Mountain colonisation, ecological evolution and miniaturisation in a radiation of direct developing New Guinea Frogs (*Choerophryne*, Microhylidae)

Paul M. Oliver[1, 2], Amy Iannella[3], Stephen J. Richards[4], Michael S.Y. Lee[4,5]

[1] *Division of Evolution and Ecology, Research School of Biology & Centre for Biodiversity Analysis, The Australian National University, Building 116, Daley Road, Canberra, Australian Capital Territory 2061, Australia.*

[2] *Department of Zoology, University of Melbourne, Parkville, Vic 3052, Australia*

[3] *School of Biological Sciences, University of Adelaide, Adelaide SA 5005, Australia*

[4] *South Australian Museum, North Terrace, Adelaide SA 5000, Australia.*
[5] *School of Biological Sciences, Flinders University, GPO Box 2100 Adelaide SA 5001, Australia.*

Corresponding author: Paul Oliver. Research School of Biology, Australian National University, Building 116, Canberra, 2601, Australia.
Email: Paul.oliver@anu.edu.au

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ABSTRACT

Aims. Mountain ranges in the tropics are characterised by high levels of localised endemism, often-aberrant evolutionary trajectories, and some of the world's most diverse regional biotas. Here we investigate the evolution of montane endemism, ecology and body size in a clade of direct-developing frogs (*Choerophryne* Microhylidae) from New Guinea.
Methods. Phylogenetic relationships were estimated from a mitochondrial molecular dataset using Bayesian and Maximum Likelihood approaches, ancestral state reconstructions were used to infer the evolution of elevational distribution, ecology (indexed by male calling height), and body size, and phylogenetically corrected regression was used to examine the relationship between these three traits.
Results. We obtained strong support for a monophyletic lineage including the vast majority of taxa sampled. Within this clade we identified one subclade that appears to have diversified primarily in montane habitats of the Central Cordillera (> 1000 m. a.s.l.), with subsequent dispersal to isolated North Papuan Mountains. A second subclade (which is also largely supported by a morphological synapomorphy) appears to have diversified primarily in hill forests (< 1000 m a.s.l.), with inferred independent upwards colonisations of isolated montane habitats, especially in the North Papuan Mountains. We found no clear relationship between small body size (adult SVL less than 15 mm) and elevation, but a strong relationship with ecology – smaller species tend to be more terrestrial.

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Conclusions. Orogeny and climatic oscillations have interacted to generate high montane biodiversity in New Guinea via both localised diversification (including upslope colonisation) and periodic dispersal across lowland regions. The correlation between extreme miniaturisation and terrestrial habits reflects a general trend in frogs, suggesting that ecological or physiological constraints limit niche usage by miniaturised frogs, even in extremely wet environments such as tropical mountains.

Keywords Central Cordillera, endemism, montane cradle, montane museum, North Papuan Mountains, terrestrial

INTRODUCTION

Tropical mountains contain some of the most diverse regional biotas in the world, with high levels of localised endemism and often fine elevational turnover in biodiversity (Mayr & Diamond, 1976; Fjeldså et al., 2012; Merckx et al., 2015; Rosauer & Jetz, 2015). The processes responsible for this exceptional diversity are of great scientific interest, both for improved understanding of the drivers of biological diversity (Janzen, 1967; Hutter et al., 2013; Graham et al., 2014), and for understanding how these highly diverse biotas will be affected by anthropogenic climatic change (Williams et al., 2003; La Sorte & Jetz, 2010; Freeman & Class Freeman, 2014).

Recently in an analysis of the biota of Mt Kinabalu on Borneo (Merckx et al., 2015) suggested that montane endemics could be broadly dichotomised into centric endemics (derived from upslope colonisation of lowland taxa) and eccentric endemics (derived via long distance colonisation of cool adapted taxa) (Merckx et al., 2015). They found evidence that both processes played an important role, with a dominance of centric endemics in lower montane habitats and eccentric endemics at higher elevations. More broadly, two paradigms to explain high diversity in tropical mountains have been advanced, and received support from different studies: a) mountain uplift and climatic change have elevated local rates of speciation (the 'cradle' hypothesis), or b) mountains have provided refugia for often specialised taxa that would have otherwise died out due to competition or climatic change (the

Mark D. Scherz 3/10/2016 23:05 **Deleted:** i 'museum' hypothesis) (Weir, 2006; Fjeldså et al., 2012; Hutter et al., 2013; Price et al., 2014).

The vast tropical island of New Guinea has arguably 'the most complex orogeny in the world' (Baldwin et al., 2012). The collision of the leading edge of the northwards-moving Australian plate with the westwards-moving southern edge of the Pacific Plate has uplifted a high Central Cordillera (> 4000 m a.s.l.) extending nearly the length of the island (Baldwin et al., 2012) (Fig. 1). However, much (or most, depending on authority) of these ranges may date to the late Miocene at the earliest, and high elevation habitats are even younger (Hall, 2002; van Ufford & Cloos, 2005; Baldwin et al., 2012). Additional smaller and more isolated montane regions scattered along northern New Guinea are the uplifted remnants of island arcs that have accreted onto the northern edge of the Australian plate, beginning in the Miocene and continuing with the rapid uplift of the Huon and Finnisterre Ranges (Hall, 2002; Polhemus, 2007).

The biota of New Guinea has been profoundly shaped by this complex orogeny. The uplift of the Central Cordillera has isolated lowland vicars to the north and south of New Guinea (Rawlings & Donnellan, 2003; Unmack et al., 2013; Georges et al., 2014). It has also been suggested that emerging elevation gradients may have increased speciation rates in some New Guinea radiations, inflating regional alpha diversity (Toussaint et al., 2013, 2014)—a species pump model similar to the uplift of the northern Andes (Weir, 2006; Santos et al., 2009). In contrast the endemic montane fauna of the smaller, younger and more isolated mountains of northern New Guinea is particularly poorly known, and there have been few phylogenetically informed assessments of the origins of endemic taxa in these ranges (Beehler et al., 2012; Oliver et al., 2012).

