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Phylogeny and phylogeography of *Mantophryne* (Anura: Microhylidae) reveals cryptic diversity in New Guinea

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ABSTRACT

New Guinea is one of five high biodiversity wilderness areas, and frog diversity is exceptionally large, with more than 400 species described to date. The microhylid frog genus *Mantophryne* is endemic to New Guinea and consists of four species, three of which have narrow geographic distributions and a fourth, *M. lateralis*, with a broad range that spans the eastern half of the island. Here, we sequence 104 *Mantophryne* samples for three mitochondrial and three nuclear loci to reconstruct the first phylogeny of the genus and to examine spatial patterns of diversity within *M. lateralis*. Results indicate that the wide-ranging *M. lateralis* is composed of at least nine geographically separated and well-supported lineages that represent putative species. Biogeographic analysis suggests that *Mantophryne* evolved on the eastern Papuan peninsula with subsequent dispersal westward, as well as overwater dispersal events to the Louisiade and D'Entrecasteaux archipelagos.

1. Introduction

New Guinea is the world's largest and highest tropical island and is one of the most biodiverse regions on the planet (Beehler, 1993; Dinerstein and Wikramanayake, 1993; Myers et al., 2000). The complex geological history of New Guinea has played a major role in the high level of biodiversity, especially in reptiles and amphibians (Heads, 2002). Relatively recent tectonic activity, including island accretion, montane uplift, and volcanism, have created a complex landscape with extreme habitat heterogeneity and topographic relief along a steep elevational gradient from sea-level to over 5000 m. Accretion of the Inner and Outer Melanesian Island Arcs onto the Australian plate formed the central mountain range approximately 5–10 million years ago (Pigram and Davies, 1987; Abbott et al., 1994; Allison, 1996; Hall, 1997; Tregoning et al., 1999; Heads, 2002).

The frog diversity on New Guinea represents approximately 8% of global diversity, despite accounting for only about 0.6% of global land area. However, frog biodiversity is extremely underestimated; species accumulation curves demonstrate that the true number on New Guinea is likely double the current 410 described species (Allison, 1996; Austin et al., 2008; Allison et al., 2010). If this prediction is correct, it would mean that New Guinea would represent an astonishing biogeographic focal center of frog diversity. The family Microhylidae is the most diverse group of frogs in New Guinea with over 218 described species representing 53% of New Guinean amphibian diversity, almost all species being endemic (Allison et al., 2010).

Based predominantly on call structure and subtle differences in morphology, Günther recently described six new species from the previously monotypic microhylid *Hylophorbus*, showing that the widespread "*H. rufescens*" is actually composed of multiple geographically separated species (Günther, 2001). However, only a handful of populations in the westernmost portion of the range were examined and *Hylophorbus* also extends into the eastern part of New Guinea. Another microhylid frog with a similarly broad range is *Mantophryne lateralis*, which occurs throughout the lowlands of eastern New Guinea (Boulenger, 1897; Zweifel, 1972; Burton, 1986; Menzies, 2006). This broad range is concordant with *Hylophorbus* and with many other amphibian species and makes *M. lateralis* a good candidate for examining patterns of genetic diversity in eastern New Guinea. *Mantophryne* currently includes only three other species, all with restricted geographic ranges (Fig. 1): *M. axanthogaster* (Sudest Island) (Kraus and Allison, 2009); *M. infulata* (Arau and the Huon Peninsula) (Zweifel, 1972; Burton, 1986); and *M. louisiadensis* (Rossel Island) (Parker, 1934; Zweifel, 1972; Burton, 1986).

Here we use data from multiple loci to reconstruct the phylogeny of the genus *Mantophryne* in order to examine spatial and topological patterns of diversity. In addition, we conduct a biogeographic analysis using ancestral reconstructions to decipher areas

