



Resolving the deep phylogeny: Implications for early adaptive radiation, cryptic, and present-day ecological diversity of Papuan microhylid frogs

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ABSTRACT

The microhylid frogs of the New Guinea region are the largest and most ecologically diverse subfamily (Asterophryinae) of one of the largest anuran families in the world and can live in communities of up to 20 species. While there has been recent progress in resolving the phylogenetic relationships of Asterophryinae, significant uncertainties remain, impeding further progress in understanding the evolution of microhabitat use, parental care, and life history variation in this group. In particular, the early divergences at the base of the tree remain unclear; as does the monophyly of some genera; and recent studies have discovered that species with wide geographic distribution are instead cryptic species complexes. In this study, we fortified geographic sampling of the largest previous phylogenetic effort by sequencing an additional 62 taxa and increased data quality and quantity by adding new layers of data vetting and by filling in previously incomplete loci to the five gene dataset (2 mitochondrial, 3 nuclear protein-coding genes) to obtain a dataset that is now 99% complete in over 2400 characters for 233 samples (205 taxa) of Asterophryinae and 3 outgroup taxa, and analyzed microhabitat use data for these taxa from field data and data collected from the literature. Importantly, our sampling includes complete community complements at 19 sites as well as representatives at over 80 sites across New Guinea and its offshore islands. We present a highly resolved molecular phylogeny which, for the first time, has over 95% of nodes supported (84% highly supported) whether using Maximum Likelihood or Bayesian Inference, allowing clarification of all genera (whether monophyletic or clearly not), their sister genera relationships, as well as an age estimate for the Asterophryinae at approximately 20MYA. Early generic diversification occurring between 17 and 12 MYA gave rise to a surprising diversity of about 18 genera as well as the 5 putative microhabitat types. Our tree reveals extensive cryptic diversity calling any widespread taxa into doubt, and clearly demonstrates that complex multispecies communities of Asterophryinae are ecologically diverse, are numerous, and of ancient origin across New Guinea. We discuss the implications of our phylogeny for explaining the explosive diversification of Asterophryinae as the result of adaptive radiation, niche conservatism, and non-adaptive radiation.

1. Introduction

A robust phylogenetic hypothesis is central to the study of biodiversity (Hunt et al., 2007; Vieites et al., 2009; Jetz et al., 2012; Feng et al., 2017). Simpson (1953) viewed adaptive radiation as special periods in evolutionary history when lineages shift into new adaptive zones that promotes phenotypic change and lineage diversification, an

idea that we can now address with modern phylogenetic approaches (eg., Schlüter, 2000; Grant and Grant, 2008; Gillespie et al., 2020). Nearly all modern investigations of evolutionary processes rely on phylogenies with clear timings and orders of branching events to inform evolutionary models, such as when exploring the geospatial history of clades (Ree et al., 2005; Van Dam and Matzke, 2016), community assembly (Webb et al., 2002; Emerson and Gillespie, 2008), or rates of

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diversification (Ricklefs, 2007; Alfaro et al., 2009), among other processes. At the same time, clades with the greatest diversity provide the greatest phylogenetic challenges, with their large numbers of taxa and short branch lengths during periods of rapid diversification, often precisely where the evolutionary process is most interesting. One such example is the hyperdiverse Asterophryinae frogs, which has been the subject of phylogenetic revision and re-revision for decades (Savage, 1973; van Boclaer et al., 2006; Frost et al., 2006; Köhler and Günther, 2008; van der Meijden et al., 2007; Rivera et al., 2017; Tu et al., 2018; Dubois et al., 2021).

The Asterophryinae subfamily of microhylid frogs is exceptionally diverse, with over 340 species and counting (AmphibiaWeb, 2020), representing over half of the world's diversity of the family Microhylidae on a comparatively tiny fraction of the earth's surface. Centered in the Papuan region, they are the dominant anuran fauna of New Guinea and its surrounding islands (including the D'Entrecasteaux islands, other small satellite islands, and the Bismarck and Louisiade Archipelagos) and also extending into Malaysia, the Philippines, along the northeastern coast of Australia, and recently described from Thailand and Vietnam. Asterophryinae are exceptionally diverse in ecology with arboreal, scansorial, terrestrial, fossorial, and semi-aquatic lifestyles evolving multiple times within the clade (Zweifel and Tyler, 1983; Köhler and Günther, 2008; Rivera et al., 2017) and can exist in complex communities of up to 20 species per site (Bickford, 2002), features that are common to adaptive radiations. However, given the turbulent geological history of the Papuan region, with landmasses variously colliding and creating mountain ranges, being pulled apart into islands, or eroded, there exist ample opportunities for allopatric speciation for the astounding accumulation of biodiversity. Further progress on the explanation of biodiversity, however, has been hampered by the lack of a robust phylogeny.

While the subfamily Asterophryinae is clearly monophyletic, it has long been recognized that the systematic treatment of its genera is challenging (Savage, 1973; van Boclaer et al., 2006; Frost et al., 2006; Köhler and Günther, 2008; van der Meijden et al., 2007; Rivera et al., 2017; Tu et al., 2018; Dubois et al., 2021). Part of the reason is the lack of phylogenetically informative morphological characters. Some of the features used in the original phylogenetic treatments were later discovered to be homoplastic, such as digital disk development (i.e., toe pad size) and toe and tibiofibula length (Boulenger, 1890; Zweifel, 1972), all characters associated with lifestyle; or have involved multiple independent loss or reduction as in the clavicles, procoracoids, and the pectoral girdle (Duellman and Trueb, 1985; Burton, 1986; Burton, 1990). In addition, traits associated with the skull are highly variable, such as the maxillary bones, dentaries, nasal development, vomer expansion, and vomer spikes (Parker, 1934; Zweifel, 1972), rendering their inclusion of limited phylogenetic value.

Recent molecular phylogenies for Asterophryinae have begun to clarify generic relationships with inclusion of additional taxa and molecular markers (Köhler and Günther, 2008; van der Meijden et al., 2007; de Sa et al., 2012; Peloso et al., 2016; Rivera et al., 2017; Tu et al., 2018). There is now strong evidence for the sister clade relationship of *Mantophryne* and *Hylophorus*, with *Callulops* sister to both genera (Rittmeyer et al., 2012; Oliver et al., 2013; Peloso et al., 2016; Rivera et al., 2017). *Xenorhina* has strong support as the sister clade to *Callulops* + *Mantophryne* + *Hylophorus* (Rivera et al., 2017). There is strong evidence for the synonymization of *Albericus* and *Choerophryne* (Peloso et al., 2016; Rivera et al., 2017). While earlier studies suggested that *Cophixalus* was not monophyletic (Köhler and Günther, 2008; Pyron and Wiens, 2011; Rittmeyer et al., 2012), the monophyly of *Cophixalus* was later confirmed with the genus arising early in the history of the subfamily (Peloso et al., 2016; Rivera et al., 2017).

Despite tremendous progress, intergeneric relationships along the backbone of the Asterophryinae phylogeny remains largely unresolved. Importantly, this means that the placement of most genera within the larger phylogeny is uncertain, and their order of diversification is not

known. Furthermore, the monophyly of several genera remains unresolved. The most enigmatic clade is the geographically widespread genus *Oreophryne*, ranging across New Guinea and its satellite islands and portions of Southeast Asia. While Köhler and Günther (2008) found *Oreophryne* to be monophyletic, the addition of species has since complicated the taxonomy, with various workers suggesting the existence of two or three clades (with the inclusion of *Aphantophryne* and possibly *Paedophryne*; Rivera et al., 2017; Tu et al., 2018), and disagreement on whether one of the *Oreophryne* clades is the most basal clade of Asterophryinae. Additional questions remain for *Austrochaperina*, *Copiula*, *Liophryne* and *Genyophryne*.

Another issue potentially contributing to systematic challenges is cryptic diversity – defined as two or more morphologically similar species that are mistakenly classified under a single species (Bickford et al., 2007; Funk et al., 2012). At the most basic level, without the proper recognition of cryptic species, biodiversity is substantially underestimated (e.g., Hebert et al., 2004; Bickford et al., 2007; Lahaye et al., 2008; Vieites et al., 2009), leading to an incomplete understanding of diversity in all aspects of the group – including life history, evolutionary history, biogeography, and genetic diversity. While many studies focus on coalescent-based approaches at the interface of population genetics and phylogenetics to delineate relatively recent cases of cryptic speciation (Heled and Drummond, 2010; Liu et al., 2010; Chifman and Kubatko, 2014), even for studies involving deep evolutionary divergences, the existence of unrecognized candidate species is increasingly recognized as a common occurrence that cannot be ignored (Fouquet et al., 2007; Rovito et al., 2013a). In systematic studies, unrecognized cryptic diversity can lead to undersampling of lineages, potentially missing taxa that could break up long branches if they were recognized as distinct species (Felsenstein, 2004). Frogs have long been recognized as a key target for cryptic species investigations (Bickford et al., 2007; Funk et al., 2012), and cryptic diversity is increasingly recognized in Asterophryinae (Oliver et al., 2013; Oliver et al., 2017). In addition, many New Guinean microhylid species remain unnamed, further complicating the assessment of the biodiversity within communities and the subfamily at large as well as difficulty in elucidating its organization.

To facilitate meaningful evolutionary studies of Asterophryinae, we revisit the phylogeny, importantly expanding geographic and species sampling to 233 samples comprising 205 taxa, including several from the Philippines and Western Indonesia. We evaluate the monophyly of all currently recognized genera, as well as resolving their timing and order of branching. We begin with the assumption that cryptic diversity is present and include multiple sites for nominal species, and evaluate the spatial scale over which candidate species in our phylogeny vary. Using our phylogeny, we analyze the evolution of microhabitat use as well as characterize complex microhylid communities. We discuss whether ecological variation evolves early in the history of Asterophryinae with subsequent community assembly, or whether it arises by the colonization of a new area with subsequent diversification. And finally, we consider whether our notions of lineage diversification have influenced our taxonomy.