The New Guinea frog biota is exceptionally diverse, with > 450 recognised species, and many more awaiting description (Oliver et al., 2013, Rittmeyer and Bulisa, in prep.)—far more diverse than nearby landmasses such as Borneo or Australia. This frog diversity is most remarkable for being dominated by just two major radiations, of which the most species rich and ecologically variable is a clade of nearly 250 recognised species of direct developing microhylids—the Asterophryninae Günther, 1858 (Frost et al., 2006). Their reproductive ecology (direct development, not limited by standing water), wide elevational distribution and high diversity suggest that microhylid frogs will provide an excellent system for understanding how the mountains may have shaped diversification in New Guinea.

Choerophryne (including the previously recognised genus *Albericus* [see Peloso et al., 2015]) is a moderately diverse clade (31 recognised taxa) of small to miniaturised microhylid frogs endemic to New Guinea. This genus occurs from hill to upper montane habitats across much of Central Cordillera and North Papuan Mountains (although they appear to absent in most of the west and southern lowlands of the island) (Günther, 2000; Richards et al., 2000). They are mostly climbing frogs with well developed pads, but range in ecology from largely arboreal to terrestrial, and include at least one highly derived montane form (complete loss of pads, very large size, bright belly colourations) (Kraus & Allison, 2000; Richards et al., 2007; Günther & Richards, 2011).

Choerophryne also includes many miniaturised species (i.e. maximum recorded adult SVL < 15 mm; Yeh, 2002) that approach minimum size limits for tetrapods (Kraus, 2010a; Rittmeyer et al., 2012). The water-permeable skin of frogs plays a critical role in shaping both local and regional patterns of diversity and habitat use (Scheffers et al., 2013), with smaller species tending to be more desiccation

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susceptible than larger species (Tracy et al., 2010). So a further prediction might be that smaller species of *Choerophryne* would tend to occur in cloud forest habitats at higher elevations that are less prone to desiccation (Nix, 1982).

Here we present an analysis of the phylogenetic relationships and evolution of key traits within *Choerophryne*. We initially focus on the origins of montane endemism, with a specific prediction being that the older Central Cordillera will be dominated by *in situ* diversification processes (centric endemism) while the younger North Papuan mountains may show evidence of colonisation from the older Central Cordillera (eccentric endemism). We also test the related prediction that ecological (arboreal to terrestrial) and body size shifts (extreme miniaturisation) may be correlated with living in novel habitats and climatic regimes at higher elevations.

METHODS

Specimen Selection

This study utilised whole specimens and tissue samples deposited in the South Australian Museum collection. As this work was done on preserved museum material, ethics approval was not sought. The taxonomic assignation of *Choerophryne* species is challenging, especially in the absence of calls – so taxonomic assignations should be considered provisional. We recognised lineages as candidate species if they met any two of the following three criteria (largely following Vieites et al., 2009); a) males with distinctive advertisement calls, b) evidence of morphological differentiation or c) evidence of genetic differentiation. Mitochondrial DNA sequences of an additional 11 *Choerophryne* were downloaded from GenBank, along with 14 outgroup sequences from 6 other New Guinean microhylid genera. Full details of all samples included are given in Appendix S1.

DNA extraction, amplification, sequencing and alignment

Whole genome DNA was extracted from frozen or alcohol preserved liver samples using the Gentra Puregene kit protocol (QIAGEN 2011). Sequence data from the 12S and 16S mitochondrial genes was PCR amplified with an annealing temperature of 58°C using the primers 12SAL and 12SBH (Palumbi et al., 2002) and 16SL3 and 16SAH (Vences et al., 2003), then purified on MultiScreen PCR_{384} Filter Plates. Sanger sequencing (forward and reverse) of purified PCR product used the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), purified using MultiScreen SEQ_{384} Filter Plates and sent to the Australian Genome Research Facility (AGRF) for capillary separation.

Geneious Pro v5.5.2 (Kearse et al., 2012) was used to align forward and reverse sequence traces and reviewed by eye. The consensus sequences along with sequences from GenBank (Appendix S1) were aligned with 8 iterations of the MUSCLE algorithm under default parameter settings (Edgar, 2004). Hypervariable regions with poor local alignment were removed using Gblocks v0.91b (Castresana, 2000); of the original 1556 aligned positions, 1347 were retained in final analyses.

Phylogenetic Analysis

To assess congruence of topology and support values across methods, we estimated phylogenetic relationships using Bayesian and Maximum Likelihood approaches. Based on the output of the model selection program MrModeltest (Nylander, 2004) all analyses were performed using the general time-reversible model, allowing for variation in the rate of evolution among sites including invariable sites (GTRig). Both genes were treated as a single partition due to the relatively short sequence length and similar features (i.e. mitochondrial).

The Maximum Likelihood tree with bootstrap values was produced using RAxML v 8.0.26 (Stamatakis, 2006) with bootstrap scores calculated using the rapid booststrap (-f a) function with 1000 replicates. The Bayesian consensus tree was generated by Mr Bayes 3.2.20 (Ronquist et al., 2012) using an unconstrained branch length prior, 4 chains (incrementally heated at temperature 0.2), each of 5 million generations with a 1 million generation burn-in and sampling every 200 generations.

These topology-only analyses with dense sampling across populations were compared to analyses where we simultaneously estimated phylogeny, divergence dates and trait evolution, on species-level trees (see below).

Trait scoring

We scored each taxon for three traits of interest: a) adult male body size, b) elevation and c) maximum calling height of males (as a proxy for arboreality vs terrestriality) (Appendix S1). These data were scored from genotyped specimens, or extracted from primary literature.

We used the maximum recorded size for males (females for many species are unknown). We used the typical measure of size in anurans – i.e. the distance from the tip of the snout to the vent or urostyle tip (SUL) which has been previously used in *Choerophryne* (Kraus & Allison, 2000; Richards et al., 2007; Günther, 2008). Although some *Choerophryne* have unusually long snouts, at most these comprise 10% of the total body length, and similar analyses undertaken which corrected for this gave similar results.