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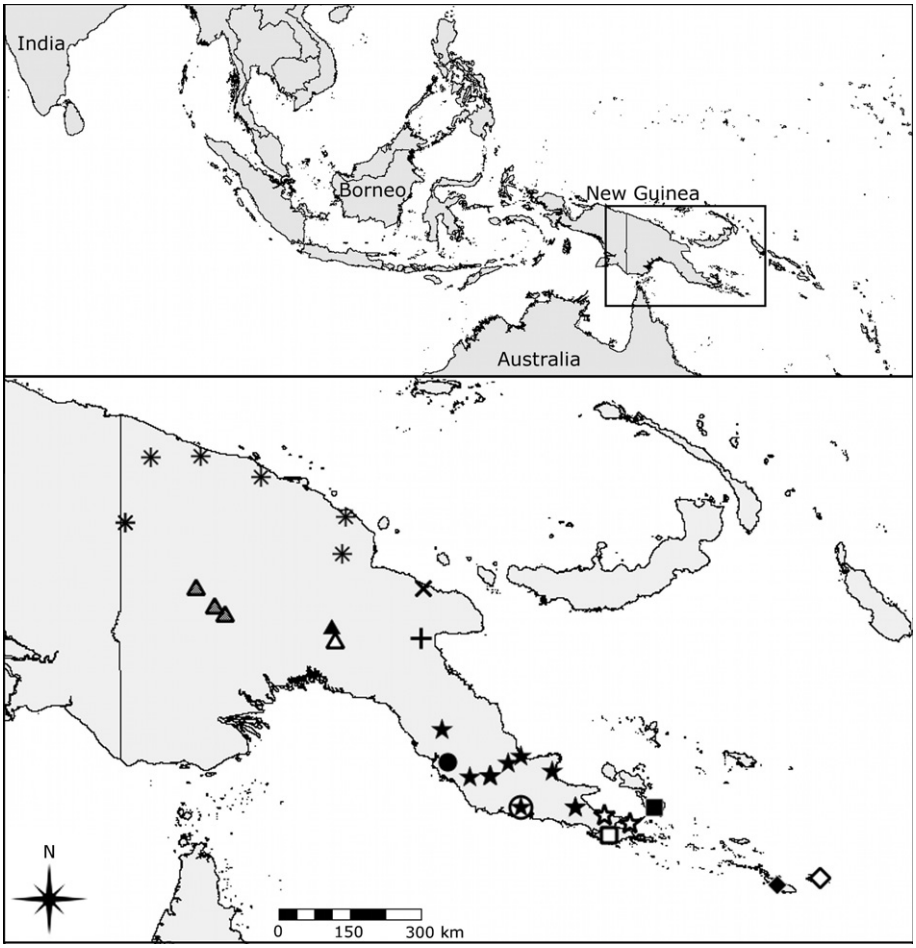

Fig. 1. Distribution map of all putative *Mantophryne* putative species. Symbols correspond to those in Fig. 3. Two species occur in Amau, indicated by an open circle and a closed star.

of origin and patterns of dispersal. Our analyses, while confined to a single genus, may provide a template for examining cryptic diversity in other frogs in New Guinea.

2. Materials and methods

2.1. Sampling

A total of 104 ingroup samples were examined, representing all four described species of *Mantophryne*: 94 samples spanning the distribution of *M. lateralis*, four *M. louisiadensis*, five *M. axanthogaster*, and one *M. infulata* (Fig. 1). To test the monophyly of *Mantophryne*, we also included 24 samples representing four described and multiple undescribed species of *Hylophorbus*, one sample of the monotypic genus *Pherohapsis* (*P. menziesi*) and eight samples of a putative new species of *Mantophryne* from Amau Village, Central Province, Papua New Guinea. Based on higher-level asterophryine phylogenetics (Köhler and Günther, 2008), we also included outgroup samples of the genera *Austrochaperina, Callulops*, *Choerophryne*, and *Sphenophryne*. Voucher numbers and collection localities are provided in Supplementary Appendix 1.

2.2. DNA isolation, amplification, and sequencing

Whole genomic DNA was extracted from liver tissue using the Qiagen DNeasy Blood and Tissue Kit (Valencia, California, USA). Three mitochondrial gene regions (*12S*, *16S*, and *cytochrome b*) and three nuclear loci (*Tyrosinase, c-myc* exon 2, and *c-myc* exon 3) were selected based on their utility in previous studies that included microhylid frogs and sequenced using previously published primers (Table 1) (Richards and Moore, 1996; Bossuyt and Milinkovitch, 2000; Wiens et al., 2005; Köhler and Günther, 2008). Target gene regions were amplified and as in Austin et al. (2010a,b) and purified as in Austin et al. (2011). Purified amplicons were then cycle sequenced in both directions with BigDye v. 3.1 Terminator Sequencing Kit (Applied Biosystems, Foster City, CA, USA) using previously published protocols and sequenced on an ABI 3100 automated capillary sequencer (Austin et al., 2010a,b).

Complementary strands were assembled and visually edited in Sequencher v4.7 (Gene Codes Corp., Ann Arbor, MI, USA) and aligned in ClustalX2 (Larkin et al., 2007). The number of variable sites for each locus was computed using Mega 5.0 (Tamura et al., 2011). All sequences were deposited in GenBank; accession numbers are available in the Supplementary Appendix 1.

