and we recommend when this is reported that those species are transferred immediately. If, however, *A. robusta* is shown to be part of this clade, additional nomenclatural acts may be necessary (i.e. to account for the generic placement of, at least, *A. adelphe* and *A. palmipes*). By implication, our action requires a re-evaluation of *Oxydactyla* as well. The type species of the genus is *O. brevicrus*, which was not sampled by us or by previous workers. Köhler and Günther (2008), Pyron and Wiens (2011) and Rittmeyer et al. (2012) all sampled only *O. crassa* whereas we have sampled *O. alpestris*. Zweifel (2000), based on the evaluation of several phenotypic characters, have flirted with the idea that *Oxydactyla* may be paraphyletic with the inclusion of *O. alpestris* in the genus. Based on the assertion by Zweifel (2000) we can only speculate on the monophyly of *Oxydactyla* and suggest only that *O. alpestris* be transferred to *Copiula*. Further investigation of the position of remaining *Oxydactyla* species will determine the fate of the generic name.

Finally, we found *Oreophryne* Boettger, 1895, to be paraphyletic in relation to *Cophixalus* Boettger 1892. Köhler and Günther (2008) reported a polyphyletic *Cophixalus*, with *C. sphagnicola* as the sister of a clade containing *Albericus laurini* and four species of *Choerophryne*; and three other species of *Cophixalus* plus *Barygenys exsul* (nested in the clade) as the sister clade to a monophyletic *Oreophryne*. A somewhat different arrangement was found by Pyron and Wiens (2011), who also found *Cophixalus* to be polyphyletic. Pyron and Wiens (2011) found *C. sphagnicola* to be the sister of *B. exsul* while the remaining three species of *Cophixalus* were paraphyletic with respect to *Oreophryne* and the clade recovered as the sister to *Aphantophryne pansa*. Rittmeyer et al. (2012) found *Oreophryne* to be monophyletic and the sister of all remaining asterophryines. *Cophixalus* was found to be polyphyletic by Rittmeyer et al. (2012) and arising in three parts of the tree: (1) *C. sphagnicola* as the sister of *Albericus* + *Choerophryne*; (2) *C. humicola* and *C. tridactylus* found as sister taxa and the sister of *A. pansa*; and (3) *C. balbus* as the sister of *Paedophryne* and *B. exsul*. De Sá et al. (2012) found *Cophixalus* to be polyphyletic, but because five of his six *Cophixalus* samples are labelled simply as *Cophixalus* sp. (probably unnamed or unidentified taxa), their tree has no important systematic implications regarding this issue. It is noteworthy, however, that De Sá et al. (2012) did not find any of their "*Cophixalus*" to be related to *Oreophryne*. The only identified species, *C. sphagnicola*, was found as the sister of *Austrochaperina derongo*.

As shown, previous work has come to conflicting results regarding relationships between *Cophixalus* and *Oreophryne* but most were conclusive that *C. sphagnicola* may not be related to either genus (at least not to those species sampled). Given our results, we consider *Oreophryne* Boettger, 1895 as a junior synonym of *Cophixalus* Boettger, 1892. However, for the following reasons, we refrain from taking this action at the present time. (1) We did not sample *Cophixalus sphagnicola*, which seems to be unrelated to the other species of *Cophixalus* that have been sampled (see discussion above). (2) We did not sample the type species of *Cophixalus* [*C. verrucosus* (Boulenger, 1898)] or of *Oreophryne* [*O. moluccensis* (Peters and Doria, 1878)]. (3) Taking any nomenclatural action here would, by implication, demand further reviews of the status of three other genera—*Aphantophryne*, *Barygenys* and *Paedophryne*—flagged by Rittmeyer et al. (2012) to be possibly nested within *Cophixalus*. Two of these genera were not sampled and our sampling of *Barygenys* differs from that of Rittmeyer et al. (2012), who only sampled *Barygenys exsul*. Pyron and Wiens (2011) found *Barygenys* to be polyphyletic, with *Barygenys exsul* as the sister taxon of *Cophixalus sphagnicola*, and *Barygenys flavigularis* (also sampled by us) as the sister of a clade containing *Xenorhina*, *Asterophrys*, *Metamagnusia* and *Pseudocallulops* (the last two labelled in their tree as *Callulops*). (4) *Cophixalus* and *Oreophryne* are the two most species-rich genera of Microhylidae and our taxon sampling is limited—five species in total. (5) Examination of the types of *Oreophryne monticola* (seven syntypes, BMNH 1947.2.12.26–1947.2.12.32) revealed a frog that deviates slightly in body shape and colour pattern from that of typical *Oreophryne*. The vast majority of *Oreophryne* reside in New Guinea and satellite islands, whereas *O. monticola* is found on the islands of