2. Materials and methods

2.1. Taxon sampling and data collection

This study includes 233 samples of Asterophryinae containing 205 taxa (including 122 named and an additional 83 putative taxa) representing all known genera. The phylogeny was rooted using three out-group taxa include the hypothesized sister taxon and more distantly related lineages – *Dyscophus antongilii*, *Scaphiophryne marmorata*, and *Platypelis grandis* (van der Meijden et al., 2007; Tu et al., 2018). The Asterophryinae samples span over 80 sites across New Guinea (Fig. 1; see Table 1 in Hill et al., submitted for publication), the Philippines, and Indonesia. A previous study included 157 Asterophryinae samples

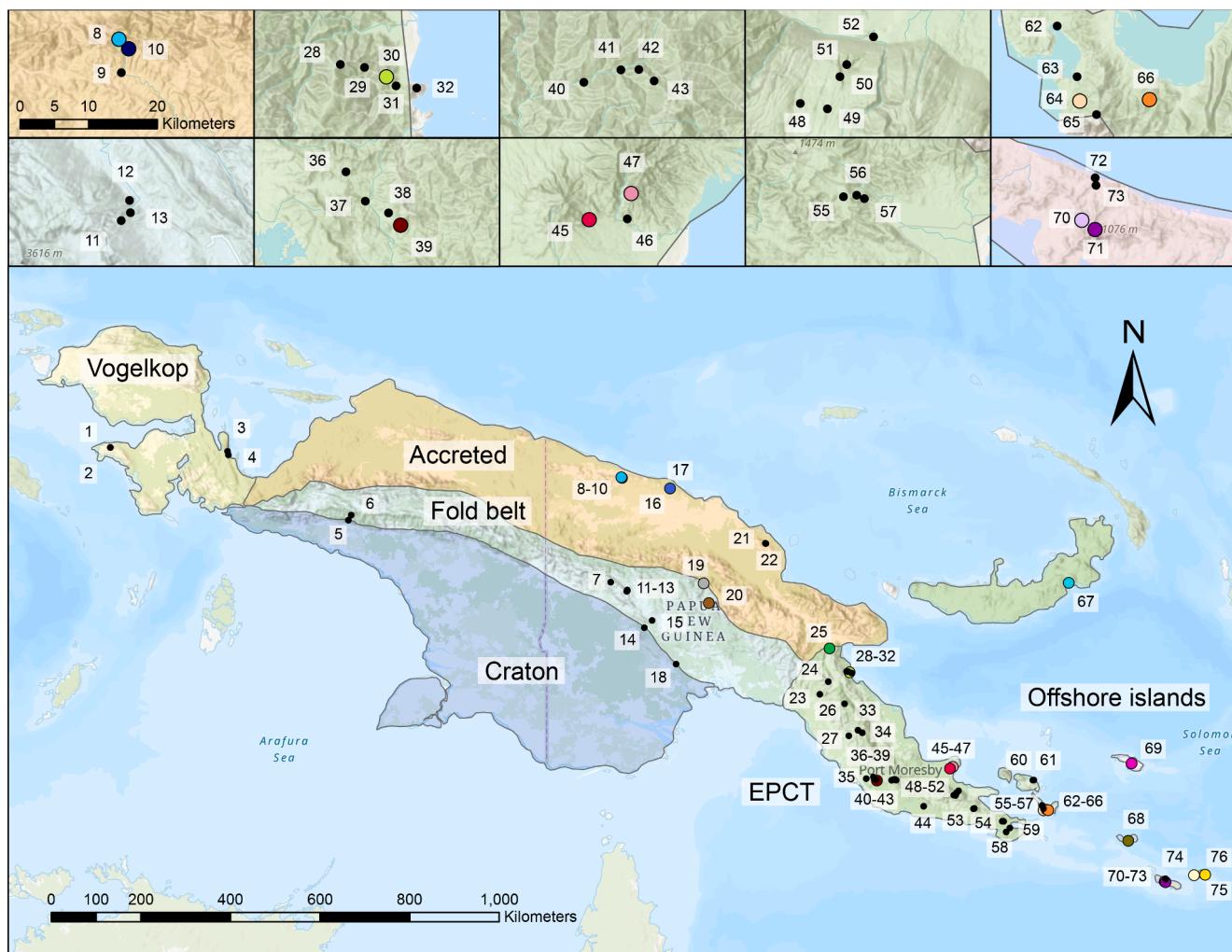


Fig. 1. Sampling sites across PNG and its satellite islands. The island of New Guinea has a composite history formed by the amalgamation of multiple geologic terranes. The major geologic terranes of New Guinea are labeled following Davies (2012): the Eastern Papuan Composite Terrane, the Accreted Terrane, the Fold Belt, the Australian Craton, and the Vogelkop Peninsula. Insets magnify areas with tight clusters of sites to show more detail. Site numbers with the names of their respective localities are as follows: sites 8–10 Torricelli Mountains, 11–13 Mt. Itukua, 28–32 Bowtutu Mountains, 36–39 Mt. Vorivori and Mt. Gerebu, 40–43 Mt. Obree, 45–47 Mt. Victory and Mt. Trafalgar, 48–52 Mt. Dayman and Mt. Suckling, 55–59 Owen Stanley Mountains, 62–66 Normanby Island, 70–73 Sudest Island. Site names, GPS coordinates, and metadata are provided in Table 1 in Hill et al. (submitted for publication).

Rivera et al. (2017), the current study expands this dataset by 74 samples. Forty-seven taxa were obtained from new field collections which targeted multi-species communities at six sites across Papua New Guinea: five mainland multi-species community sites (Kamiali [site 30], Morobe Province; Gerebu [site 39], Central Province; and Rondon Ridge [site 20], Western Highlands Province), one mainland site with a single species (Baiyer River [site 19], Western Highlands Province), and two satellite island multi-species community sites (Mwatebu [site 66] and Buyetai [site 64], on Normanby Island, Milne Bay Province).

Field workers collected frogs after sunset, slowly walking through the habitat listening for frog calls, and capturing frogs by hand. Animals were euthanized using MS-222 following UH IACUC protocol 12–1458 to M. Butler with liver samples stored in 70% EtOH and specimens preserved for morphological study and deposited at the Bishop Museum or University of Michigan Museum of Zoology. Previously reported samples Rivera et al. (2017) were obtained via tissue loan from the Bishop Museum, Honolulu, Hawaii (BPBM), Museum of Vertebrate Zoology at Berkeley (UMZ), University of Michigan Museum of Zoology (UMMZ), Zoolisches Museum Berlin (ZMB), and University of Kansas Biodiversity Institute and Natural History Museum (KU). Specimen accession numbers and metadata are reported in Table 1 of Hill et al. (submitted for publication).

Total DNA was extracted from liver samples stored in 70% ethanol using the Qiagen DNeasy extraction kit following manufacturer's protocols. Five rare samples were repaired using the NEB "preCR Repair Mix" following manufacturer's protocols prior to PCR amplification. These included samples which may have been formalin fixed (*Aphantophryne pansa*.1 BPBM5299 and *Aphantophryne pansa*.2 BPBM8312) or slightly degraded during shipment from the field (Rondon Ridge: *Asterophrys* sp. AA24862 & *Callulops wilhelmanus*.sp.1 AA24828; Baiyer River: *Hylophorus rufescens*.sp.2 AA24930).

The five locus dataset (~2500 bp) contains three nuclear (Seventh in Absentia (SIA), Brain Derived Neurotrophic Factor (BDNF), Sodium Calcium Exchange subunit-1 (NXC-1)), and two mitochondrial loci (Cytochrome oxidase b (CYTB), and NADH dehydrogenase subunit 4 (ND4)), selected in a previous study (Rivera et al., 2017) to span a range of evolutionary rates sufficient to infer a phylogeny with time depth of approximately 25MY. We redesigned all primers for improved performance (see Table 2 in Hill et al., submitted for publication) based on Asterophryinae sequences downloaded from GenBank (Sayers et al., 2022) including whole mitochondrial genomes Tu et al. (2018) as well as our previous sequencing efforts. Primers for the nuclear loci were designed using NCBI Primer-BLAST software (Ye et al., 2012), and for the mitochondrial loci degenerate primers were designed against the

multiples alignment using HYDEN (Linhart and Shamir, 2002) using default settings. Standard PCR protocols (initial denaturation 1.5 m at 94° followed by 30 cycles of 94° for 30s, annealing temperatures for 30s and extension at 72° for 60s) were used with GoTaq Green Master Mix (Promega) protocols (0.4uM-0.8uM of each primer with ~5 ng of genomic DNA per 25uL reaction). Touchdown PCR and/or nested PCR was used to improve amplification for difficult targets. For touchdown PCR, annealing temperature generally started at 5° above Tm (see Table 2 in Hill et al., submitted for publication) and decreased by 1° per cycle for the first 10 cycles, followed by 25 cycles at the lowest annealing temperature. Some combinations of NXC-1 and ND4 primers required the touchdown window of 55–45°. A 15 degree touchdown window was used for CytB, as necessary, to improve yield likely due to the degeneracy or the greater Tm disparity between forward and reverse primers. The PCR product was cleaned using NEB Monarch spin columns (PCR DNA cleanup kit protocol). Sanger sequencing followed standard protocols using Applied Biosystems BigDye terminator chemistry on an ABI 3730XL sequencer at the University of Hawaii at Manoa's Advanced Studies of Genomics, Proteomics and Bioinformatics facility (<https://www.hawaii.edu/microbiology/asgpb/>).

High-quality sequence data were filtered using multiple quality checks: quality scores (>40%), BLAST confirmation of loci, curation by pairwise alignment between taxa for each locus, and scrutinizing any distances that did not align with initial assignments to genera. Furthermore, we examined the placement of taxa in phylogenetic trees, by locus and other subsets of the data with the aim of identifying for further scrutiny any samples that were far from their putative relatives. Any suspect data were confirmed by resequencing with new PCR primers and increased PCR stringency as appropriate. In all, 250 sequences were resequenced, about one-fifth of the dataset, significantly reducing the number of odd phylogenetic placements. All sequences were deposited in GenBank with accession numbers MZ634561 - MZ635501 and MZ647717 - MZ647945 (see Table 1 in Hill et al., submitted for publication).