2.3. Phylogenetic analysis

To estimate phylogenetic relationships, Bayesian inference, maximum likelihood (ML), and maximum parsimony (MP) were implemented for individual loci as well as for a concatenated dataset of all six loci. Maximum parsimony analyses were conducted in PAUP* ver.4.0b10 (Swofford, 2003) using PAUPrat (Sikes and Lewis, 2001) to implement the parsimony ratchet (Nixon, 1999). Fifty replicates of 5000 ratchet iterations and 20% character perturbation

Table 1
List of loci, PCR primers, and annealing temperatures.

Locus	Primer	Primer sequence 5′–3′	Temp. (°C)	References
12S	*L2519*	AAACTGGGATTAGATACCCCACTAT	55	Richards and Moore, 1996
	H3296	GCTAGACCATKATGCAAAAGGTA		Richards and Moore, 1996
16S	*16S-L*	TCGAACTTAGAGATAGCTGGTT	55	
	16S-H	GCGAATGTTTTTGGTAAACA		
Cytb	*CytbA*	CCATGAGGACAAATATCATTYTGRGG	46	Bossuyt and Milinkovitch, 2000
	CytbB	CTTCTACTGGTTGTCCTCCGATTCA		Bossuyt and Milinkovitch, 2000
Tyrosinase	*Tyr1A*	AGGTCCTCTTRAGCAAGGAATG	55	Bossuyt and Milinkovitch, 2000
	Tyr1F	TCATCTCCCGYCAYCTTCTGGAT		Bossuyt and Milinkovitch, 2000
C-myc exon 2	*cmyc2F*	ACVGARTTCCTGGGAGGGGACATGG	55	Wiens et al., 2005
	cmyc-ex2d R	TCATTCAATGGGTAAGGGAAGACC		Wiens et al., 2005
C-myc exon 3	*c-myc-ex3F*	CCCACCAGTCCAGACCTCACCACAG	48	Wiens et al., 2005
	c-myc-ex3R	GTTCTCTTTTGAGTTTTAACTGTTC		Wiens et al., 2005

were conducted using tree bisection–reconstruction (TBR) branch swapping to find the most parsimonious trees using the CIPRES portal (Warnow, 2008). Branch support for ML and MP was assessed with 1000 bootstrap (BS) pseudoreplicates. For Bayesian and ML analyses, a variety of partitioning strategies were tested, including partitioning by locus and by codon position (for the protein-coding cytochrome b gene). The best-fit model of sequence evolution for each partition was selected in jModeltest v0.1.1 (Posada, 2008) using the corrected Akaike Information Criterion (AICc; Table 2). ML analyses were implemented in Garli v2.0 (Zwickl, 2006) using default parameters and five search replicates per partitioning strategy. The best-fit partitioning strategy was then selected using the AIC, and 50 total search replicates were conducted on the best-fit partitioning strategy. Bayesian analyses were implemented in MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). As in ML analyses, a variety of partitioning strategies were tested and the best-fit strategy was selected by the AIC. For each partitioning strategy, Bayesian analyses consisted of two independent runs, each with four chains, run for 25 million generations sampling every 1000 generations. Substitution model parameters were unlinked among partitions, and rate priors were set to "variable" to allow differing substitution rates among partitions. The branch-length prior was set to an unconstrained exponential distribution with a mean of 50; all other priors were left at default settings. Convergence was assessed by examining the potential scale-reduction factors in MrBayes, as well as by examining the traces of all parameters and the effective sample sizes in Tracer v1.5 (Rambaut and Drummond, 2007) and by comparing the Bayesian posterior probabilities (BPPs) of all splits in Are We There Yet (AWTY) (Nylander et al., 2008).

Because heterogeneity among gene genealogies may be substantial (Degnan and Rosenberg, 2006, 2009; Edwards et al., 2007), in addition to concatenated phylogenies, we also estimated the underlying species tree using *BEAST (Heled and Drummond, 2010), which accounts for heterogeneity among gene trees due to incomplete lineage sorting. Species tree analyses, such as *BEAST, require samples to be assigned *a priori* to species that accurately represent independently evolving evolutionary lineages. In this case, we suspected that *M. lateralis* represents a cryptic complex of multiple species. We generated four species trees assuming that well-supported (>0.95 BPP, >75 MLBS, >75 MPBS) clades in the concatenated phylogenetic analyses with 3%, 4%, 5%, and 6% mitochondrial divergence represented distinct lineages. Analyses were conducted using an uncorrelated lognormal molecular clock for 250 million generations, sampling every 1000 generations. The first 50,000 samples were discarded as burn-in. Mitochondrial loci were partitioned as in the concatenated analysis, but were constrained to a single gene genealogy due because recombination within the mitochondrial genome is unexpected. Each nuclear locus was allowed to evolve independently under its own best-fit model. Convergence and stationarity were assessed by examining traces and effective sample sizes of all parameters in Tracer 1.5.