Lombok and Bali, in the Lesser Sunda Islands. It is possible that *Oreophryne monticola* deserves its own genus to account for its genetic, morphological and geographical distinctiveness in contrast to the remaining *Oreophryne*. This is, however, a matter that must be treated in future work.

Subfamily Cophylinae

Of the seven genera of cophylines we were able to sample six, missing only the monotypic (and rare) *Madecassophryne*. Surprisingly, of the six sampled genera only *Anodonthyla* and *Plethodontohyla* are monophyletic.

The relationships of *Cophyla* Boettger 1880 and *Platypelis* Boulenger, 1882 differ in both parsimony and ML analyses, but from both it is clear that the differentiation between *Cophyla* and *Platypelis* is not well delimited. Rakotoarison et al. (2012) commented briefly on this issue but nonetheless rooted their phylogenetic tree of several *Platypelis* with a single *Cophyla* (*C. berara*), therefore assuming the monophyly of *Platypelis* in relation to *Cophyla*. The two genera are almost indistinguishable on the basis of external morphology (Glaw and Vences, 2007) and putative differentiation is limited to a few osteological characters (particularly the arrangement of the vomer and clavicles, which are known to vary widely in Microhylidae). The presumed phylogenetic distinctiveness of the two is based on analyses with limited taxon and character sampling. Wollenberg et al. (2008) found a sister taxon relationship between *Cophyla* and *Platypelis*, but the arrangement received low support values. On what is perhaps the most rigorous test of the relationships of *Cophyla* and *Platypelis*, Pyron and Wiens (2011) found both of them to be monophyletic but not the sisters of each other, as did previous studies (Andreone et al., 2005; Wollenberg et al., 2008). Our result does not support reciprocal monophyly of both genera, and hence we consider *Platypelis* a junior synonym of *Cophyla*.

The non-monophyly of *Stumpffia* was reported by Wollenberg et al. (2008) and later confirmed by Pyron and Wiens (2011) and Perl et al. (2014), although in different topologies. Wollenberg et al. (2008) found *Rhombophryne* to be nested in *Stumpffia*. More precisely, *S. helenae* + and an unnamed species (*Stumpffia* sp. 8) formed the sister of all *Rhombophryne*, with that clade being sister of all remaining *Stumpffia*. Pyron and Wiens (2011) found *S. helenae* to be sister of a monophyletic *Rhombophryne*, while the remaining *Stumpffia* were sister of a clade containing *Cophyla* + the (*S. helenae* + Rhombophryne) clade. Wollenberg et al. (2008) had suggested that *S. helenae* and "*Stumpffia*" sp. 8 could be recognized as a new genus, but this was never formally proposed. Here, we consider *Stumpffia* Boettger, 1881 to be a junior synonym of *Rhombophryne* Boettger, 1880.

One of the specimens included in our analyses (AMNH 167315) is the same one included by Frost et al. (2006) and originally labelled as *Plethodontohyla* in their analysis. The sample was recovered nested within the *Rombophryne*/*Stumpffia* clade, and is therefore treated as an unnamed species of *Rhombophryne*.