2.2. Phylogenetic analyses

The five coding genes were aligned using the MUSCLE algorithm (Edgar, 2004) in Mesquite (Maddison and Maddison, 2019) after amino acid translation using TranslatorX (Abascal et al., 2010). Amino acid positions 14 – 21 of the ND4 sequence produced an ambiguous alignment and were removed before back-translation to nucleic acids for phylogenetic analysis. PartitionFinder2 (Lanfear et al., 2016) was used to simultaneously fit evolutionary models and data partitions by locus and codon. The best model was a 13 partition model identified by AIC score (Akaike Information Criterion; Akaike, 1974), (see Table 3 in Hill et al., submitted for publication). Time-calibrated trees were reconstructed using the timing of six geological events separating sister taxa: five from Rivera et al. (2017) with the addition of 25±10 MY for the docking of the EPCT dating the genus *Paedophryne*. The maximum likelihood (ML) tree was estimated using IQTREE (Nguyen et al., 2014). Nodal support was assessed using 2000 bootstrap replicates. The Bayesian inference (BI) tree was estimated using BEAST2 (Bouckaert et al., 2014). Two independent Monte Carlo (MCMC) runs were performed for 100,000,000 generations and sampled every 10,000 generations. The phylogeny was co-estimated with associate divergence times using log-normal priors for the geological calibration points. Chain stationarity was assessed using Tracer v1.7.1 (Rambaut et al., 2018). The runs were combined using LogCombiner with a burn-in of 30% and the consensus tree was generated using TreeAnnotator (Drummond and Rambaut, 2007). Nodes were recognized as highly supported if BS support values ranged between ≥90 and PP ≥0.90. Moderately supported nodes possessed BS support of 80 ≤x < 90 and PP.8 ≤x < .9. Weakly supported nodes had BS support of 70 ≤x < 80 and PP.7 ≤x < .8. Nodes below < .70 PP and < 70 BS support were considered unsupported.

Cryptic diversity is increasingly recognized across anurans (Vieites et al., 2009; Padial et al., 2010) and known from this subfamily of frogs (Oliver et al., 2013). While the present study was designed as a phylogenetic analysis across the subfamily, we nevertheless checked for the possibility that samples in fact represent multiple distinct lineages. Oliver et al. (2013) tested a range of cutoffs and found that 3% mitochondrial divergence was effective in delineating distinct evolutionary lineages in the genus *Mantophryne*. This study used different mitochondrial markers, therefore we examined species pairs falling below cutoffs of 5%, 4%, 3%, 2%, and 1% mitochondrial divergence for correspondence with their phylogenetic (combined nuclear and mitochondrial) divergences. We used genetic divergence criteria to designate candidate species, but additional work is necessary to confirm these hypotheses.

We pruned the phylogeny down to single representatives of each species at each site, resulting in a phylogeny with 218 taxa using the R package ape (Paradis et al., 2004), and used this tree in downstream analyses.

2.3. Community analysis

Because Asterophryinae have received little ecological study, we took an empirical approach to defining “communities”. Over decades of research, field workers have set up camps at various distances to sample biodiversity (based on fieldwork described here and from previous expeditions and recorded in the BPBM database). We analyzed the spatial clustering of collection sites and calculated the pairwise distance between each recorded GPS coordinate using the R package geodist (Padgham and Sumner, 2021), and found that a cutoff of ~1 km was sufficient to separate all collection sites used by field workers. Our working definition of communities are therefore the collection of candidate species that live in those sites, and we compared the phylogenetic resolution between species within sites vs. between sites. We assessed the diversity of lifestyles contained in eighteen multispecies communities for which we have reasonably complete species sampling based on our own fieldwork or that of our close colleagues (see Table 1 in Hill et al., submitted for publication).

2.4. Evolution of lifestyle

For each species included in our phylogeny, we collected habitat use information. Five anuran lifestyle descriptors are recognized for Asterophryinae based on the perch type noted during collection and their behavior (Zweifel and Tyler, 1983; Köhler and Günther, 2008): arboreal (found on tree trunks or tree canopy, >2 m above the ground), scansorial (shrubs 2 m above the ground), terrestrial (ground, on the forest floor or leaf litter), fossorial underground (underground in holes or burrows), and semi-aquatic (stream, associated with streams and will swim for escape). We assigned species to a lifestyle based on microhabitat type collected from our own fieldwork or from published work including field guides, species descriptions, and museum databases (Menzies, 2006, see citations listed in Table 1 in Hill et al., submitted for publication).

We reconstructed maximum likelihood ancestral states for lifestyle using the R package geiger (Pennell et al., 2014). ML models for the evolution of lifestyle along the phylogeny were fit assuming that rates of evolutionary transition are equal (equal rates), different for each pairwise comparison (all rates different), and different but symmetric (i.e., the probability of arboreal to terrestrial transition = the probability of terrestrial to arboreal; symmetric). The best fit model was identified using AICc (Akaike Information Criterion; Akaike, 1974) and used to generate 2000 stochastic mappings onto the phylogeny using SIMMAP (Bollback, 2006) in the R package phytools (Revell, 2012). A majority of nodes (excepting 10) were reconstructed with >= 70% support for a single lifestyle. The branches descending from these nodes were painted by this lifestyle. Where there is some support at the nodes for other alternatives (the dominant state was < 70%), pie charts indicate

proportional support. For the purpose of counting transitions, we assigned states to the 10 nodes that fell below the 70% threshold using majority rule ($> 50\%$ support).

2.5. Rate through time analysis

We investigated the tempo of lineage diversification using a lineage-through-time (LTT) analysis (Nee et al., 1992) from the phytools package (Revell, 2012). The γ -statistic describes the mean diversification rate (Pybus and Harvey, 2000). A parameter value of zero represents a constant rate of diversification through time, while significant negative values ($\gamma < 0$) indicate early diversification, and positive values ($\gamma > 0$) indicate late diversification.

Unless otherwise stated, all analyses were conducted in the R statistical computing environment (R Core Team, 2022). Phylogenetic plots were created using the R packages ggtree, ouch, treeio, and dyphylyr (King and Butler, 2009; Wang et al., 2019; Yu, 2020; Wickham et al., 2021), and with a custom script based on modifications of ouch.

3. Results

We assembled a 233 sample dataset (205 taxa) using three nuclear and two mitochondrial genes (GenBank accession numbers listed in Table 1 in Hill et al. (submitted for publication)). With the new primers, 174 loci were added which were missing from Rivera et al. (2017), as well as the addition of 74 samples. The matrix contains 2474 nt and is 99% complete at the five loci, missing only 1 SIA, 2 BDNF, 5 CYTB and 7 ND4 sequences.

3.1. Increase in phylogenetic support

We obtained a consensus topology that was highly supported, whether reconstructed using Bayesian time-calibrated or Maximum Likelihood methods (Fig. 2). This phylogeny received substantially more highly supported nodes than previous studies (Rivera et al., 2017). Of the 236 nodes present in the tree, 84% of the nodes were highly supported with BS support values ≥ 90 and PP ≥ 0.90 (see full tree presented in Figure 1 of Hill et al., submitted for publication); the phylogeny pruned to 218 species – one species per site is presented in Fig. 2). Five percent of the nodes were moderately supported ($80 \leq x < 90$ and PP.8 $\leq x < .9$), and 6% were weakly supported ($70 \leq x < 80$ and PP.7 $\leq x < .8$). Only 5% of the nodes are unsupported with BS support < 70 and PP support < 0.7 . Along the backbone, nearly all of the nodes show some degree of support, with about half strongly supported (4 strong, 4 weak, 2 unsupported). The weakly supported nodes occur in regions with increased rates of genera diversification. We also note there were two places in the tree with minor topological differences between the ML and BI phylogenies consisting of swapping the order of adjacent nodes with short branches (within *Hylophorus* and *Xenorhina*). These do not have any impact on the intergeneric relationships established within the phylogeny.

3.2. Timing and rate of clade evolution

Both ML and BI time-calibrated reconstructions estimate the age of Asterophryinae at ~ 20 MY old (Fig. 2), roughly 5 MY younger than the estimate by Rivera et al. (2017). The genera have deep histories, originating early in the history of the clade, between 20 – 12 MYA (Fig. 3). We do see an early burst of diversification with many short branches near the base of the tree (Fig. 2), coincident with generic diversification and microhabitat use transitions, consistent with the pattern expected for an adaptive radiation. Early diversification is also supported by the shape of the lineage through time plot and the large and negative γ statistic ($\gamma = -5.174$, P-value = 2.29×10^{-7} , Fig. 3). The more recent era between 10 MYA and the present was another time of substantial species accumulation with most genera diversifying extensively.

3.3. Cryptic species and sites

Gene flow appears to be low, with nearly all samples phylogenetically distinct. Where we had multiple samples of nominal species from the same site, in each case these individuals were resolved as phylogenetically distinct with significant nodal support and short, significant branch lengths separating tips. Oliver et al. (2013) reported that 3% mitochondrial distance was effective in delineating species. In our pairwise analysis of mitochondrial divergence, samples separated by 3–4% mitochondrial divergence shared a common ancestor 0.9–1.4 MY ago by combined nuclear and mitochondrial phylogenetic distance. Five pairs of samples separated by 2–3% mitochondrial divergence were similarly separated by 0.7–1.3 MY of phylogenetic distance. Samples below 2% mitochondrial divergence possessed short branch lengths, ranging from 0.1–0.6 MY. Therefore we found that species pairs which satisfy both criteria were reliably identified as distinct: $> 2\%$ mitochondrial divergence and > 0.7 MY of phylogenetic distance.

A rough spatial cutoff of ~ 1.1 km worked well to describe distinct sites containing separate candidate species, providing a spatial scale similar to that produced by elevational variation of ~ 100 m, producing sites that correspond well with what field collectors labeled as “camps” or collection sites. In general, taxa at distinct sites were also distinct candidate species. Only a handful of taxa were found to be truly distributed between sites that were over 1 km apart. These included candidate species shared between Mt. Gerebu and Mt. Obree region, a separation of ~ 34 km (*Choerophryne gunnari*, *Hylophorus cf. rufescens.12*, and *Mantophryne cf. lateralis.4*). In the Bowutu mountains, *Callulops personatus* ranged to Mt. Shungol, a distance of ~ 69 km, and *Hylophorus cf. rufescens.5* ranged ~ 5 km from Cliffside camp to the Suzuki track site. Four Normanby Island species are separated by a phylogenetic distance of only about 200,000 years, producing little mitochondrial divergence. We considered these as the same species occurring across sites separated by ~ 10 km across a mountain range: 3 species on Normanby Island shared between Mwatebu and Buyetai sites: *Callulops doriae complex sp.1*, *Cophixalus desticans complex sp.1*, and *Hylophorus cf. rufescens.6*; one species (*Mantophryne cf. lateralis.1*) was shared between three sites: Mwatebu, Buyetai, and Sibonai. All remaining candidate species (~ 195) were unique at each site.