2.4. Biogeographic reconstruction

To examine the biogeographic history of *Mantophryne,* we used maximum likelihood ancestral state reconstruction (MLASR) (Schluter et al., 1997), Statistical Dispersal–Vicariance Analysis (S-DIVA) (Yu et al., 2010), and Dispersal–Extinction Cladogenesis (DEC) (Ree et al., 2005) analyses. These methods require discrete regions of occurrence, which we defined by geologic history (Fig. 2): (0) North Coast (including accreted portions of Outer Melanesian Island Arc), (1) Papuan Peninsula (East Papuan Composite Terrane), (2) D'Entrecasteaux Islands, (3) Louisiade Islands (an extension of the Owen Stanley Terrane on the Papuan Peninsula),

Table 2
List of the length, number of variable sites, and partitioning scheme for each locus.

Partition	Length	All samples No. variable sites (No. parsimony informative sites)	Ingroup samples No. variable sites (No. parsimony informative sites)	Best-fit model
12S	687	347 (301)	218 (188)	TIM3 + G
16S	445	298 (271)	212 (184)	HKY + G
Cyt b pos. 1	163	65 (60)	36 (32)	TrN + G
Cyt b pos. 2	163	28 (24)	12 (11)	TrN + G
Cyt b pos. 3	163	160 (159)	139 (132)	TIM1 + G
Tyrosinase	503	155 (95)	76 (45)	HKY + G
c-myc exon 2	330	52 (28)	20 (12)	
c-myc exon 3	356	70 (43)	32 (16)	