Subfamily Gastrophryninae

De Sá et al. (2012) recently provided a thorough systematic review of this clade and our results are largely congruent with the taxonomic arrangement proposed therein, and thus endorsed herein. The only major topological conflict pertains to the clade that includes species of *Chiasmocleis*. De Sá et al. (2012) transferred several species of *Chiasmocleis* to *Syncope*. This result was, however, not supported by a larger analysis of the group by Peloso et al. (2014), which placed *Syncope* in the synonymy of *Chiasmocleis*. Whereas De Sá et al. (2012) found *C. bassleri* to be nested within his *Syncope* clade, Peloso et al. (2014) recovered *C. bassleri* as the sister species of two clades, one containing all species of *Chiasmocleis* and the other containing all species of *Syncope* (*sensu* De Sá et al., 2012), thus suggesting the name *Chiasmocleis* should be applied to the whole clade. Here, we recovered *C. bassleri* in a different position from that found by both De Sá et al. (2012) and Peloso et al. (2014). This new position of *C. bassleri*, however, requires no addendums to the taxonomy proposed by Peloso et al. (2014).

Subfamily Microhylinae

Taxon sampling within microhylines is sufficient to tackle several recurrent problems in the group's taxonomy. *Kaloula* is again recovered as paraphyletic, with *K. taprobanica* being the sister taxon of a clade containing *Ramanella* and *Uperodon* (both endemic to India). The other two species of *Kaloula* sampled were found as the sister clade of *Metaphrynella sundana*. Similar results were reported in previous inferences with different taxa and character sampling (Kurabayashi et al., 2011; Pyron and Wiens, 2011; De Sá et al., 2012). To provide a monophyletic taxonomy for microhylines, we are left with the following options: (1) lump the whole clade into a single name, for which *Kaloula* Gray, 1831, would have priority over the remaining sampled members (i.e. *Metaphrynella* Parker, 1934; *Ramanella* Rao and Ramanna, 1925; and *Uperodon* Duméril and Bibron, 1841); (2) provide a new generic name for *Kaloula taprobanica*; (3) or lump *K. taprobanica*, *Ramanella* and *Uperodon* into a single name, for which *Uperodon* would have priority. We have not sampled *Phrynella* for this study, which appears to be related to *Metaphrynella* (Kurabayashi et al., 2011). On the other hand, the position of *K. taprobanica* as the sister of *Uperodon* and *Ramanella* is stable even with distinct datasets and analytical methods (see Van Bocxlaer et al., 2006; Kurabayashi et al., 2011; Pyron and Wiens, 2011; De Sá et al., 2012). The clade containing *K. taprobanica* + *Uperodon* + *Ramanella* is consistently recovered as the sister taxon of all other *Kaloula* + (*Phrynella* and *Metaphrynella*) (Pyron and Wiens, 2011; De Sá et al., 2012; our TE142 analyses). Ramaswami (1936) had provided evidence that the characters separating *Ramanella* and *Kaloula* (mostly based on characteristics of the vomer) are non-existent and suggested that the two be considered synonyms (note that most of Ramaswami's observations on *Kaloula* are based on *K. taprobanica*). Curiously, the proposal was largely ignored in subsequent work. Thus, the most reasonable option given our topology seems to be placing *Ramanella* Rao and Ramanna, 1925, and *Kaloula tatrobanica* Parker, 1934, into *Uperodon* Duméril and Bibron, 1841.

On another problematic issue within microhylines is *Glyphoglossus molossus* nested within *Calluella* (*C. guttulata* and *C. yunnanensis*). Paraphyly of *Calluella* with respect to *Glyphoglossus* was also reported by Matsui et al. (2011) and Das et al. (2014) both based on denser samplings of *Calluella*.

The position of *Calluella yunnanensis* found by us, as well as by Matsui et al. (2011) and Daas et al. (2014), differs markedly from that found by De Sá et al. (2012) who reported the species to be nested within *Microhyla*. On the other hand, *Microhyla,* which is monophyletic in all of our TE142 analyses, was reported to be paraphyletic by De Sá et al. (2012), with *M. achatina* (the type species of the genus) found isolated from all other *Microhyla* and sister of *C. guttulata* + *G. molossus*. We investigated the source of this incongruence more deeply. Nucleotide BLAST searches (Altschul et al., 1990) against the NCBI database (GenBank) of De Sá et al. (2012) sequences provide decisive evidence that their sequences were misidentified. The *M. achatina* samples from De Sá et al. (2012) blasts with 99% identity (0.0 E-value) against two sequences of *C. yunnanensis* (FMNH 232988 [KC822481] and KUHE 44148 [AB634684]), while it has only 89% identity (0.0 E-value) with the only other sequence of *M. achatina* available at the database (RMB 2629 [KC822492]). The *C. yunnanensis* sample of De Sá et al. (2012) blasts