The maximum elevational range obtained for any species was just over 1000 metres, in both cases involving widespread hill forest taxa that range into lower montane forests. To score elevation as a continuous character (for use in phylogenetic regressions) we used the mid-point of records for each lineage (to the nearest 100 m). For discrete classification we used the forest classification system presented by Johns (1982): hill forest and lowlands (< 1000 m a.s.l.), lower montane (1000–2000 m a.s.l.), mid-montane (2000–3000 m a.s.l.) and upper montane (> 3000 m a.s.l.). These bands broadly reflect how reducing mean temperatures with elevation shapes the transition from megathermal to microthermal vegetative communities (Nix, 1982). For most taxa, the majority of records were focused in just one of these bands. The small number of taxa whose distributions spanned bands were placed in the band in which the majority of records were concentrated. Finally, *Choerophryne laurini* is only known from typical lower montane forest on mossy ridge tops in the Wondowoi mountains between 800-950 metres, so was coded as lower montane for discrete analyses.

Male *Choerophryne* show extensive variation in the typical calling height from largely terrestrial (e.g. *Choerophryne alpestris*) to more than 3 metres off the ground (e.g. *Choerophryne pandanicola*) (Günther & Richards, 2011). To score calling height as a continuous trait we used the maximum recorded calling height of males, either from the literature or personal observations. To easily visualise calling

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ecology on the phylogenetic tree we further divided taxa into two broad guilds: a) *Terrestrial* - species that called predominantly on or very close to the ground on leaf litter or duff (generally less than 50 cm high), and b) *Scansorial* - species that usually climb into vegetation and call from (generally more exposed) positions up to several metres high. Two taxa (*C. arndtorum* and *C. microps*) for which the majority of calling records are terrestrial but which have occasionally been recorded calling a metre or more above the ground (Günther, 2008), were coded as terrestrial in the discrete character analyses, while the maximum recorded calling height was used in continuous trait based analyses.

Ancestral state analyses.

We used BEAST v 1.8.2. (Drummond & Rambaut, 2007) to co-estimate trait evolution (including ancestral states) with phylogeny and divergence dates. These analyses used a reduced dataset comprising a single exemplar of all genetically and/or morphologically divergent lineages identified in earlier phylogenetic analyses (i.e. recognised or candidate species). We also excluded two samples from a highly divergent clade at the base of the *Choerophryne* radiation (see results), so as to ensure these analyses were focused on a strongly supported and well sampled monophyletic group. Size was $\log_{10}$ transformed. The two discrete variables (elevation and calling ecology) were coded using the MK + strict clock model, which assumes that transformations between states are reversible and occur at the same rate throughout the tree; more complex models were not feasible due to the relatively small tree and number of transformations. Elevation states were ordered – e.g. shifts from lower- to upper-montane habitats were constrained to involve moving through mid-montane habitats. The original molecular data for each exemplar was also included. Each BEAST analysis was repeated four times to check for stationarity (convergence). Analyses were run for 50 million generations, sampling every 100,000 generations. The first 20% of trees were discarded as burnin and the remaining 3200 postburn trees were pooled to generate the final consensus topology. The final xml file is given in Appendix S2.

BEAST automatically produces an ultrametric tree – however there are no fossil calibrations within *Choerophryne*, and there has been no recent thorough assessment of rates of mitochondrial DNA evolution in frogs. To provide a rough timescale for *Choerophryne,* we used a molecular evolutionary rate for mitochondrial genes of between 1-2% pairwise per million years, which was incorporated into the rate prior. Rates of molecular variation vary extensively (Eo & DeWoody, 2010), and thus the resultant dates from this are interpreted with caution. The ancestral state analyses (above) only require relative rather than absolute branch lengths (e.g. they could still be performed if root age was arbitrarily scaled to 1).

Phylogenetic Least Squares Regression

The relationship of a) body size to calling ecology and/or elevation and b) calling ecology to elevation was analysed using BayesTraits v 2.0 (Pagel & Meade, 2013)**,** across the concatenated 3200 post-burnin trees from BEAST. For these analyses all variables were included as $\log_{10}$-transformed continuous states. We only included data for lineages in two well-sampled clades of *Choerophryne* (see below), other species in the trees were scored as missing data. The Bayesian MCMC implementation of the Continuous module was used to regress a) body size against ecology and elevation,

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and b) ecology against elevation; 11 million steps were used with the first 1 million for burn-in, and 4 runs of BayesTraits were performed and checked for convergence. Pagel and Meade (2013) suggested that the significance of a variable could be assessed either by comparing harmonic means (for analyses with and without the variable), or whether the estimated distribution of that variable (e.g. 95% HPD) excludes 0. Because of issues with using harmonic means to estimate marginal likelihoods (Xie et al., 2011), we use the second approach.

RESULTS

Phylogenetic relationships and lineage diversity.

All analyses identified three major lineages of *Choerophryne* (Fig. 2). Clade A comprised the majority of sampled taxa that were formerly placed in the genus *Albericus*, Clade B included all taxa with a moderate to pronounced rostral projection formerly placed in *Choerophryne sensu stricto*. Clade C comprised two scansorial taxa lacking distinctive rostral projections and respectively occurring to south of the Central Cordillera in hill forest, and on the Finnistere Ranges (north-east New Guinea) in hill to lower montane forest.

A sister taxon relationship between Clades A and B was strongly supported in all analyses. Clade C was more divergent and there was no evidence that it forms the sister group to Clade A+B (or any other microhylid lineage). All basal relationships between the New Guinea microhylid genera we sampled were poorly supported.

Within Clade A we identified two strongly supported primary lineages, with the major split being between a clade of two lower montane taxa and hill forest taxa from the south side of Central Cordillera, and several clusters of species from across the Central Cordillera and North Papuan Mountains, including derived near terrestrial taxa from mid to upper montane habitats (*C. alpestris* and *C. brevicrus*).

Within Clade B there were three well supported primary lineages: one comprising three deeply divergent taxa (two unnamed) from hill forest to mid-montane habitats on the Central Cordillera; a further lineage of large-bodied and very long-snouted taxa from hill and lower montane forest in northern New Guinea; and finally a diverse conglomeration including lineages from hill and lower montane forests in northern New Guinea, in addition to one taxon from south of the Central Cordillera (*C. gracilirostris*).