3.4. Communities and their phylogenetic structure

Our eighteen multi-species communities varied in number from two to 15 species (Fig. 4B). More than half of these have six or more species each. The members of each community span the phylogeny, generally with one species per genus, resulting in communities whose member species are typically not closely related to one another (Fig. 5). In the instances where there are two or more species per genus, most commonly they are *Cophixalus* or *Choerophryne* (Fig. 4C). Exceptions include the sites in the Torricelli mountains which have multiple *Hylophorus*, *Astrochaperina*, or *Choerophryne*.

3.5. Evolution of lifestyle

The best fit model for reconstructing the ancestral states for lifestyle assumed a symmetric transition rate matrix $AIC_{SYM} = 309$, two points lower than the next best model: all rates different $AIC_{ARD} = 311$ and nine points lower than the equal rates model: $AIC_{EQR} = 318$. Lifestyles at internal nodes were reconstructed with very high support throughout the tree (the vast majority above 90% probability). The ancestor to Asterophryinae was inferred as terrestrial with 59% probability, and there is some support for arboreal with 35% probability (Fig. 2). ArboREAL and scansorial lifestyles evolved within the first two MY after the genesis of the clade (by ~ 18 MYA; Fig. 2). The last two: semi-aquatic and fossorial lifestyles evolved shortly thereafter (by ~ 16 MYA). All lifestyles have repeatedly and independently evolved throughout the clade's history.

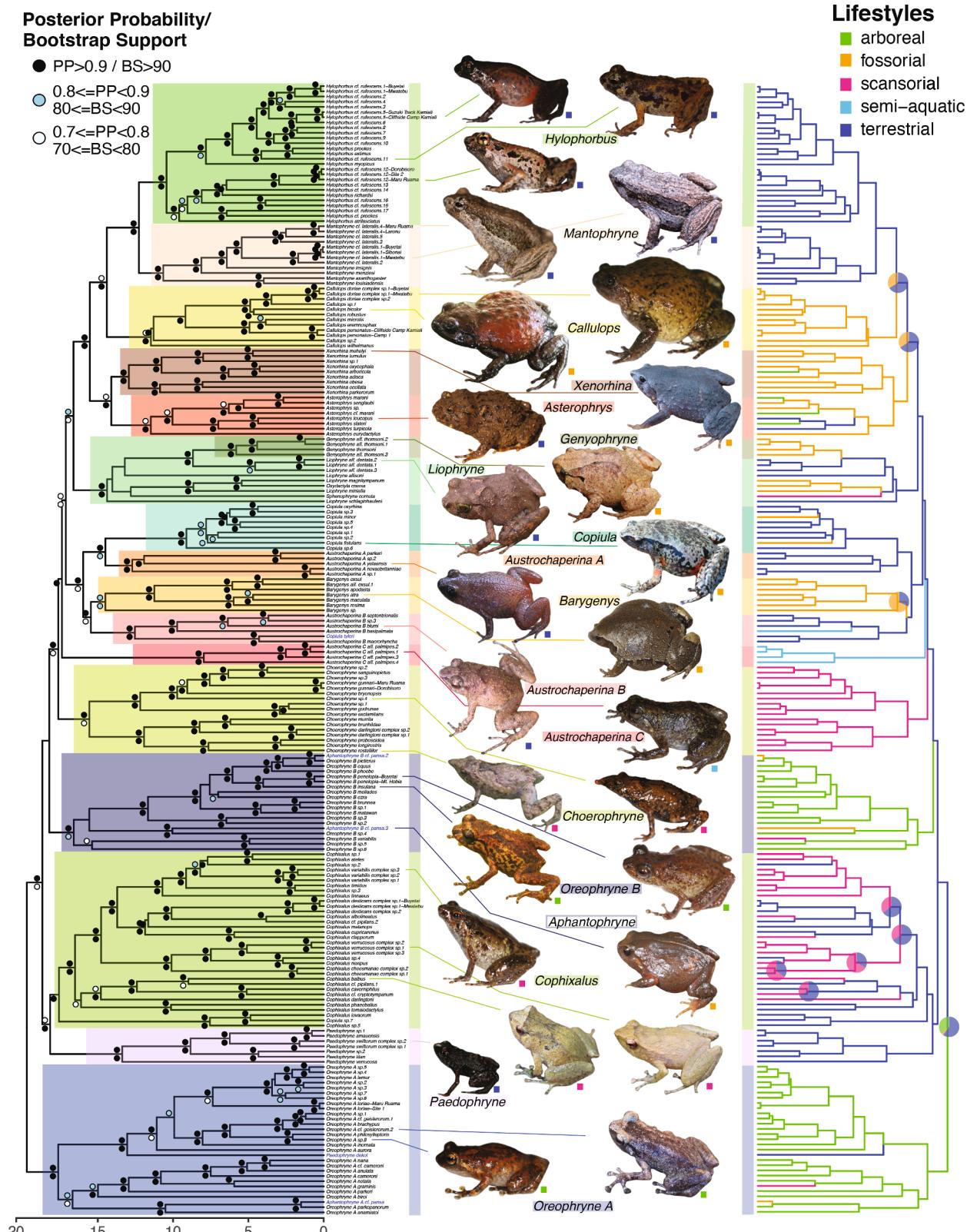


Fig. 2. (Left) Time Calibrated BI phylogenetic reconstruction of 218 species of Asterophryinae using BEAST2. Dots indicate nodal support: BI posterior probability above branch, ML bootstrap support below branch where ML reconstruction has identical topology. The ML tree contains more supported nodes within *Hylophorus* and *Xenorhina* than indicated here, but with local rearrangement of adjacent nodes (see Fig. 1 vs. 2 in Hill et al., submitted for publication). Genera highlighted by color. (Middle) Photos of representative Asterophryinae species for each genus. Line color indicates genus, colored square near hind leg indicates lifestyle. (Right) The same phylogeny painted by lifestyle. Support for a single ancestral state was near 100% for most nodes. Where the dominant ancestral state received < 70% support, pies indicate relative support for each lifestyle. See text for more details.

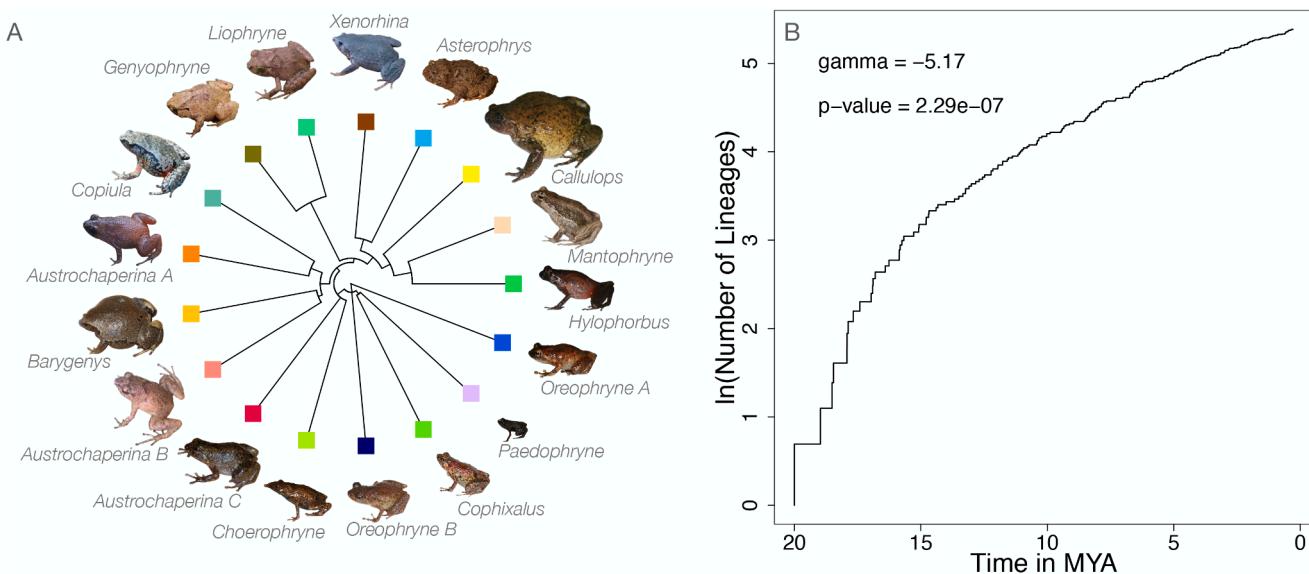


Fig. 3. (A) A circular time-calibrated phylogeny of the diverse genera of Asterophryinae. Highlighted are the early diversification events from the base of the phylogeny to the origin of each genus. See Fig. 2 for full tree including intra-generic branches. (B) Lineage through time plot for the Asterophryinae phylogeny. The natural-logarithm of the number of lineages versus time since the origin of the subfamily (in Millions of Years Ago) is shown with the γ statistic and corresponding *p*-value.

Overall there is a pattern of niche conservatism: across the history of Asterophryinae, we found 33 transitions in lifestyle for 217 nodes (Fig. 2). Genera primarily have a single microhabitat association, but in some instances, secondary microhabitat transitions occur most notably within *Asterophys* and *Cophixalus*. Many of these secondary transitions occur between ~15 MYA and the present day.

4. Discussion

Asterophryinae has long been recognized as an unusually speciose clade of frogs with diverse lifestyles (Zweifel and Tyler, 1983; Köhler and Günther, 2008; Rivera et al., 2017), however, the lack of phylogenetic resolution in the early-to-middle history of the group has prevented further progress. Here, with 95% of nodes supported in the largest and most completely resolved phylogeny for Asterophryinae to date, our results show that this group arrived in New Guinea about 20 MYA and rapidly diversified (Fig. 3) accompanied by early ecological diversification (Fig. 2), as expected for adaptive radiation (Schluter, 2000). Below we discuss Asterophryinae communities, cryptic diversity and phylogenetic inference, and the taxonomic implications of this work.