with 99% identity (0.0 E-value) against *Chaperina fusca* (RMB 3031 [KC180012]). The same specimen of *C. yunnanensis* sequenced by De Sá et al. (2012: FMNH 232988) was later sequenced by Blackburn et al. (2013: accession number KC822481), and the two sequences show an identity value of only 87% (E-value 2e-169). When the sequence by De Sá et al. (2012) is blasted against the other *C. yunnanensis* specimen (KUHE 44148: AB634684) the same occurs (87% identity, E-value 5e-175). Clearly, the results for this part of the tree of De Sá et al. (2012) cannot be taken into account as they appear to be laden with misidentified, or mixed samples.

Given the above evidence, we follow our results and conclude that *Glyphoglossus* is nested within *Calluella*. Thus, *Calluella* Stoliczka, 1872 is here considered a junior synonym of *Glyphoglossus* Günther, 1869.

Appendix 2

Treatment of microhylid species names revised herein.

Subfamily	Current name and authority	Revised taxonomy (new combinations)
Asterophryinae	*Albericus alpestris* Kraus, 2010	*Choerophryne alpestris*
	Albericus brevicrus Günther and Richards, 2012	*Choerophryne brevicrus*
	Albericus brunhildae Menzies, 1999	*Choerophryne brunhildae*
	Albericus darlingtoni (Loveridge, 1948)	*Choerophryne darlingtoni*
	Albericus exclamitans Kraus and Allison, 2005	*Choerophryne exclamitans*
	Albericus fafniri Menzies, 1999	*Choerophryne fafniri*
	Albericus gudrunae Menzies, 1999	*Choerophryne gudrunae*
	Albericus gunnari Menzies, 1999	*Choerophryne gunnari*
	Albericus laurini Günther, 2000	*Choerophryne laurini*
	Albericus murritus Kraus and Allison, 2009	*Choerophryne murrita*
	Albericus pandanicolus Günther and Richards, 2012	*Choerophryne pandanicola*
	Albericus rhenaurum Menzies, 1999	*Choerophryne rhenaurum*
	Albericus sanguinopictus Kraus and Allison, 2005	*Choerophryne sanguinopictus*
	Albericus siegfriedi Menzies, 1999	*Choerophryne siegfriedi*
	Albericus swanhildae Menzies, 1999	*Choerophryne swanhildae*
	Albericus tuberculus (Richards, Johnston, and Burton, 1992)	*Choerophryne tubercula*
	Albericus valkuriarum Menzies, 1999	*Choerophryne valkuriarum*
	Albericus variegatus (Van Kampen, 1923)	*Choerophryne variegatus*
	Austrochaperina derongo Zweifel, 2000	*Copiula derongo*
	Austrochaperina guttata Zweifel, 2000	*Copiula guttata*
	Austrochaperina rivularis Zweifel, 2000	*Copiula rivularis*
	Oxydactyla alpestris Zweifel, 2000	*Copiula alpestris*

(Continued)