In all three major clades we identified lineages (candidate species) that were deeply divergent from, and could not be confidently assigned to, recognised species. This was most pronounced in Clade A - which includes a number of scansorial species that are difficult to diagnose on the basis of external morphology.

Ancestral States analyses

The phylogenetic tree for the ancestral states analysis (Fig. 3) was consistent with and very similar to the better-sampled trees (Fig. 2). Clade C was not included in ancestral state analyses due to uncertainty about its phylogenetic placement and its relatively small number of lineages.

Ancestral states analyses highlighted the contrasting evolutionary trajectories of the two 'core' clades of *Choerophryne* (A & B) for which we were able to obtain moderate taxon sampling (Fig. 4). Hill forest habitats (largely distributed between 0-1000 m a.s.l) were inferred to be the ancestral habitat for both Clades A and B. Clade

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A was inferred to have diversified primarily within montane habitats during the late Miocene (~14 nominal taxa), including more recent upslope shifts into mid and upper-montane zones. Independent colonisation of isolated mountains in the north coast is also inferred (specifically the Foja and Wondiwoi Mountains). In contrast Clade B was centred on hill forest habitats, but with 2–4 relatively recent upslope shifts into montane habitats in mostly distantly related taxa, again mainly occurring in isolated North Papuan Mountains (specifically Japen Island and the Foja and Torricelli Mountains).

Extremely small species (<15 mm) were scattered across the phylogeny - implying that multiple lineages of *Choerophryne* have independently evolved very small body size. Taxa in the predominantly scansorial Clade A tended to be larger than those in the more terrestrial clade B.

Calling ecology was relatively labile across the genus, with multiple shifts between predominately terrestrial to predominately scansorial calling (the latter inferred as the ancestral state for the common ancestor of clades A and B). However there was again somewhat contrasting patterns across the two clades; while clade A was inferred to be largely scansorial with a small number of shifts towards terrestrial calling, Clade B included a majority of taxa that call from on or close to the ground, and this state was accordingly inferred as ancestral for this clade, but has undergone at least three reversals to scansorial calling.

Phylogenetic Regressions

All BayesTraits runs converged well before the burn-in, and the concatenated runs yielded ESSs of all parameters >1000. In the analysis relating body size to ecology and/or elevation, both ecology and elevation (considered together: Pagel and Meade 2013) exhibited significant phylogenetic structure, as expected (Lambda was significantly positive: mean 0.55; 95% HPD 0.12, 0.98). Ecology (as indexed by calling height) was positively associated with body size, with a regression coefficient that was always estimated as positive (mean = 0.09, 95% HPD = 0.03, 0.15). However, elevation was not related to body size, with a regression coefficient centred almost exactly on 0 (mean = 0.01, 0. 95% HPD = -0.11, +0.11).

In the analysis relating ecology to elevation, calling height was found to be weakly negatively related to elevation, with a 95% HPD which did not quite exclude 0 (mean = -0.6534, 95% HPD = -1.273, 0.0445). Subsequent investigation suggested that this could be attributable to the effect of three extremely high elevation taxa (>2500 m a.s.l.) in Clade A that live in mossy grasslands and are largely terrestrial. Subsequent re-analyses with these taxa removed weakened this relationship further, resulting in a 95% highest probability posterior distribution that more broadly included 0 (mean = -0.47, 95% HPD = -1.0585, 0.1589).

DISCUSSION

Despite the biological wealth and high endemism of the New Guinea Mountains (Tallowin, in review) and emerging evidence for major evolutionary radiations (Toussaint et al., 2014; Givnish et al., 2015), only a small number of phylogenetic studies of lineages with distributions centred on the montane regions of New Guinea have been published (Meredith et al., 2010; Toussaint et al., 2013; Irestedt et al., 2015). Our study complements this recent work by focusing on a lineage of small, direct-developing frogs that may reasonably be presumed to have comparatively low

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vagility. Here, we discuss the implications of our study for understanding the biodiversity of New Guinea and the processes that may have shaped this, and also more broadly for understanding correlates with the evolution of extreme miniaturisation in frogs.

Unrecognised species diversity and phylogeny

Molecular assessments of amphibian diversity on tropical islands over the last decade have revealed exceptionally high levels of previously unrecognised diversity (Meegaskumbura et al., 2002; Vieites et al., 2009). However, while New Guinea already has the most diverse insular frog fauna in the world (over 450 recognised species [Rittmeyer and Bulisa in prep]), molecular assessments of frog diversity in this region are scarce. We uncovered 12 lineages that we currently consider to be candidate species, in addition to three others we recently named (Iannella et al. 2014, 2015). A recent molecular study of another genus of New Guinea microhylid frogs (*Mantophryne*) also revealed a high diversity of unrecognised deeply divergent lineages (Oliver et al., 2013). Further fieldwork and integration of molecular, morphological and acoustic analyses seem certain to cement New Guinea's position as a global hotspot of amphibian diversity.

The taxa we sampled within *Choerophryne* formed three strongly supported major lineages. These three major clades within *Choerophryne* make sense in light of morphological data. Clades A and C are largely scansorial lineages that were formerly placed in the now synonymised genus '*Albericus*', while Clade B corresponds to taxa to which the genus *Choerophryne* was formerly restricted, and are all characterised by an extended maxillary process.

Clades A and B together formed a strongly supported monophyletic group, but the overall monophyly of all three lineages of *Choerophryne* we sampled was not strongly supported (or rejected). As there are however morphological synapomorphies uniting all three lineages of *Choerophryne* (see Burton & Zweifel, 1995), and the monophyly of the lineages was recently supported based on a phylogenomic study including exemplars of all three major lineages (Peloso et al., 2015), we consider the non-monophyly of *Choerophryne* in our analyses most likely an artefact of rapid diversification and the short rapidly saturating loci we used. At this stage we see no reason to reject the morphological and phylogenomic evidence for the monophyly of this genus.

