

# Mitochondrial genes reveal cryptic diversity in plant-breeding frogs from Madagascar (Anura, Mantellidae, *Guibemantis*)

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## Abstract

One group of mantellid frogs from Madagascar (subgenus *Pandanusicola* of *Guibemantis*) includes species that complete larval development in the water-filled leaf axils of rainforest plants. This group consists of six described species: *G. albolineatus*, *G. bicalcaratus*, *G. flavobrunneus*, *G. liber*, *G. pulcher*, and *G. punctatus*. We sequenced the 12S and 16S mitochondrial rRNA genes (~1.8 kb) from multiple specimens (35 total) of all six species to assess phylogenetic relationships within this group. All reconstructions strongly supported *G. liber* as part of the *Pandanusicola* clade, even though this species does not breed in plant leaf axils. This result confirms a striking reversal of reproductive specialization. However, all analyses also indicated that specimens assigned to *G. liber* include genetically distinct allopatric forms that do not form a monophyletic group. Most other taxa that were adequately sampled (*G. bicalcaratus*, *G. flavobrunneus*, and *G. pulcher*) likewise consist of several genetically distinct lineages that do not form monophyletic groups. These results suggest that many of the recognized species in this group are complexes of cryptic species.

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## 1. Introduction

Madagascar contains a disproportionately large portion of Earth's biological diversity and nearly all Malagasy frogs (all except one introduced species) are endemic to the island. Considering described species only, the amphibian fauna of Madagascar now includes over 200 frog species in five families (nearly 4% of the world's total; Frost et al., 2006; Glaw and Vences, 2003). Since the early 1990s, herpetologists have uncovered dozens of new frog taxa from Madagascar and this rapid rate of description shows no sign of slowing. Many of these new species are

rather striking in appearance and are clearly different from other known forms. Others are minimally morphologically differentiated from one another and have been detected only by careful combination of morphological comparisons, bio-acoustic analysis, and molecular genetic tools.

Many of these frogs have now been subject to molecular phylogenetic analysis (e.g., Andreone et al., 2005; Glaw and Vences, 2006; Vences and Glaw, 2001; Vences et al., 2003). Based on the phylogenies obtained, it became clear that various genera as previously understood were non-monophyletic (Richards et al., 2000; Vences et al., 2003). Consequently, the classification of various groups has been recently revised, especially in the endemic Malagasy family Mantellidae (e.g., Glaw and Vences, 2006). Because the Malagasy poison frogs of the genus *Mantella* were deeply nested within the genus *Mantidactylus* as formerly recog-

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nized (e.g., Richards et al., 2000), the latter has now been partitioned into the eight genera *Blommersia*, *Boehmantis*, *Gephyromantis*, *Guibemantis*, *Mantidactylus*, *Spinomantis*, *Wakea*, and the recently discovered *Tsingymantis*. Within the Mantellidae, however, one problematic group has received little attention thus far: the plant-breeding frogs currently classified in the subgenus *Pandanusicola* in the genus *Guibemantis* (Mantellidae; Glaw and Vences, 2006). Most of these species lay their eggs on the leaves of *Pandanus* plants (or less commonly other phytotelmata), and the tadpoles develop in the tiny water bodies in the leaf axils of these plants (Lehtinen, 2002). Parental care in the form of amphisexual egg attendance is confirmed for two species in this group (Lehtinen, 2003), and the tadpoles are specialized in a number of ways for life in a small, desiccation-prone, and food-poor environment (Lehtinen, 2004).

There are six species currently assigned to the subgenus *Pandanusicola*. These include *G. albolineatus* (Blommers-Schlösser and Blanc, 1991), *G. bicalcaratus* (Boettger, 1913), *G. flavobrunneus* (Blommers-Schlösser, 1979), *G. liber* (Peracca, 1893), *G. pulcher* (Boulenger, 1882), and *G. punctatus* (Blommers-Schlösser, 1979). Many of these species (as currently understood) show substantial variation in coloration patterns; this is particularly notable in *G. liber*. *Guibemantis liber* was tentatively placed with these species in the phenetic *pulcher* group by Blommers-Schlösser (1979) and Blommers-Schlösser and Blanc (1991), but was placed by later authors (e.g., Andreone, 2003; Glaw and Vences, 1994) into the subgenus *Guibemantis* (the *depressiceps* group of Blommers-Schlösser and Blanc, 1991). Members of the subgenus *Guibemantis* (currently composed of four species), as far as is known, lay their eggs on vegetation overhanging ponds, lack parental care and have unspecialized, typical pond-type tadpoles.