Subfamily	Current name and authority	Revised taxonomy (new combinations)
Cophylinae	*Platypelis alticola* (Guibé, 1974)	*Cophyla alticola*
	Platypelis barbouri Noble, 1940	*Cophyla barbouri*
	Platypelis cowanii Boulenger, 1882	*Cophyla cowanii*
	Platypelis grandis (Boulenger, 1889)	*Cophyla grandis*
	Platypelis karenae Rosa, Crottini, Noël, Rabibisoa, Raxworthy, and Andreone, 2014	*Cophyla karenae*
	Platypelis mavomavo Andreone, Fenolio, and Walvoord, 2003	*Cophyla mavomavo*
	Platypelis milloti Guibé, 1950	*Cophyla milloti*
	Platypelis olgae Rakotoarison, Glaw, Vieites, Raminosoa, and Vences, 2012	*Cophyla olgae*
	Platypelis pollicaris Boulenger, 1888	*Cophyla pollicaris*
	Platypelis ravus Glaw, Köhler, and Vences, 2012	*Cophyla ravus*
	Platypelis tetra Andreone, Fenolio, and Walvoord, 2003	*Cophyla tetra*
	Platypelis tsaratananaensis Guibé, 1974	*Cophyla tsaratananaensis*
	Platypelis tuberifera (Methuen, 1920)	*Cophyla tuberifera*
	Rhombophryne matavy D'Cruze, Köhler, Vences, and Glaw, 2010	*Plethodontohyla matavy*
	Stumpffia analamaina Klages, Glaw, Köhler, Müller, Hipsley, and Vences, 2013	*Rhombophryne analamainus*
	Stumpffia be Köhler, Vences, D'Cruze, and Glaw, 2010	*Rhombophryne be*
	Stumpffia gimmeli Glaw and Vences, 1992	*Rhombophryne gimmeli*
	Stumpffia grandis Guibé, 1974	*Rhombophryne grandis*
	Stumpffia hara Köhler, Vences, D'Cruze, and Glaw, 2010	*Rhombophryne hara*
	Stumpffia helenae Vallan, 2000	*Rhombophryne helenae*
	Stumpffia madagascariensis Mocquard, 1895	*Rhombophryne madagascariensis*
	Stumpffia megsoni Köhler, Vences, D'Cruze, and Glaw, 2010	*Rhombophryne megsoni*
	Stumpffia miery Ndriantsoa, Riemann, Vences, Klages, Raminosoa, Rödel, and Glos, 2013	*Rhombophryne miery*
	Stumpffia psologlossa Boettger, 1881	*Rhombophryne psologlossus*
	Stumpffia pygmaea Vences and Glaw, 1991	*Rhombophryne pygmaeus*
	Stumpffia roseifemoralis Guibé, 1974	*Rhombophryne reseifemoralis*
	Stumpffia staffordi Köhler, Vences, D'Cruze, and Glaw, 2010	*Rhombophryne staffordi*
	Stumpffia tetradactyla Vences and Glaw, 1991	*Rhombophryne tetradactylus*
	Stumpffia tridactyla Guibé, 1975	*Rhombophryne tridactylus*
Microhylinae	*Calluella brooksii* (Boulenger, 1904)	*Glyphoglossus brooksi*
	Calluella capsa Das, Min, Hsu, Hertwig, and Haas, 2014	*Glyphoglossus capsa**
	Calluella flava Kiew, 1984	*Glyphoglossus flavus*
	Calluella guttulata (Blyth, 1856)	*Glyphoglossus guttulatus*
	Calluella minuta Das, Yaakob, and Lim, 2004	*Glyphoglossus minutus*
	Calluella smithi (Barbour and Noble, 1916)	*Glyphoglossus smithi*
	Calluella volzi (Van Kampen, 1905)	*Glyphoglossus volzi*
	Calluella yunnanensis Boulenger, 1919	*Glyphoglossus yunnanensis*
	Kaloula taprobanica Parker, 1934	*Uperodon taprobanicus*
	Ramanella anamalaiensis Rao, 1937	*Uperodon anamalaiensis*
	Ramanella minor Rao, 1937	*Uperodon minor*
	Ramanella montana (Jerdon, 1854)	*Uperodon montanus*
	Ramanella mormorata Rao, 1937	*Uperodon mormoratus*
	Ramanella nagaoi Manamendra-Arachchi and Pethiyagoda, 2001	*Uperodon nagaoi*
	Ramanella obscura (Günther, 1864)	*Uperodon obscurus*
	Ramanella palmata Parker, 1934	*Uperodon palmatus*
	Ramanella triangularis (Günther, 1876)	*Uperodon triangularis*
	Ramanella variegata (Stoliczka, 1872)	*Uperodon variegatus*

* We interpret the species name "*capsa*" (Das et al., 2014) to be used as a noun and not an adjective. Therefore, the epithet remains unchanged despite the fact that *Glyphoglossus* is a masculine name.