In light of this phylogenetic uncertainty in the dataset, we reiterate that we focused evolutionary analyses (see below) on the strongly supported monophyletic lineage comprising clades A and B. Furthermore, we emphasise that while there was uncertainty for many nodes within this clade, the key conclusions we present below about the evolution of ecology, body size and elevation distribution span divergent, morphological and ecologically differentiated lineages that are well supported.

Geographic and taxonomic considerations

Before discussing evolutionary patterns we also consider the potential effects of taxonomic and geographic sampling gaps. Our sampling of taxa in Clade B (which can be readily distinguished from most other *Choerophryne* by a skeletal character) includes all recognised species and several candidate taxa, while our sampling of clades A and C is more incomplete. Museum records of specimens that can be assigned to Clade B are primarily from hill forest elevations, while taxa

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Comment [12]: This paragraph made no sense as it was previously written (one sentence was incomplete); I have intervened to clarify what you were trying to say.

NGS data was only available from two of the three lineages in Peloso et al.; not enough information to justifiably say that the monophyly of Albericus was refuted based on 'phylogenomic data'; the two Albericus species that clustered with Choerophryne were based on the seven Sanger loci only.

I would therefore recommend rewording the statement that the relationship was supported by phylogenomic data

More importantly in my opinion, a more informative taxonomic decision would have been to give the C. exclamitans lineage its own name, and to maintain Albericus and Choerophryne as more or less reliably distinguishable genera. You have shown nicely that they are monophyletic with high support.

morphologically consistent with clade's A and C are mostly found in montane forest, especially in regions of the Central New Guinea where they overlap with Clade B. These data suggest that our characterisation of one group as primarily hill forest frogs and the other as primarily montane frogs is robust (Fig. 5).

Potentially important distributional gaps in our genetic sampling included the western portion of the Central Cordillera and the Papuan Peninsula. Recent surveys in western New Guinea (upper Mamberamo, Fak Fak mountains) have indicated that *Choerophryne* (which are usually easy to locate) are absent or rare, suggesting this is not an important area of endemism for this genus (Günther, 2000; Richards et al., 2000). Endemic *Choerophryne* are found in the Papuan Peninsula, but none of these taxa are shared with Central New Guinea, suggesting that taxa in this region—which is geologically very distinctive—will have their own history.

While the inclusion of extra taxa and filling sampling gaps will certainly refine patterns, we are confident it will not change the picture of broadly reciprocal patterns of elevational distribution and montane colonisation between clades A and B in Central New Guinea upon which our results hinge.

Montane cradle or museum?

Newly uplifting tropical mountains have been shown to be 'cradles' of young diversification in diverse regional bird communities (Weir, 2006; Price et al., 2014). Recent work on beetles, mammals and birds has suggested a similar association between recent uplift of mountains in New Guinea and diversification (Meredith et al., 2010; Toussaint et al., 2014; Irestedt et al., 2015). Our study supports this, and indicates that Clade A in particular is moderately diverse (15 nominal taxa and probably many more yet to be named), and almost entirely endemic to the New Guinea Highlands (>1000 m). In the absence of reliable calibrations for our molecular dataset, dates should be regarded as a preliminary estimate at best, but we note that our dates suggest lower montane habitats were colonised by the mid-Miocene, while higher altitude taxa (i.e. > 2000 m a.s.l.) in Clade A are relatively young (Pliocene). This pattern is broadly consistent with progressive upslope colonisation as the Central Cordillera gained height through the late Miocene and Pliocene.

The converse hypothesis is that montane areas have functioned as 'museums' for lineages that have been extirpated from surrounding regions by climatic or biotic change (Fjeldså et al., 2012; Hutter et al., 2013). Montane habitats in New Guinea are home to a suite of deeply divergent bird lineages and also show high phylogenetic endemism of mammals (Jønsson et al., 2010; Rosauer & Jetz, 2015). In *Choerophryne* one clade in the Central Cordillera region (*burtoni*, *sp*B2 and *sp*B3) shows outwardly disjunct distributions and deep divergences (estimated 10 mya in the tree). This is suggestive of 'marooned' relict lineages that have persisted through uplift, but not extensively diversified—especially when compared to the broadly sympatric and much more diverse Clade A. However given that there are relatively few of these lineages, these data do not provide support for the museum hypothesis playing a major role in inflating mountain diversity in New Guinea frogs at this stage.

Contrasting origins of montane endemics in northern New Guinea

In the older Central Cordillera, Clade A appears to have colonised montane habitats early in its radiation, and shows evidence of multiple further upslope diversification events (Fig. 4), with a tendency for diversification in higher elevations to be younger,

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and just one highly derived (terrestrial) lineage having successfully colonised upper montane habitats (*C. alpestris*). These patterns are all consistent with an overall trend towards upslope migration and diversification as the Central Cordillera rose through the Miocene and Pliocene (Meredith et al., 2010; Baldwin et al., 2012; Irestedt et al., 2015)

The younger, lower elevation, more isolated and poorly known North Papuan Ranges show a more multifaceted pattern. These ranges are home to numerous endemic or isolated populations of montane taxa (Beehler et al., 2012; Oliver & Richards, 2012), but in most cases these are clearly related to, or even conspecific with, montane taxa occurring elsewhere in New Guinea (e.g. 100% of birds are allopatric isolates of lineages occurring in montane habitats elsewhere; Beehler et al., 2012). In *Choerophryne* two lineages in Clade A show a similar pattern—they appear to be endemic to montane habitats in the north Papuan Mountains (not found below around 1000 m), related to taxa otherwise known only from montane Central Cordillera habitats, and thus far unknown from the intervening lowlands (Richards & Suryadi, 2003). These apparent disjunct distributions suggest that lower montane forests in New Guinea have a dynamic climate history, possibly including periods of major elevational depression similar to those inferred elsewhere in the tropics (Colinvaux et al., 1996; Zhuo, 1999).