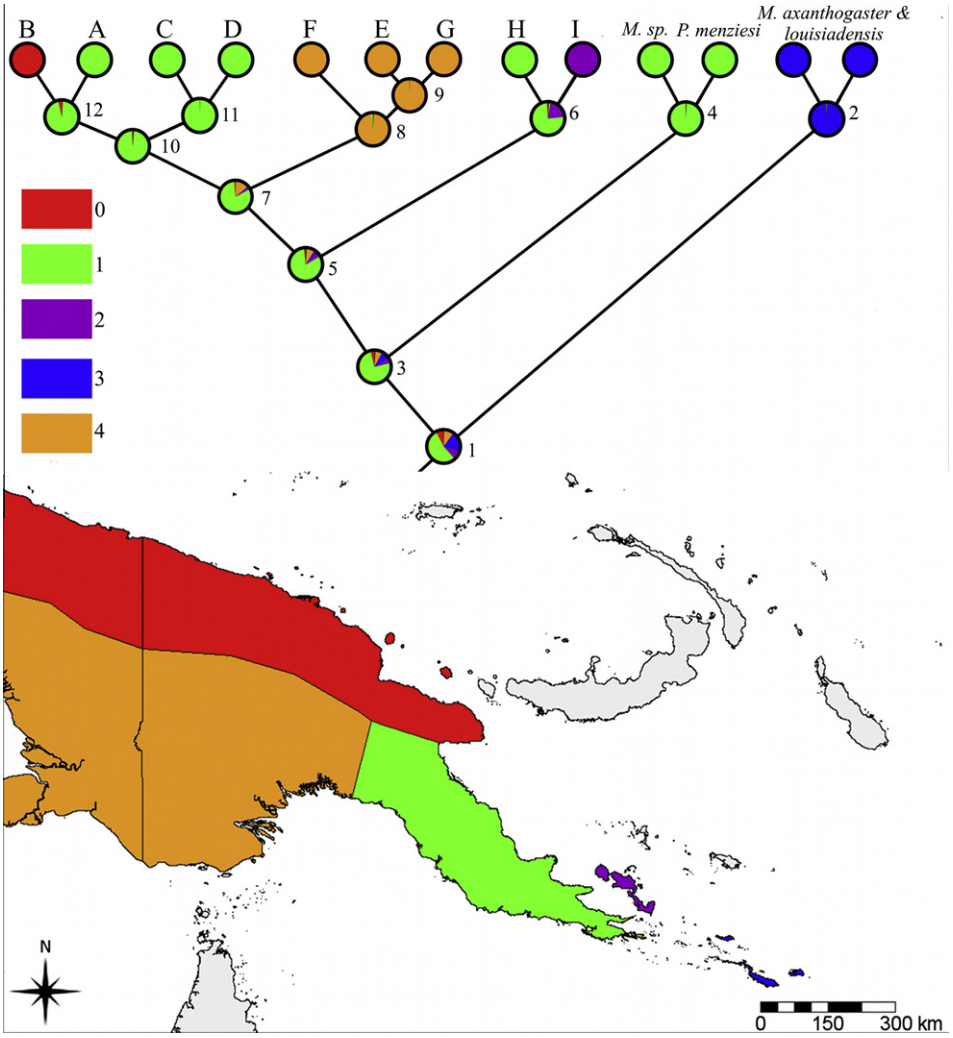

Fig. 2. Biogeographic reconstruction of *Mantophryne*. Biogeographic regions: 0-North Coast (includes accreted portions of Outer Melanesia Island Arc), 1-Papuan Peninsula (East Papuan Composite Terrane), 2-D'Entrecasteaux Islands, 3-Louisiade Islands, and 4-Southern and Western New Guinea (Australian Plate). Support values for each node are listed in Table 3.

and (4) Southern and Western New Guinea (Australian Plate). MLASR was conducted in Mesquite v. 2.74 (Maddison and Maddison, 2010) using the maximum clade credibility species tree estimated in *BEAST. However, this method assumes that each species (extant and ancestral) occurred in a single region, whereas, in reality, it is possible that some ancestral species spanned multiple regions. Thus, we also investigated biogeographic history using S-DIVA and DEC analyses. Both S-DIVA and DEC accomodate more realistic, though more complex, models by allowing distributions to span multiple regions and by limiting dispersal between distant regions. S-DIVA was implemented in RASP ver.1.107 (Yu et al., 2010, 2011) using a posterior distribution of 20,000 species trees from *BEAST analysis (thinned to one per 10,000 iterations) to integrate reconstructions over phylogenetic uncertainty. DEC was conducted using lagrange ver.2.0.1 (Ree and Smith, 2008) in Python ver.2.72, using the maximum clade credibility species tree estimated in *BEAST. In both S-DIVA and DEC analyses, we constrained ancestral distributions to adjacent areas, i.e. 0–1, 1–2, 1–3, 1–2–3, or 1–4. We did not allow a 0–4 distribution because unsuitable montane habitat occurs between regions 0 and 4, making dispersal directly between these regions highly unlikely. Similarly, we also constrained dispersal between island systems and either North Coast or southern/western populations, since these dispersals are unlikely without traversing the mainland Papuan peninsula.

3. Results

3.1. Phylogenetic analyses

The final aligned length of the combined mitochondrial and nuclear dataset was 1621 bp. The length of each partition, number of variable and parsimony informative sites, and models for nucleotide substitution are provided in Table 2. Putative species groupings generated using 3% and 4% divergence were identical, and all analyses yielded identical topologies with similar support; thus, we present the results from the 4% divergence analysis. The phylogenetic reconstruction (Fig. 3) suggests that *M. lateralis* is monophyletic and split into nine clades: (A) Mt. Shungol, Morobe Province, (B) North Coast New Guinea – Huon Peninsula (Outer Melanesian Arc), (C) Northern Milne Bay Province, (D) Central and Oro Province, (E) Southwestern New Guinea and Southern Highlands Province, (F) Haia, Gulf Province (G) Sobo and Haia, Gulf Province, (H) Southern Milne Bay Province, (I) the D'Entrecasteaux islands. Each *M. lateralis* clade is strongly supported with 100 MPBS, >89 MLBS and 1.0 BPP, and is geographically isolated, with one exception: samples from Haia, Gulf Province were recovered in both clades F and G. *Pherohapsis* and a putative new species of *Mantophryne* from Amau Village, Papua New Guinea (*M.* Amau sp.) are sister taxa and were recovered as embedded within