Previous analysis of mitochondrial genes indicated that *Pandanusicola* and *Guibemantis* are sister clades (Richards et al., 2000). Consequently, both subgenera were included in one genus (*Guibemantis*) in a recent comprehensive classification of the mantellids (Glaw and Vences, 2006). However, the analysis by Richards et al. (2000) included only a single member of *Pandanusicola* and *Guibemantis* (*G. flavobrunneus* and *G. kathrinae*, respectively). Lehtinen et al. (2004) extended this analysis by including all known members of *Pandanusicola* (except *G. punctatus*, for which tissues were unavailable) and two additional species of *Guibemantis* (*G. liber* and *G. tornieri*). These results suggested that *G. liber* may be properly placed in *Pandanusicola*, rather than *Guibemantis*. Based on their tree, Lehtinen et al. (2004) also suggested that plant-breeding evolved only once in this group, but there may have been a reversal back to the ancestral reproductive mode (pond-breeding) in *G. liber*. Even with this work, however, only a single specimen from each putative species was used, leaving open the question of whether cryptic diversity within the recognized species was obscuring the real evolutionary history of this group.

To address this problem, we use mitochondrial sequence data from these previous studies and add sequences for 30 additional specimens attributed to this problematic group (including *G. punctatus*, which was not included earlier). By using a more comprehensive sampling of the available taxa, we herein reconstruct the evolutionary history of this group.

## 2. Materials and methods

### 2.1. DNA sequences/laboratory methods

A subset of the 12S and 16S mitochondrial rRNA sequences used in this study was taken from Richards et al. (2000) and Lehtinen et al. (2004). New sequences were added to these using standard laboratory protocols. DNA was extracted from frozen tissue samples using Qiagen DNeasy<sup>®</sup> tissue kits. The 12S and 16S rRNA genes were amplified via the polymerase chain reaction with the primers reported in Richards et al. (2000). PCR products were purified using Qiagen MinElute<sup>™</sup> PCR column purification kits. Purified PCR products were sequenced using the amplification primers on an automated sequencer (ABI 3100) at the MCIC at the Ohio Agricultural Research and Development Center. A subset of the sequencing was done using similar protocols in the Cannatella Lab at the University of Texas.

Sequence chromatograms were directly imported into Codon Code Aligner 1.5.1 where bases called using Phred software and contigs were assembled. Multiple sequence alignment was performed using ClustalX 1.81 (Thompson et al., 1997). Alternative alignments were explored by systematically varying gap opening and gap extension penalties in varying ratios. Alternative alignments were compared visually for positional homology. Hypervariable regions of the sequences (corresponding to secondary structure) were excluded from analysis. The best alignment was found by setting the gap creation/extension penalties to 10/5. Novel sequences were submitted to GenBank (Table 1).

### 2.2. Phylogenetic analysis

To estimate of the phylogenetic relationships of the species in the subgenus *Pandanusicola*, we used maximum parsimony, maximum likelihood and Bayesian analyses. Partition homogeneity was examined with the ILD test in PAUP\* version 4b10 (Swofford, 1998), using a heuristic search strategy and 1000 replicates.

The maximum parsimony (MP) and maximum likelihood (ML) analyses were implemented in PAUP\* 4.0b10 using heuristic searches and 100 random addition sequence replicates. The tree-bisection-reconnection algorithm was used for branch swapping with the steepest descent option off. In the MP analyses, clade support was assessed using 2000 bootstrap replicates and characters were unordered and unweighted. In the ML analysis, we used the

Table 1

Collection localities of specimens used in this study and GenBank accession numbers of sequenced gene fragments (12S and 16S, respectively)