In contrast, ancestral state analyses of well sampled Clade B provide strong evidence for at least two and potentially three independent derivations of North Papuan montane endemics from surrounding lowland taxa (Fig. 4). More detailed fine scale sampling is required to understand the processes that have shaped this endemism; elevational segregation may be an outcome rather than a driver of speciation (Caro et al., 2013; Freeman, 2015). However, regardless of the exact process, this pattern represents the first evidence that montane endemic vertebrates have arisen *de novo* in northern New Guinea from largely lowland lineages. These contrasting origins of endemism suggest that the suite of young and isolated North Papuan Mountains may provide excellent opportunities for comparative analyses of the processes driving montane endemism in young tropical mountains.

Mountain Uplift and Vicariance

Mountain uplift may also inflate regional diversity at lower elevations by isolating formerly continuous populations of lowland taxa (vicariance). In New Guinea the uplift of the Central Cordillera has isolated northern and southern vicars in lowland and aquatic taxa (Rawlings & Donnellan, 2003; Georges et al., 2014), and potentially also lower montane taxa (Irestedt et al., 2015). Our sampling of *Choerophryne* did not reveal extensive north-south vicariance that can be clearly linked to the uplift of the Central Cordillera—although one possible exception is a recently described pair of potential sister taxa in Clade B from hill and lower montane forest (*C. gracilirostris* [south] and *C. grylloides* [north]) that are estimated to have diverged around 10 mya. This general lack of signal for north-south vicariance is perhaps not surprising given the majority of species in the two clades are associated with hill and montane forest—mountain uplift would have isolated these taxa less effectively than lowland or aquatic taxa. Thus, while orogeny has clearly been a major driver of diversification patterns across New Guinea, how this process may have affected rates of lineage formation, and in particular morphological and ecological diversification, will likely be highly taxon-dependent.

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At the lower size limits of vertebrates: correlates of repeated miniaturisation

A number of new lineages of tiny frogs that approach minimum size limits for vertebrates have discovered recently (Wollenberg et al., 2008; Kraus, 2010a; Rittmeyer et al., 2012), and it has been suggested that miniaturised frogs may represent an often overlooked, but important ecological guild in tropical areas (Rittmeyer et al., 2012). Broadly, three patterns are globally apparent in miniaturised frogs; most lack a free-swimming tadpole stage (Estrada & Hedges, 1996), most occur in wet tropical and usually insular regions and most are more-or-less terrestrial (Kraus, 2010a; Rittmeyer et al., 2012). Across the six different genera of Papuan microhylids that contain miniaturised taxa (*Aphantophryne*, *Austrochaperina*, *Choerophryne*, *Cophixalus*, *Oreophryne* and *Paedophryne*) all three of these correlates are supported.

Our analyses further indicate that within *Choerophryne* there have been at least three relatively recent shifts towards extremely small body size (three lineages ~15 mm or less), all of which are inferred in lineages that call on or close to the ground. The plasticity of body size and ecology of *Choerophryne* contrasts with conservatism of these same features in another miniaturised genus of Papuan microhylids, *Paedophryne* (Rittmeyer et al. 2012). Patterns of evolution across both genera do however strongly support the hypothesis that physiological or ecological constraints limit miniaturised taxa to a terrestrial lifestyle. Most recognised taxa missing from our analyses are moderate sized and scansorial species that are likely in Clades A and C. Their inclusion would also be unlikely to change the correlation between terrestriality and small size.

Contra our initial prediction, we did not find a strong positive correlation between elevation and either ecology (calling height) or body size, as might be expected if desiccation risk is decreased at higher elevations (Scheffers et al., 2013). This lack of pattern may indicate that for frogs of extremely small size, physiological or ecological pressures associated with microhabitat use are a bigger constraint on body sizes than variation in climates over elevations. Unlike the correlation between terrestriality and small size in which we are confident and which mirrors a broader pattern, further analysis including both *Choerophryne* taxa missing from our dataset, and other genera of microhylid is probably needed to refine understanding of the potentially much more nuanced three-way relationships between body size, ecology and elevation.

Finally, *Choerophryne* provides a striking example of an insular frog lineage that has undergone ecological diversification with repeated shifts between scansorial and relatively terrestrial ecologies, reflected in significant reduction or even loss of terminal discs and shortening of limbs (Günther, 2008; Kraus, 2010b; Günther & Richards, 2011). Similar ecological diversity and morphological plasticity has also observed in other microhylid lineages in New Guinea, as well as in Madagascar and Philippines (Andreone et al., 2005; Köhler & Günther, 2008; Blackburn et al., 2013). In contrast, microhylids generally seem to be peripheral (and usually terrestrial or fossorial) components of frog diversity in continental regions (see Duellman, 1999). This suggests that microhylids might be comparatively good colonists of islands (in some cases perhaps associated with direct development) and have great adaptive potential in these regions, but may be poorer competitors in diverse continental frog communities (perhaps due to their specialised feeding apparatus: Meyers et al., 2004).

CONCLUSIONS

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Comment [18]: This in fact argues for a more flexible feeding apparatus than other frogs; it is not really more specialised.

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Our new phylogeny and ecophenotypic data for the microhylid frog genus *Choerophryne* indicates that montane areas have been colonised via a complex suite of biogeographic processes—especially upslope colonisation and speciation in presumably novel highland habitats and dispersal between montane islands—and that the relative importance of these processes has differed across even closely related lineages. *Choerophryne* also shows a correlation between extremely small size and utilisation of terrestrial habitats, mirroring a global pattern that suggests that, in frogs, ecological or physiological constraints largely limit extremely miniaturised taxa to terrestrial microhabitats in tropical areas.

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Supporting Information.

Appendix S1. Supplementary Tables. Specimens numbers, locality information and GenBank accession numbers for *Choerophryne* specimens included in analyses (Table S1); GenBank accession details for outgroup samples (Table S2); and summary data on body, elevational distribution and calling height for *Choerophryne* (Table S3).

Appendix S2. BEAST input file for ancestral state analyses

Appendix S3. Treefile for chronogram estimated in BEAST with ancestral states.

Figure 1. Map of New Guinea showing the major montane regions, and the distribution of sampling points for the three major lineages of *Choerophryne* identified in this study.