4.1. Asterophryinae communities

One of the most distinctive, yet little-studied features of this group are their complex communities, in which adaptation has been assumed to play a key role. We describe here communities of up to 15 syntopic species of Asterophryinae. We found that the typical complex Asterophryinae community has six or more species from up to nine genera with representatives of up to five lifestyles: terrestrial, arboreal, fossorial, scansorial, and semi-aquatic (listed in order of frequency; Fig. 4). This level of within-community ecological diversity is unusual among amphibian adaptive radiations, which are reported to contain 2–3 species in microsympatry which differ in body size (*Thorius* salamanders: Rovito et al., 2013b, *Limnonectes* fanged frogs: Setiadi et al., 2011), or in microhabitat association (*Desmognathus* salamanders: Kozak et al., 2005, *Kaloula* microhylid frogs: Blackburn et al., 2013), and up to 4 species of Bolitoglossini salamanders (Wake, 1987).

Ecological opportunity has contributed to Asterophryinae's

diversification. Asterophryinae was the first frog lineage to colonize the growing New Guinea island in the Miocene, ~20 MYA. Based on our phylogeny, early diversification was rapid, with short branches along the backbone of the radiation confirmed by a highly significant gamma statistic (Figs. 2, 3). Within the first several million years of arrival to New Guinea, all five microhabitat types had evolved along with diversification into half of the present-day genera. Microhabitat transitions within Asterophryinae often coincide with the evolution of new genera, followed generally by niche conservatism within most genera (Fig. 2), a pattern that is common to amphibian radiations (see citations above). Several genera provide exceptions to the general pattern, with multiple microhabitat transitions concentrated in: *Cophixalus* (terrestrial, scansorial), *Copiula* (terrestrial, fossorial), *Asterophys* (arboreal, terrestrial, and fossorial), and *Choerophryne* reported in Oliver et al. (2017) from taxa not included here. Undoubtedly there will be more discoveries made by continuing to complete the phylogeny.

Our phylogenetic analysis shows that larger Asterophryinae communities are built up by adding species of either terrestrial or scansorial forms, with as many as five terrestrial species and as many as six scansorial species in the largest assemblages (Fig. 4). For the terrestrial forms, in nearly every case the additional species represent distinct genera (Fig. 4). For example, the Mwatebu site on Normanby Island has five species and five genera of terrestrial frogs (Figs. 4C, 5). In contrast, there are only two genera known to possess scansorial lifestyles, *Cophixalus* and *Choerophryne*, and thus at Cliffside camp there are six scansorial species from these two genera. Interestingly, there is generally only one arboreal species per site, from either *Oreophryne* A or *Oreophryne* B, which are possible to locate in the field through their calls. Furthermore, both *Oreophryne* A and B lineages co-occur at the locality level at nearby sites in the offshore islands of Normanby and Rossel, and on the mainland at Mt. Trafalgar on the EPCT.

A remaining puzzle is how such complex communities of Asterophryinae are assembled. The patterns we find here are in contrast to iconic examples of adaptive radiation such as *Anolis* lizards, Darwin's Finches, and African haplochromine cichlids, which involves a founding species colonizing an environment followed by in situ diversification into available ecological niches (Losos et al., 1998; Schluter, 2000; Gillespie, 2004; Fine et al., 2005). Asterophryinae communities clearly do not evolve de novo diversification following colonization, instead, we

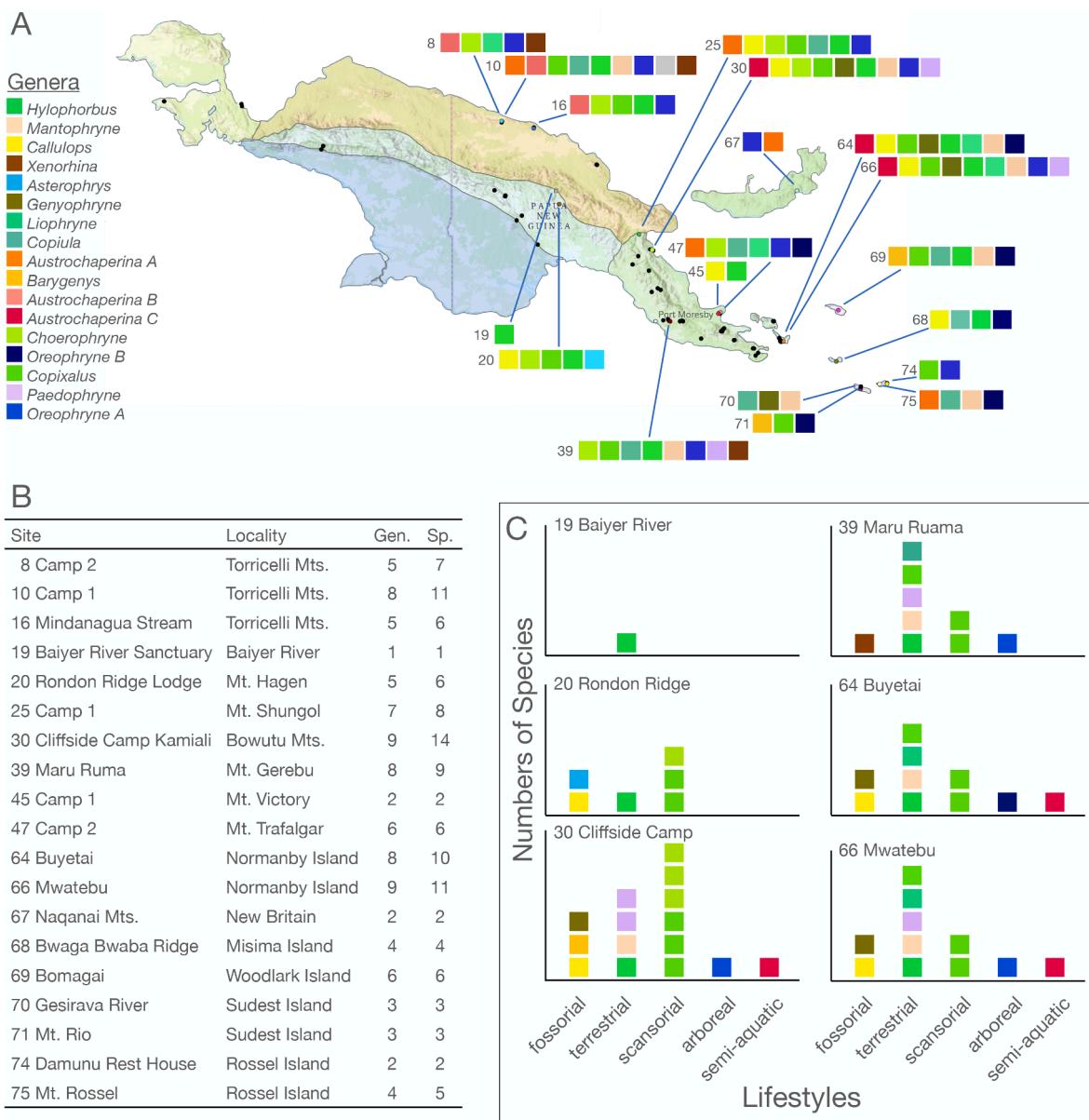


Fig. 4. Geographic distribution of sites. (A) Multispecies communities indicated on map with barplots showing high generic diversity. (B) Table of site number and name, localities, and numbers of genera and species at each site. GPS coordinates for each site and the species found at each site are given in (see Table 1 in Hill et al., 2022). (C) Lifestyle and generic diversity by lifestyle at the six sites where we completed fieldwork for this study.

find early diversification, niche conservatism, and migration, which implies community buildup by some process of assembly (Emerson and Gillespie, 2008). Lineages of Asterophryinae are characterized by low gene flow as even replicates of species at the same site are phylogenetically distinct, and species, in general, are separated over small spatial scales, producing high endemism (see also Oliver et al., 2013; Oliver et al., 2017). Over evolutionary time scales, it appears that these lineages are undergoing a continual process of fragmentation and species accumulation (Fig. 3). As nearly all of our communities trace their most recent common ancestor to the base of the tree, there appears to be little phylogenetic structure within communities (Fig. 5).

These patterns are a first description of the remarkable endemism and diversity of Asterophryinae. Clearly, more fieldwork spanning greater geographical coverage (for example, are there similarly complex communities in Western and Southern New Guinea?) is needed to gain a more complete assessment of and to understand the mechanisms producing this exceptional diversity.

4.2. Cryptic diversity and phylogenetic inference

Cryptic diversity is increasingly recognized as the dominant pattern across the tree of life (reviewed in: Bickford et al., 2007) and is a problem that affects many domains of biology including taxonomy, phylogenetics, conservation, and biodiversity studies. Of the 233 samples representing 205 taxa included in our phylogeny, only nine candidate species were found at more than one site (Fig. 2), demonstrating the overwhelming tendency toward narrow-range endemism in this subfamily. Most geographically widespread “species” were found to be complexes of distinct phylogenetic lineages, some of which were quite ancient. For example, lineages of the monotypic *Genyophryne thomsoni* diverged from their most recent common ancestor as long as 8 MY ago, and similarly so for *Austrochaperina palmipes*. *Mantophryne lateralis* and *Hylophorus rufescens* were previously thought to be single species with very large geographic ranges, yet we found five lineages of the *Mantophryne lateralis* species complex and 18 candidate species of the *Hylophorus rufescens* species complex, some of whom share a common