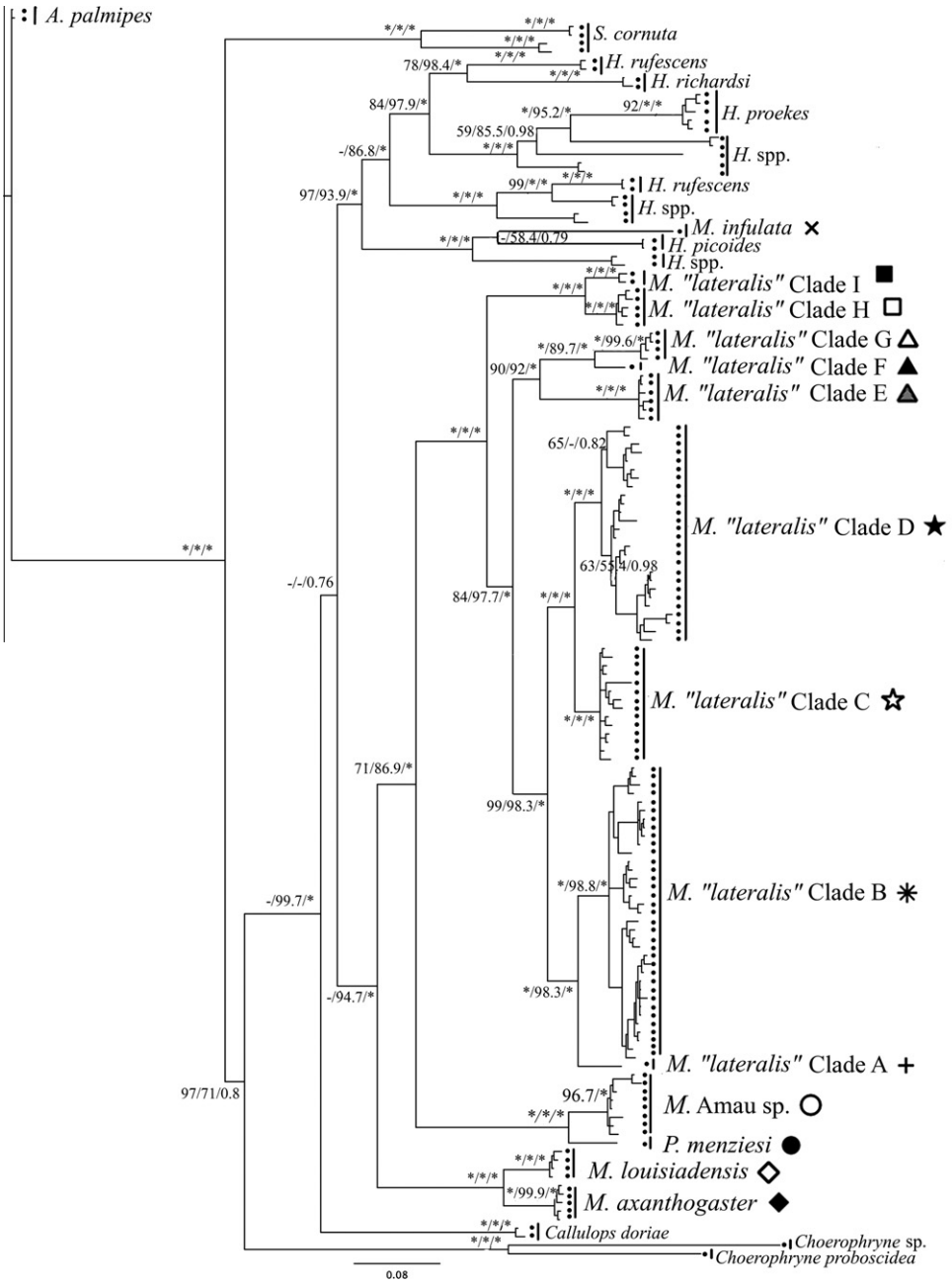

Fig. 3. Concatenated phylogeny. Branch support values are listed as maximum parsimony bootstrap/maximum likelihood bootstrap/Bayesian posterior probability (BPP). 100 bootstrap or 1.0 BPP are indicated by an ∗. Bootstraps less than 50, and BPP less than 0.75, are not listed. Symbols correspond to those in Fig. 1.

Mantophryne, the sister clade to the *M. lateralis* complex. *Mantophryne louisiadensis* + *M. axanthogaster* were recovered as sister taxa and forming the sister group to *M. lateralis* + *Pherohapsis*. Furthermore, *M. infulata* was recovered within the sister genus *Hylophorbus*, rather than within *Mantophryne*.

The species tree estimates (Fig. 4) largely corroborate the concatenated gene tree. The only exception is the relationships among *M. lateralis* clades E, F, and G. In the concatenated gene tree, E is the sister taxon to F + G. However, in the species tree, F is the sister-taxon to E + G. This E + F + G clade is strongly supported in both the species tree (BPP = 0.99) and the concatenated gene tree (MPBS = 90, MLBS = 92, BPP = 1.0). Although the sister taxon relationship of clades F + G is strongly supported in the concatenated phylogeny (MPBS = 100, MLBS = 89.7, BPP = 1.0), the sister relationship of clades E + G is only weakly supported in the species tree (BPP = 0.81). Lower-level relationships are often discordant between these two methods due to heterogeneity among gene trees (Degnan and Rosenberg, 2006, 2009; Edwards et al., 2007).