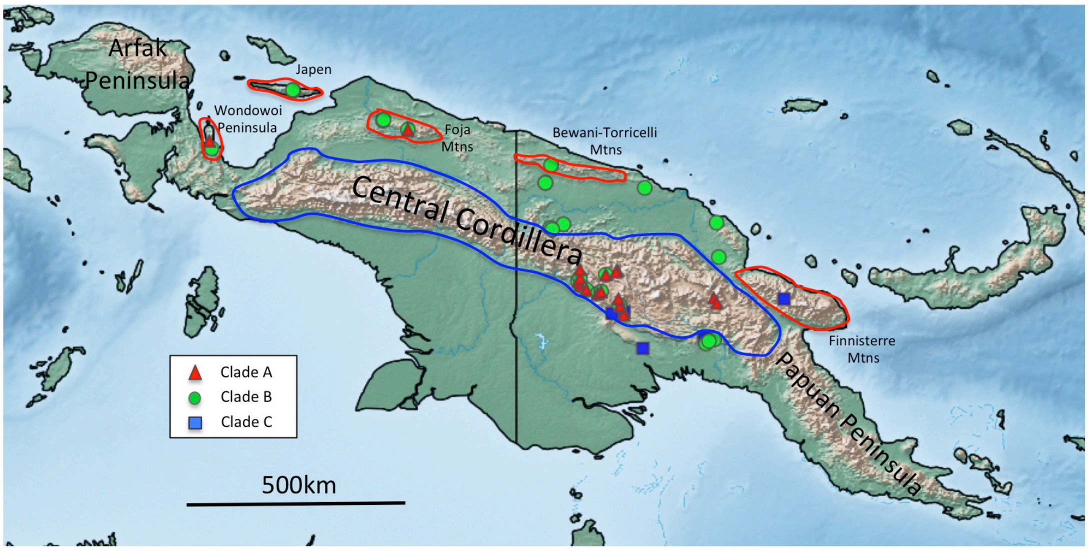


Figure 2. Summary Maximum Likelihood Tree with bootstrap nodal support above the line and Bayesian posterior probabilities below the line; * indicates <50% Bayesian posterior probability, interspecific nodes without support values were poorly resolved in both analyses, intraspecific node supports are omitted for clarity. Pictures are proportional. All pictures taken by S. Richards.

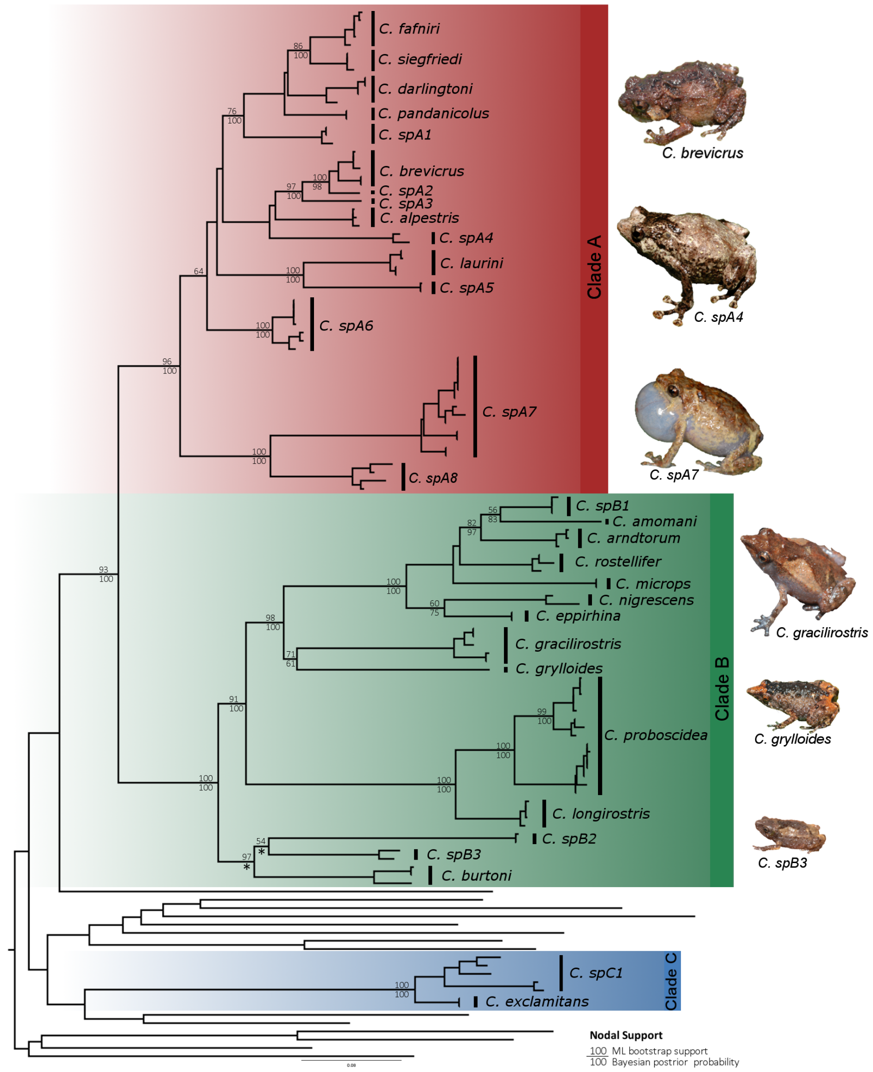


Figure 3. Chronogram for *Choerophryne* and outgroups estimated using 12S and 16S data and rate based calibration methods. Grey bars indicate 95% posterior distribution of age estimates for three well supported basal nodes. Node values are Bayesian Posterior Support values from BEAST analysis. Axes along bottom indicate time in millions of years ago. Branches colour coded to reflect maximum body size, with red

corresponding to the smallest size and four taxa under 15 mm further annotated by an asterisk.