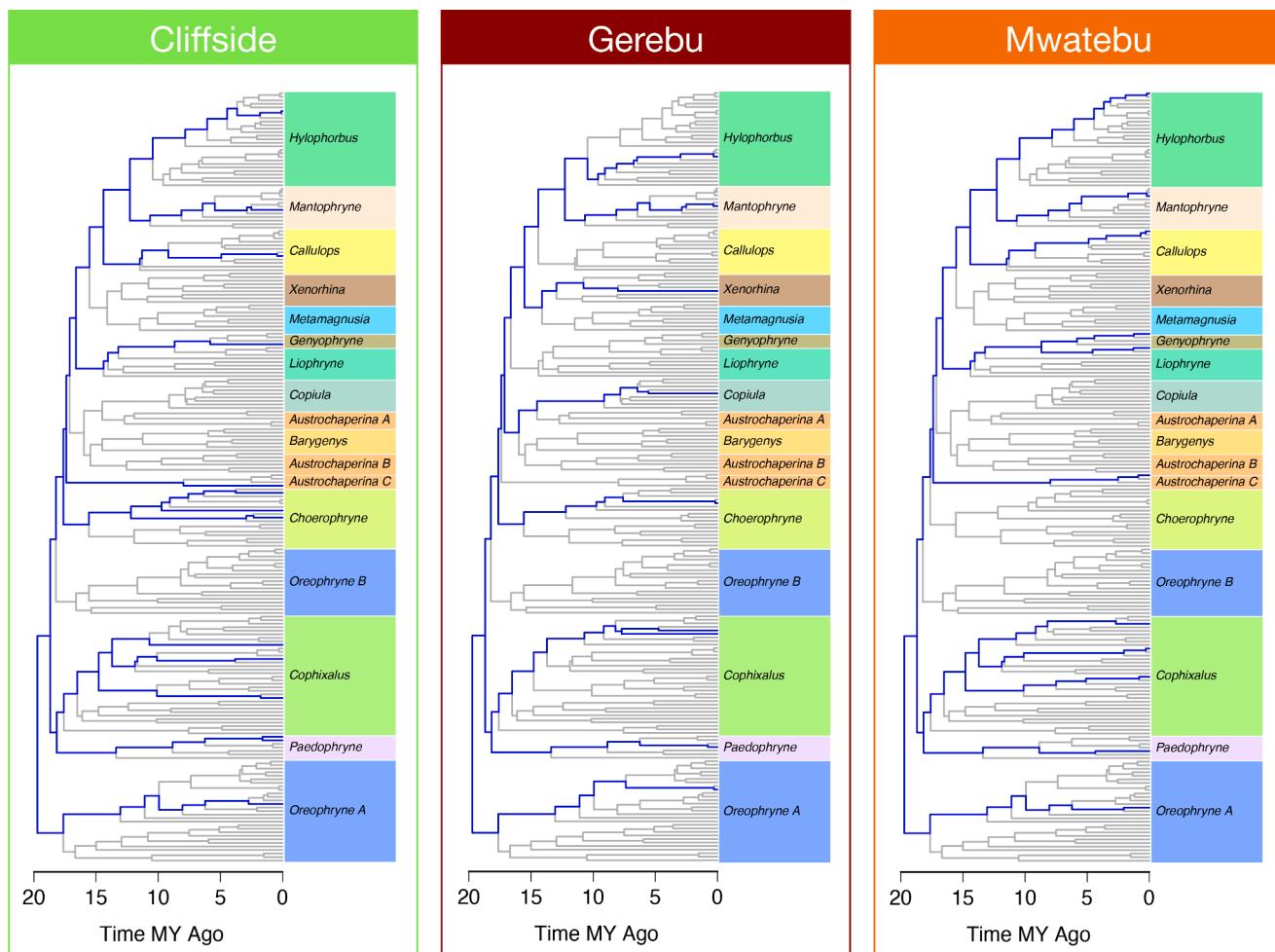


Fig. 5. The phylogeny of communities within the context of the larger phylogeny illustrating the deep divergences within each community. Three example communities are traced here, two on the mainland of New Guinea: Cliffside camp, Kamiali, Bowtutu Mountains [site 30], and Maru Ruama, Mt. Gerebu [site 39]; and one on an offshore island: Mwatebu, Normanby Island [site 66]. Color codes indicating sites follow Fig. 4.

ancestor as long as 10 MY ago. All of these examples are morphologically cryptic species complexes. Several studies have found multiple cryptic species complexes within genera (Zweifel, 1979; Oliver et al., 2013; Oliver et al., 2017). Here, we found cryptic diversity in at least 12 genera, within: *Callulops doriae*, *Genyophryne thomsoni*, *Liophryne dentata*, *Copiula* sp., *Barygenys exsul*, *Austrochaperina palmipes*, *Choerophryne* sp., *Choerophryne darlingtoni*, *Aphantophryne pansa*, *Hylophorus*, *Mantophryne*, several lineages of *Cophixalus*, several lineages of *Oreophryne* A and B, and *Paedophryne swiftorum*. Our analyses clearly demonstrate that any widespread species of Asterophryinae should be considered suspect until verified. Especially when studying biodiversity in remote localities, the possibility of cryptic diversity should be considered when developing a sampling strategy to make the best use of limited time and resources.

Until now, one mystery of this group is how we obtain so many widely-distributed species of diminutive frogs, often across sites separated across the open ocean from the mainland of New Guinea to its satellite islands. The short answer is that they are not widely-distributed species. The vast majority of them have very small distributions (less than ~1 km), resulting in extremely high endemism. This pattern is reasonable given the ecological heterogeneity of New Guinea – separation of ~1 km often corresponds to sites at different elevations or habitats. A point of debate in our own fieldwork was whether it was worth the investment in resources to obtain more samples of *Hylophorus rufescens*, *Mantophryne lateralis*, *Liophryne dentata*, *Austrochaperina palmipes* and other species across both mainland and island sites. We

decided to sample specimens from every site. Had we not done so, we would have never discovered the extent of the ancient extensive cryptic diversity within Asterophryinae.

Furthermore, by adding more representatives from “wide-spread species”, portions of the tree topology which were previously ambiguous became stabilized. Whereas it was previously unclear where many genera fit into the larger phylogeny, their sister clade relationships are now clarified bringing resolution to previously intractable nodes (compare Rivera et al., 2017, with Figure 2). As it turned out, with incomplete sampling, many formerly monotypic lineages tended to be at the end of long branches, which were broken up by adding geographic sampling. That the inclusion of multiple geographic samples of “species” would stabilize the subfamily-level phylogeny was a surprising and serendipitous result.

4.3. Asterophryinae phylogenetics

It became clear early on in the biological exploration of New Guinea, that lineage diversity was low compared to adjacent parts of SE Asia and other tropical areas (Allison, 2009). This helped create an impression that species diversity was also low and this was seemingly confirmed by early workers. For example, Richard Zweifel's pioneering taxonomic work beginning in the 1950s on the thousands of frog specimens collected during the Archbold Expeditions demonstrated that many morphologically diagnosed species had fairly large geographic ranges (Zweifel, 1956; Zweifel, 1963; Zweifel, 1971). However, with the

development of portable sound recorders in the 1960s, it quickly became clear that many of these widespread taxa were actually composite species, many of them range-restricted endemics (Nelson, 1973; Menzies and Tyler, 1977). Molecular work, including our findings reported herein, has shown that even frogs with similar calls (e.g., *Genyophryne thomsoni*) are composite species, suggesting that frog species richness in New Guinea is exceptional.

As a case in point, the semi-aquatic *Austrochaperina palmipes* does not call. Zweifel (2000) diagnosed *Austrochaperina palmipes* based on morphological similarities to other members of *Austrochaperina* however it has never allied with any other species of *Austrochaperina* in molecular phylogenetic treatments (Rittmeyer et al., 2012; Peloso et al., 2016; Rivera et al., 2017; Tu et al., 2018). Here we show that it is not a single species distributed across the mainland and offshore islands, but is actually a cryptic species complex. The fossorial species complex *Genyophryne thomsoni* is monotypic and has a similar widespread distribution pattern. It produces simple one or two-note (low-information) calls, and only during heavy rains, providing limited opportunities for collecting the large amounts of data required to discover any distinctions. Both of these have derived lifestyles with no clear morphological alliance to other groups: *Austrochaperina palmipes* is an excellent swimmer among direct-developing terrestrial frogs, is able to climb seeps and waterfalls, and hops along stream rocks. *Genyophryne thomsoni* is fossorial with short muscular legs and disc-shaped bodies, seemingly well suited for a burrowing lifestyle. We showed that both *A. palmipes* and *G. thomsoni* are actually cryptic species complexes that originated at least ~8 Ma with *G. thomsoni* sister to the cryptic complex of *Liophryne dentata*, and *A. palmipes* is sister to a major clade including *Austrochaperina A* and *B* + *Barygenys* + *Copiula* + *Liophryne* + *Genyophryne* + *Xenorhina* + *Asterophys* + *Callulops* + *Mantophryne* + *Hylophorus*.

The earliest efforts in molecular phylogenetics of Asterophryinae used mitochondrial 12S and 16S loci (Köhler and Günther, 2008), which are efficient for diagnosing species, but nuclear genes are clearly required to find support for deeper nodes in this group (de Sa et al., 2012) as well as across the Anuran tree of life (Feng et al., 2017). However, the mere inclusion of a few nuclear genes is not sufficient to find nodal support for taxa at the end of long branches, which require increased sampling to break up long branches.

For the first time, we were able to clarify the intergeneric organization of Asterophryinae with strong support: establishing the monophyly of most genera and the non-monophyly of the remaining genera, and clearly identifying their sister clade relationships, including the timing and order of diversification. This is a significant step forward from a previous paper by some of us where a little less than half of the nodes had some support, and importantly, most of the deeper nodes were not resolved including those along the backbone (Rivera et al., 2017). In the current phylogeny, the problematic non-monophyletic genera *Oreophryne* and *Austrochaperina* are now clearly resolved into 2 or 3 monophyletic clades, respectively, with clear placement relative to their sister taxa.

We accomplished this nearly fully resolved tree partly by improvements to the data. We added 74 tips to the tree bringing the total tips to 233. The vast majority of these broaden geographic sampling, which we suspect helped to break up some of the long branches in the previous tree. We completed the dataset of loci sequenced for each sample (now 99% complete) of our five-locus dataset. Given that our subfamily spans ~20MY of evolution, it is important to select loci with mutation rates to inform the various depths of the tree, from slowly evolving nuclear (e.g. BDNF and SIA), moderately evolving nuclear (NXC1) to fast-evolving mitochondrial (e.g. CYTB and ND4). In particular, the ND4 dataset and to a lesser extent the NXC1 dataset had many holes in 2017; developing improved primers specific to Asterophryinae enabled the completion of the faster-evolving datasets that likely improved support and reduced odd placements. Improved data curation confirmed or replaced suspect data by reexamining locus-by-locus pairwise similarity matrices between taxa and re-sequencing any loci that were outliers.