3.2. Biogeography

MLASR, S-DIVA, and DEC produced similar results (Table 3). MLSAR suggests that *Mantophryne* originated in the Papuan Peninsula ($p = 0.54$) over the Louisiade Islands ($p = 0.19$), while S-DIVA reconstructs a root distribution spanning the Papuan Peninsula and the Louisiade Islands ($p = 0.999$). DEC ($p = 0.73$) estimates the initial split within *Mantophryne* occurring between lineages in the Louisiade Islands and in the Papuan Peninsula ($p = 0.739$).

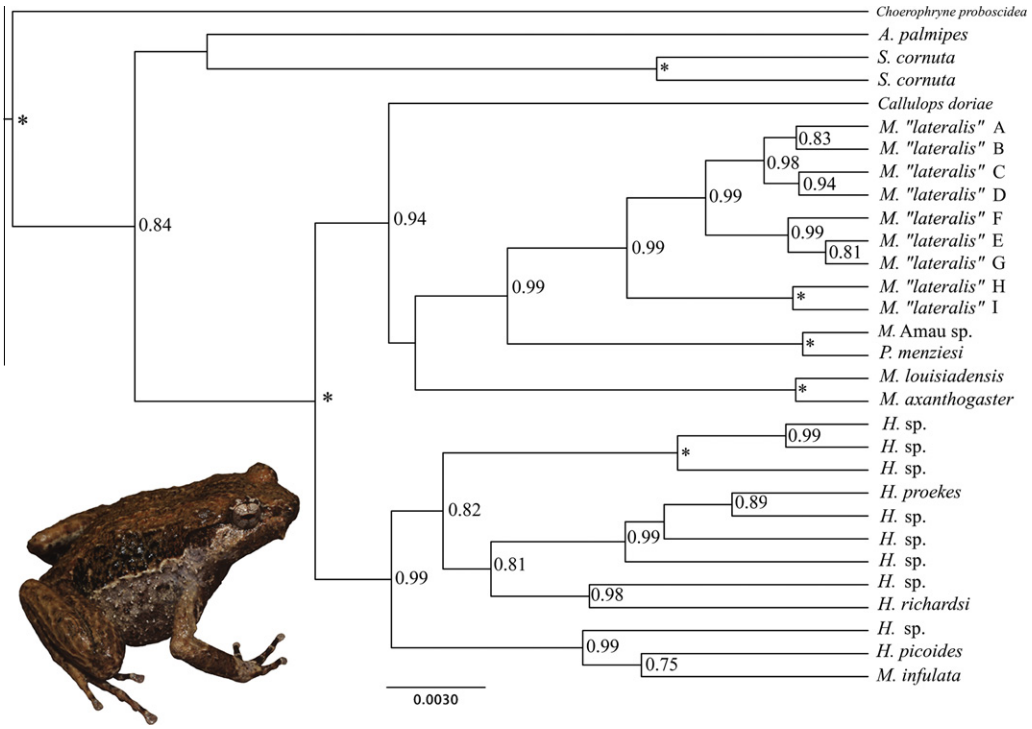

Fig. 4. Species tree from *BEAST. Node supports are listed as Bayesian posterior probabilities. Support values <0.75 are not listed and values of 1.0 are listed as *.

Table 3
Biogeographic reconstruction probabilities. 0-Northern New Guinea (accreted portions of the Outer Melanesian Island Arc), 1-Papuan Peninsula (East Papuan Composite Terrane), 2-D'Entrecasteaux Islands, 3-Louisiade Islands and 4-Southern and Western New Guinea (Australian Plate).

Node	Reconstructed distribution	MLASR support	Reconstructed distribution	S-DIVA support	Reconstructed distribution	DEC support
1	[1]	0.542	[13]	0.999	[3|1]	0.739
	[3]	0.198			[3|12]	0.131
	[4]	0.097				
	[2]	0.087				
	[0]	0.075				
2	[3]	0.985	[3]	1.000	[3|3]	0.992
3	[1]	0.752	[1]	0.996	[1|1]	0.792
	[3]	0.079			[12|1]	0.143
	[4]	0.072				
	[2]	0.058				
4	[1]	0.994	[1]	1.000	[1|1]	0.996
5	[1]	0.817	[1]	0.998	[1|1]	0.559
	[4]	0.083			[1|12]	0.217
	[2]	0.055			[14|1]	0.192
6	[1]	0.758	[12]	1.000	[1|2]	0.968
	[2]	0.196				
7	[1]	0.817	[14]	1.000	[4|1]	0.942
	[4]	0.137				
8	[4]	0.972	[4]	1.000	[4|4]	0.984
9	[4]	0.999	[4]	1.000	[4|4]	0.998
10	[1]	0.977	[01]	0.518	[1|1]	0.757
			[1]	0.482	[01|1]	0.238
11	[1]	0.997	[1]	1.000	[1|1]	0.999
12	[1]	0.953	[01]	1.000	[1|0]	0.941