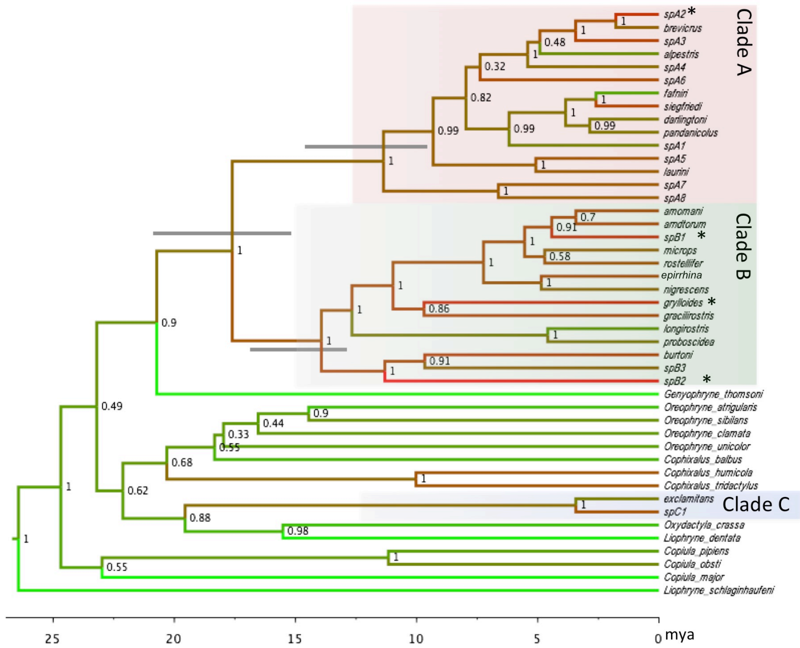


Figure 4. Trait evolution in the major lineages of *Choerophryne* estimated using BEAST based on discrete characters codings. Ancestral states with a probability of greater than 60% are indicated with an asterisk. Branch widths on both trees are proportional to maximum recorded adult male SVL and very small taxa (<15 mm) are indicated with bold and an asterisk. Taxa with distributions in the Central Cordillera are highlighted in blue, while those from northern New Guinea are in red. Three inferred upslope shifts in Clade B into lower montane forest in the North Papuan Mountains are indicated by arrows.

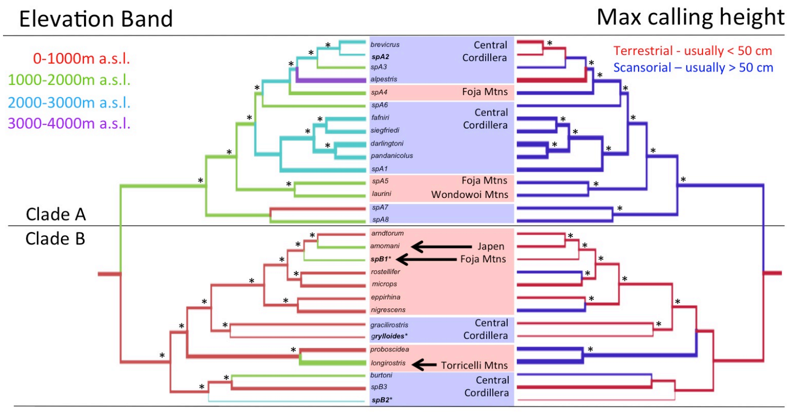
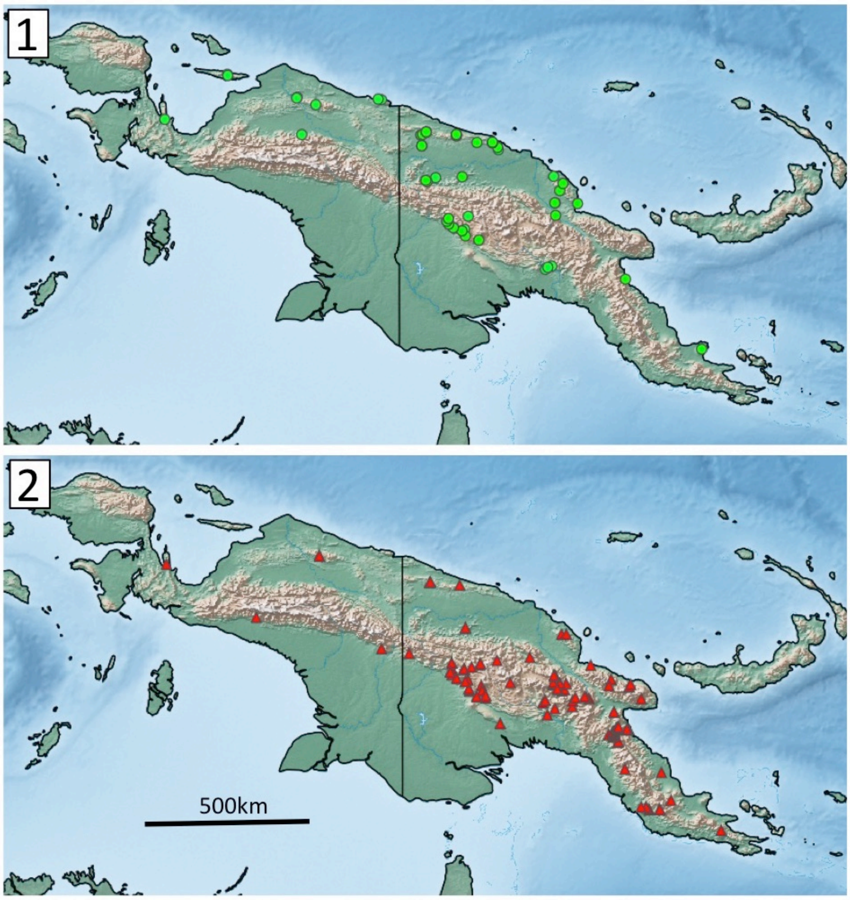


Figure 5. Summary of museum records for *Choerophryne* grouped by phenotype. 1) taxa with an extended maxillary process (in large part corresponded to Clade B, but see Kraus, 2013 for a probable exception from Milne Bay Province), and 2) taxa lacking an extended maxillary process, rendering them morphologically consistent with genotyped samples in Clade A and C.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