Finally, we reconstructed phylogenies with improved evolutionary models (partitioned by locus and codon and selected by PartitionFinder2). Phylogenetic problems with more taxa are inherently more difficult to resolve. In groups such as Asterophryinae with a deeply nested history, a tendency toward fragmentation among morphologically cryptic lineages with known taxonomic issues, it is important to have a wide representation of samples across time and space to ensure that the broadest possible sampling of the diversity of the group is included.

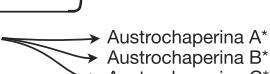
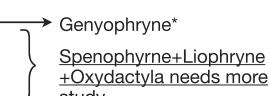
4.4. Taxonomic findings

We are now closer to a phylogenetic taxonomy for the Asterophryinae. We recovered 16 monophyletic clades of Asterophryinae, leaving only the possible paraphyly of *Liophryne* and its relationship to *Sphenophryne*, *Oxydactyla*, and *Genyophryne* unclear and requiring further study. Our new recommendations include synonymization of the monotypic *Oninia* with *Asterophys*, identifying *Aphantophryne* for further study, and finding strong support for dividing *Oreophryne* (and possibly *Aphantophryne*) into two reciprocally monophyletic clades (Fig. 1). Our primary additions are establishing the monophyly of previously unsupported genera and establishing sister genera relationships with strong support, in addition to clarifying many taxa previously thought to be widespread species into phylogenetically distinct lineages (summarized in Table 1). A full list of taxonomic recommendations is given in Table S1.

The taxonomy of Asterophryinae has had a turbulent history over its long 120 years of study with numerous name changes and reversals, which are included here for workers new to this nomenclature while reviewing taxonomic changes for each genus-level clade. Asterophryinae, a subfamily of Microhylidae, was established by Albert

Table 1

Summary of recommendations to changes in generic taxonomy and comparison to Rivera et al. (2017). Underlined taxa require more study.

| Former Taxonomy | Rivera et al. 2017 | This Study |
|---|--|---|
| <i>Aphantophryne</i> <i>Oreophryne</i> | <i>Aphantophryne</i> + <i>Oreophryne-2</i> , no support for rest of <i>Oreophryne</i> | <i>Aphantophryne A</i> + <i>Oreophryne A*</i> <i>Aphantophryne B</i> + <i>Oreophryne B</i> |
| <i>Asterophys</i> <i>Metamagnusia</i> <i>Pseudocallulops</i> <i>Oninia</i> | <i>Asterophys</i> | <i>Asterophys*</i> |
| <i>Austrochaperina</i> | --- |  |
| <i>Barygenys</i> | <i>Barygenys</i> | <i>Barygenys*</i> |
| <i>Callulops</i> | <i>Callulops</i> | <i>Callulops*</i> |
| <i>Choerophryne</i> | <i>Choerophryne</i> | <i>Choerophryne*</i> |
| <i>Cophixalus</i> | <i>Cophixalus</i> | <i>Cophixalus*</i> |
| <i>Copiula</i> | --- | <i>Copiula*</i> |
| <i>Genyophryne</i> <i>Liophryne</i> <i>Oxydactyla</i> <i>Sphenophryne</i> | weak support at many nodes needs more study |  |
| <i>Hylophorus</i> | <i>Hylophorus*</i> | <i>Hylophorus*</i> |
| <i>Mantophryne</i> | <i>Mantophryne*</i> | <i>Mantophryne*</i> |
| <i>Paedophryne</i> | --- | <i>Paedophryne*</i> |
| <i>Xenorhina</i> | <i>Xenorhina*</i> | <i>Xenorhina*</i> |

Günther, 1858, and for a time divided into Asterophryinae and Sphenophryinae, and Asterophryinae and Genyophryinae by different workers, before being synonymized back to Asterophryinae (reviewed in: Frost, 2021). Inter-generic organization of Asterophryinae has been particularly enigmatic as evidenced by the multiple reorganizations of its ~21 genera over the last decades (Zweifel, 1956; Menzies and Tyler, 1977; Zweifel and Allison, 1982; Burton, 1986; Zweifel and Parker, 1989; Burton and Zweifel, 1995; Zweifel, 2000; van Boclaer et al., 2006; Frost et al., 2006; Günther, 2009; Kraus, 2013; Peloso et al., 2016).

Dubois et al. (2021) proposed to reclassify 350+ taxa of Asterophryinae under a single genus: *Asterophys*, citing some instances of unresolved polyphyly or paraphyly. We find that this does more harm than good. We find strong evidence for 16 monophyletic generic level clades, with only a few groups requiring further work (similar to the findings of: Rivera et al., 2017; Tu et al., 2018, Table 1). In this study, we revealed most of the intergeneric relationships as well. Therefore, categorizing all Asterophryinae as a single genus *Asterophys* would abolish all of this work over the past 120 + years to uncover the structure of this diverse group and do nothing to clarify the taxonomy. We reject this proposal and will refer to our results with respect to the pre-Dubois et al. (2021) taxonomy.

Asterophys Tschudi, 1838. Rivera et al. (2017) established the monophyly of *Asterophys* + *Metamagnusia* + *Pseudocallulops* and recommended their synonymization. Tu et al. (2018) additionally synonymized the monotypic genus *Oniria* to the enlarged *Asterophys*, suggesting the sister taxon relationship of *Asterophys* and *Xenorhina*. Our study provides strong support for the monophyly of *Asterophys* (including *Metamagnusia* + *Oniria* + *Pseudocallulops*) and its sister relationship to *Xenorhina*.

Aphantophryne Fry, 1917 is a terrestrial clade of three named species. Previous studies used only one specimen of *Aphantophryne pansa* resulting in an ambiguous placement within Asterophryinae (Köhler and Günther, 2008; de Sa et al., 2012; Tu et al., 2018). Rivera et al. (2017) included four specimens collected from a single site which were recovered as a monophyletic clade. To these four we added two *Aphantophryne pansa* specimens from different peaks of the Owen Stanley mountain range. Interestingly, these are phylogenetically distinct suggesting that *Aphantophryne* is polyphyletic with BPBM 5299 nested within *Oreophryne* A, and with BPBM 8312 nested within *Oreophryne* B. While these nodes are strongly supported; they involve only one specimen each. We recommend further study with the inclusion of more specimens.

Austrochaperina Fry, 1912 is a terrestrial group with two semi-aquatic members. The polyphyly of *Austrochaperina* was suggested by several studies that recovered *Austrochaperina* species interdigitated with *Copiula* (Köhler and Günther, 2008; Pyron and Wiens, 2011; Rittmeyer et al., 2012). Later studies found a weak signal for possibly two clades of *Austrochaperina*, one sister to *Copiula*, separate from *Austrochaperina palmipes* but whose sister relationship was ambiguous (Rivera et al., 2017; Tu et al., 2018). Our results clearly show that *Austrochaperina* is polyphyletic with three well-supported and reciprocally monophyletic clades: *Austrochaperina* A is sister to *Barygenys*, while *Austrochaperina* B is sister to *Copiula*. The semi-aquatic *Austrochaperina palmipes* is not a single widely-distributed species, but rather a monophyletic clade of species which are not related to *Austrochaperina* A nor B. Reclassification and further study of *Austrochaperina* is highly recommended.

Barygenys Parker, 1936 is a fossorial group whose monophyly was confirmed by multiple molecular studies (Sumida et al., 2000; Frost et al., 2006; Köhler and Günther, 2008; Rittmeyer et al., 2012; Peloso et al., 2016). What was unknown was its sister relationship, which varied between *Cophixalus*, *Genyophryne*, and *Paedophryne*. We confirm strong support for the monophyly of *Barygenys* and find strong support for its sister relationship to *Austrochaperina* B.

Callulops Boulenger (1888) is a fossorial group with a long history.

Peters (1867) described a new species (of now *Callulops*) and assigned it to *Phrynomantis* which has the same name as an African genus. Zweifel (1972) re-diagnosed *Phrynomantis* and assigned 15 species to it, including 5 new species. Species were named to both *Phrynomantis* and *Callulops* until 1988, when Dubois, 1988 synonymized all Papuan *Phrynomantis* into *Callulops*, 121 years after its initial description. We confirmed the monophyly of *Callulops* and its sister relationship to *Mantophryne* + *Hylophorus* along with Günther et al. (2010), Rittmeyer et al. (2012), Peloso et al. (2016), Rivera et al. (2017).

Choerophryne Van Kampen, 1914 The formerly recognized genus *Albericus* was synonymized with *Choerophryne* based on molecular evidence by Peloso et al. (2016). We confirm strong support for the monophyly of *Choerophryne* along with previous studies (Peloso et al., 2016; Rivera et al., 2017; Tu et al., 2018).

Copiula Méhely, 1901 is a group with terrestrial and fossorial members that has historically been difficult to resolve. Menzies and Tyler (1977) and Burton (1990) identified multiple morphological characters that distinguish *Copiula* from other Papuan microhylids: broad premaxillae, enlarged rostral glands, large truncated digital disks, and the origin, course, and insertion of the deltoid. Molecular studies rejected monophyly (Köhler and Günther, 2008; Tu et al., 2018) or were inconclusive (Rivera et al., 2017). We found only two species that are not part of a monophyletic *Copiula* (in common with Rivera et al., 2017): *Copiula tyleri* is nested within *Austrochaperina* B with high support while *Copiula* sp.7 is nested within *Cophixalus*, again with high support. We visually examined the specimens and found that *Copiula tyleri* bears a strong resemblance to its sister taxon *Austrochaperina* B *macrorhyncha*, and the specimen of *Copiula* sp.7 is a very small individual and difficult to assign; both specimens should receive further study. With the exception of these two specimens, we found strong support for *Copiula* as a monophyletic clade that is sister to *Austrochaperina* B.

Cophixalus Boettger, 1892 is a large group with terrestrial and scansorial members. Earlier studies concluded that *Cophixalus* is polyphyletic (Köhler and Günther, 2008; Pyron and Wiens, 2011; Rittmeyer et al., 2012), which was contradicted by Rivera et al. (2017) who found strong support for its monophyly pending the inclusion of *Copiula* sp. 7 (BPBM 38939). Visual inspection of BPBM 38939 revealed a small specimen that is potentially a juvenile. As juvenile specimens are difficult to identify, we attributed the GenBank submissions for this specimen as "Unidentified microhylid". Setting aside BPBM 38939, our analysis finds strong support for the monophyly of *Cophixalus* in agreement with Rivera et al. (2017), and a strong sister relationship to *Paedophryne*.