MLASR (p = 0.75, 0.81), S-DIVA (p = 0.99, 0.99), and DEC (p = 0.79, 0.55) all suggest that mainland *Mantophryne* and the *M. lateralis* complex originated in the Papuan peninsula. Furthermore, all analyses suggest that the North Coast and South Coast populations each originated from single dispersal events out of the Papuan Peninsula region.

4. Discussion

This is the first phylogeographic study of any New Guinean frog. Our molecular data for the widespread *M. lateralis* suggest that there may be nine or more independently evolving lineages across its range in eastern New Guinea. These data, combined with the

recent work by Günther and others on the genus *Hylophorbus* in central and western New Guinea, suggest that some, and possibly many, of the currently widespread frog species in New Guinea may instead represent cryptic complexes of multiple species (Günther, 2001; Richards and Oliver, 2007; Kraus and Allison, 2009). While Günther (2001) primarily focused his species analyses on *Hylophorbus* at the westernmost tip of New Guinea, our phylogenetic analyses included several populations of *Hylophorbus* from the central and eastern part of the island. These samples were deeply divergent, and may represent independent evolutionary lineages. *Hylophorbus* exhibits similar ecology to *Mantophryne* and could, therefore, display similar topological and spatial patterns. In addition, we included two disparate populations of the widespread *Sphenophryne cornuta*. These populations, sampled from north and south of the central cordillera, similarly show a large degree of sequence divergence (14.8% mtDNA), and may represent distinct species.

Recent work on New Guinean reptiles has also revealed cryptic diversity in wide-ranging species, including two skinks (*Sphenomorphus jobiensis* and *Carlia fusca*) and three snakes (two *Aspidomorphus* species and *Morelia viridis*) (Donnellan and Aplin, 1989; Rawlings and Donnellan, 2003; Zug, 2004; Austin et al., 2011). Preliminary results from work done by others and us involving additional lizard "species" show similar patterns (unpublished data). These data, in concert with our findings, suggest that cryptic species might be more common in New Guinea than previously thought. Current understanding has been based in large part on studies of museum specimens, where details of color and pattern are often difficult to discern. Research involving living animals clearly supports the fact that many New Guinean taxa currently treated as single species represent complexes of multiple species (F. Kraus, unpubl. data).

Biogeographic reconstructions suggest that *Mantophryne* most likely evolved in the Papuan peninsular region and subsequently dispersed outward. This would require at least two oceanic dispersal events to the Louisiade and D'Etrecasteaux archipelagos, as these islands have long remained isolated from mainland New Guinea (Luyendyk et al., 1973; Davies and Warren, 1988; Hill et al., 1992; Abers et al., 1997; Little et al., 2007). While the three biogeographic analyses may initially appear incongruent, this reflects methodological differences, rather than truly conflicting signal. MLASR suggests that the peninsular origin is the most likely but also indicates a possible Louisiade islands origin, while S-DIVA reconstructs the basal node of *Mantophryne* as occurring in both the Louisiades and the Papuan peninsula. The results from DEC indicate that at the time of the basal split, *Mantophryne* occured on both the Papuan peninsula and the Louisiade islands. Interestingly, the genus *Hylophorbus* also occurs in both the Louisiade and D'Entrecasteaux archipelagos off the eastern tip of New Guinea. This is consistent with other frog genera, such as *Litoria, Hylarana, Copiula,* and *Callulops*, suggesting that the concordant distributions of these two genera may mirror common historical and ecological processes.

Our results based on an improved understanding of phylogenetic relationships highlight the taxonomic confusion associated with the closely related genera *Mantophryne*, *Hylophorbus* and *Pherohapsis*. The monotypic *Pherohapsis* is embedded within *Mantophryne* and thus should be synonymized, changing *P. menziesi* to *M. menziesi*. Our results also show that *M. infulata* is nested within *Hylophorbus* and should be included in that genus as *H. infulatus.* We are currently analyzing call and morphological data to assess whether nine clades of the *lateralis* complex, as well as the putative new species of *Mantophryne* from Amau, are conspecific lineages or distinct species in an effort towards a comprehensive species delimitation analysis for *Mantophryne*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.02.023.

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