Genyophryne Parker, 1934 is a monotypic genus of fossorial frogs and was defined on the morphology of the pectoral girdle, maxillae, vertebral column, and tongue, but there has been debate regarding which genus it is most closely allied (Parker, 1934; Zweifel, 1971). Here we find that it is an old monophyletic species complex (~8 Ma) where geographically disparate sites contain phylogenetically distinct species nested within the clade (*Liophryne* + *Sphenophryne* + *Oxydactyla*). We discuss these genera in the *Liophryne* section.

Hylophorus Macleay (1878) originally described a new genus that contained only one species, *Hylophorus rufescens*, collected around the Binaturi River in southern New Guinea on an expedition to New Guinea from Australia in 1875 aboard the Chevert. A century later the genus was revisited by Zweifel (1972) who left *Hylophorus* unchanged (but named two subspecies). It was not until Günther (2001), nearly 130 years after the original description, that the first non-*rufescens* *Hylophorus* was described, based in part on variation in call signatures and habitat. Currently, we still have a very widespread distribution of *Hylophorus rufescens* with some researchers preferring to catalog samples as unnamed species, creating a rather confusing taxonomic situation. We confirm strong support for the monophyly of *Hylophorus* and its sister relationship to *Mantophryne*, which has been strongly supported in previous molecular phylogenetic studies (Köhler and Günther, 2008; Rivera et al., 2017; Tu et al., 2018). We furthermore confirm the

existence of many ancient cryptic lineages and recommend that *Hylophorus* receive more study to clarify species within this monophyletic genus.

Liophryne still requires further study as we cannot confidently confirm nor reject monophyly. Parker (1934) originally included *Liophryne* as a member of the genus *Sphenophryne* based on the pectoral girdle having a complete complement of bony and cartilaginous elements; he otherwise noted that the members of *Liophryne* were diverse. Tyler and Menzies (1971) upon reanalysis established *Sphenophryne* as a genus based on the inclusion of the presence of a partly free tongue and procoelous vertebral column. Zweifel (2000) separated the clade into 3 genera: *Liophryne*, *Sphenophryne*, and *Oxydactyla*, noting that his classifications may include morphological features that are homoplastic. Rivera et al. (2017) recovered a paraphyletic grouping of *Liophryne* that included *Sphenophryne*, *Oxydactyla* and *Genyophryne* and suggested further study due to weak support at some of the basal nodes of this grouping, which we confirm here. We also find that *Liophryne dentata* is not single species but a monophyletic species complex nested within the larger clade. We note, however, that some internal nodes near the base of this clade remain unsupported as did Rivera et al. (2017) and recommend further study with the inclusion of more taxa aimed to verify whether *Liophryne* + *Oxydactyla* + *Genyophryne* should be synonymized into *Sphenophryne*, or whether these genera can be recovered as separate monophyletic clades.

Mantophryne Boulenger (1897) was described as a monotypic genus with the single terrestrial species *Mantophryne lateralis*. Zweifel (1972) reclassified *Mantophryne* as *Phrynomantis*. *Mantophryne* was resurrected in 1986 (Burton, 1986) and has remained a monotypic genus until the recent description of four named species, the discovery of cryptic diversity, and the synonymization of the monotypic *Pherohapsis menziesi* into *Mantophryne* (Oliver et al., 2013). We confirmed the monophyly of *Mantophryne* and its sister relationship to *Hylophorus* along with (Oliver et al., 2013; Peloso et al., 2016; Rivera et al., 2017).

Oreophryne Boettger (1895) is a large group of arboreal frogs with an extremely large distribution (throughout mainland New Guinea, offshore islands to the west, north, and east, and parts of South East Asia), whose monophyly has long been suspect. Several research groups, using different taxa, came to differing conclusions: *Oreophryne* basal to all remaining Asterophryinae (de Sa et al., 2012), or polyphyletic with a portion of it basal with other species scattered throughout the tree (Tu et al., 2018), whereas Rivera et al. (2017) suggested that *Oreophryne* may represent three clades. With the largest sampling of *Oreophryne* to date (44 taxa), we find strong support for two evolutionarily independent clades which we denote A and B. We note tissues for the type species of *Oreophryne*, *Oreophryne senckenbergiana* (Boettger, 1895), were not available for this study, and therefore it is unclear which group should retain the name *Oreophryne*. However, it is clear that these are two reciprocally monophyletic groups. “*Oreophryne A*” is highly supported as the sister to the remainder of the subfamily, and has a large distribution extending from the EPCT and Eastern offshore islands westward through the Accreted Terranes. The unrelated younger “*Oreophryne B*” clade is restricted to the Southeastern tip of the EPCT and the Eastern offshore islands, but its precise sister taxon remains unclear with two possibilities: potentially sister to *Cophixalus*, or sister to all of the remaining clades excepting *Oreophryne A*, *Paedophryne*, and *Cophixalus*, Fig. 2). Interestingly, both clades appear in South East Asia: from Sulawesi - *Oreophryne B variabilis* and *Oreophryne B sp.5*, and from the Philippines - *Oreophryne A anulata* and *Oreophryne A nana*. As a historical note, species contained in *Oreophryne A* have been previously described as belonging to the following genera: *Phrynxalus* (Stejneger, 1908; Taylor, 1920), *Chaperina* (Taylor, 1920), *Sphenophryne* (Boulenger, 1896; Boulenger, 1898; Méhely, 1897), *Hylella* (Werner, 1989), *Hyla* (Barbour, 1912), *Mehelyia* (Wandolleck, 1910), and *Phrynomantis* (Noble, 1926), whereas we could find no synonyms for species of *Oreophryne B*.

Despite the apparent confusion, the organization of “*Oreophryne*” is

now clarifying. The “*Oreophryne*” species in de Sa et al. (2012), Peloso et al. (2016) and *O. anulata* in Tu et al. (2018) group with *Oreophryne A*, and the species in Rivera et al. (2017) condense into *Oreophryne A* or B. The Sulawesi and Philippine taxa share most recent common ancestors with New Guinean taxa ~8 – 10 MYA, nested within clades with much deeper histories, raising the possibility that the migration of *Oreophryne A* and B are coming from New Guinea to South Asia. Increasing sampling from South Asia and areas outside of New Guinea are needed to confirm their distribution and direction of dispersal.

Paedophryne Kraus, 2010 is a miniaturized terrestrial group has been allied to at least three different genera (all with low support): monophyletic and sister to *Cophixalus* (Rittmeyer et al., 2012), sister to *Cophixalus* with *P. swiftorum* nested within *Choerophryne* (Rivera et al., 2017), and monophyletic and possibly allied to *Oreophryne* (Tu et al., 2018). *Paedophryne dekot* is the only sample grouping outside of *Paedophryne* and within *Oreophryne A*, and thus should be reexamined for possible misdiagnosis (but was not available at the time of this study). Here we corroborate the monophyly of *Paedophryne* (with the exclusion of *Paedophryne dekot*) and find support for a sister relationship to *Cophixalus* (PP = 0.75 and BS = 93), the original proposal of Rittmeyer et al. (2012).

Xenorhina Peters, 1683 is a largely fossorial genus. *Xenobatrachus* was synonymized with *Xenorhina* by Frost et al. (2006). This result is supported by both morphological (Allison and Kraus, 2000) and molecular characters (this study Frost et al., 2006; Rivera et al., 2017; Tu et al., 2018). We confirm strong support for the monophyly of *Xenorhina* and its sister relationship to *Asterophrys*.

4.5. Conclusions

We reconstructed the most robust phylogeny of Papuan Asterophryinae to date that includes 205 taxa sampled from over 80 sites across both mainland New Guinea and the satellite islands. We also find support for the monophyly of 11 of the established genera; the remaining 7 genera have strong support for non-monophyly and we recommend taxonomic reclassification. Additionally, we found evidence for cryptic diversity in both monotypic genera and widespread species suggesting that the taxonomy of all “widespread taxa” should be reevaluated. The sampling of cryptic species substantially increased phylogenetic resolution by anchoring the deeper nodes within the topology, breaking up long branches, and increasing nodal support globally. We described community composition of Asterophryinae for the first time and found that multispecies communities are common and comprised of four or five lifestyles represented by diverse genera. Our phylogenetic analysis shows that early, rapid diversification gave rise to numerous genera coincident with ecological evolution. As a result, we see that the explosive speciation found within Asterophryinae is potentially due to the combination of early adaptive radiation, niche conservatism, and non-adaptive processes of community assembly.

Supplementary Materials

A full list of specimens, sites, and metadata is given in Table 1 in Hill et al. (submitted for publication). A list of generic level taxonomic revisions and all species affected is provided in Table S1.

See Figure 1 vs. 2 in Hill et al. (submitted for publication) for a comparison of Bayesian inference versus Maximum Likelihood time-calibrated phylogenetic reconstructions of the complete dataset (233 samples). The BI analysis recovered the age of the basal node as 19.7 MYA with 95% CI 24–15 MYA. A similar age was recovered by the ML analysis at 18.5 MYA with 95% CI 26–14 MYA.

See Figure 3 in Hill et al. (submitted for publication) for a nuclear-only Bayesian inference reconstruction and see Figure 4 for a mitochondrial-only BI reconstruction.

CRediT authorship contribution statement

Ethan C. Hill: Formal analysis, Data Curation, Methodology, Project administration, Writing – Original Draft, Investigation, Writing - Review & Editing, Visualization. **Claire J. Fraser:** Investigation , Data Curation, Writing – Original Draft, Writing – Review & Editing. **Diana F. Gao:** Investigation, Writing – Review & Editing, Visualization. **Mary J. Jarman:** Investigation, Writing – Review & Editing. **Elizabeth R. Henry:** Investigation, Resources. **Bulisa Iova:** Investigation, Resources. **Allen Allison:** Conceptualization, Methodology, Investigation, Resources, Supervision, Writing – Original Draft, Writing – Review & Editing. **Marguerite A. Butler:** Conceptualization, Methodology, Formal analysis, Data Curation, Supervision, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualization, Project administration, Funding acquisition, Visualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version, at <https://doi.org/10.1016/j.ympev.2022.107618>.

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