Species	Subgenus	Locality	Voucher	GenBank Accession Numbers
<i>Guibemantis</i> cf. <i>albolineatus</i>	<i>Pandanusicola</i>	Andasibe [A]	ZSM 250/2002	AY454354, AY454376
<i>Guibemantis</i> cf. <i>bicalcaratus</i>	<i>Pandanusicola</i>	Marojejy [B]	ZCMV 2044	EF468005, EF472505
<i>Guibemantis bicalcaratus</i>	<i>Pandanusicola</i>	Manongarivo Reserve [C]	UMMZ 212592	EF468006, EF472506
<i>Guibemantis bicalcaratus</i>	<i>Pandanusicola</i>	Besariaka [D]	ZCMV 932	EF468023, EF472522
<i>Guibemantis bicalcaratus</i>	<i>Pandanusicola</i>	Marojejy Reserve, Manantenina River [B]	UMMZ 212597	EF468007, EF472507
<i>Guibemantis bicalcaratus</i>	<i>Pandanusicola</i>	Sainte Luce [E]	FGZC 2588	EF468008, EF472508
<i>Guibemantis</i> (?) <i>bicalcaratus</i>	<i>Pandanusicola?</i>	Maharira [F]	ZMA 20444	EF468024, EF472523
<i>Guibemantis</i> (?) <i>bicalcaratus</i>	<i>Pandanusicola?</i>	Vohiparara [F]	ZSM 745/2003	EF468025, EF472524
<i>Guibemantis bicalcaratus</i>	<i>Pandanusicola</i>	Sainte Luce [E]	UMMZ 197485	AY454356, AY454379
<i>Guibemantis</i> cf. <i>depressiceps</i>	<i>Guibemantis</i>	Andohahela	FGZC 2434	EF468009, EF488668
<i>Guibemantis flavobrunneus</i>	<i>Pandanusicola</i>	Manombo [G]	ZMA 20119	EF468010, EF472509
<i>Guibemantis flavobrunneus</i>	<i>Pandanusicola</i>	Mangoro River area, precise locality unknown	FGZC 2655	EF468011, EF472510
<i>Guibemantis flavobrunneus</i>	<i>Pandanusicola</i>	Ambohitantely [H]	FMNH 259923	AY454367, AY454392
<i>Guibemantis flavobrunneus</i>	<i>Pandanusicola</i>	Andringitra, Iatara River [I]	UMMZ 212929	EF468012, EF472511
<i>Guibemantis flavobrunneus</i>	<i>Pandanusicola</i>	Cap Est [J]	UMMZ 212922	EF468013, EF472512
<i>Guibemantis flavobrunneus</i>	<i>Pandanusicola</i>	Ankaratra Mountains [O]	UMMZ 212919	EF468014, EF472513
<i>Guibemantis kathrinae</i>	<i>Guibemantis</i>	Manantantely forest	UMMZ 198114	AF261242, AF261260
<i>Guibemantis liber</i>	<i>Pandanusicola</i>	Montagne d'Ambre [L]	ZMA 19644	EF468029, EF472528
<i>Guibemantis liber</i>	<i>Pandanusicola</i>	Veembe [K]	ZMA 20117	EF468028, EF472527
<i>Guibemantis liber</i>	<i>Pandanusicola</i>	Montagne d'Ambre [L]	UMMZ 213064	EF468015, EF472514
<i>Guibemantis liber</i>	<i>Pandanusicola</i>	Manongarivo Reserve, Ambalafary [C]	UMMZ 213068	EF468016, EF472515
<i>Guibemantis liber</i>	<i>Pandanusicola</i>	Tsaratanana, Befosa [M]	UMMZ 213101	EF468017, EF472516
<i>Guibemantis</i> cf. <i>liber</i>	<i>Pandanusicola</i>	Veembe [K]	ZMA 20116	EF468027, EF472526
<i>Guibemantis</i> cf. <i>liber</i>	<i>Pandanusicola</i>	Ankavanana River [N]	UMMZ 213093	AY454364, AY454387
<i>Guibemantis</i> cf. <i>liber</i>	<i>Pandanusicola</i>	Marojejy Reserve, Manantenina River [B]	UMMZ 213070	EF468018, EF472517
<i>Guibemantis</i> cf. <i>liber</i>	<i>Pandanusicola</i>	Andringitra, Sahavaby River [I]	UMMZ 213314	AY454368, AY454393
<i>Guibemantis pulcher</i>	<i>Pandanusicola</i>	Samalaotra [F]	ZCMV 2002.277	EF468035, EF472534
<i>Guibemantis pulcher</i>	<i>Pandanusicola</i>	Maharira [F]	ZCMV 265	EF468034, EF472533
<i>Guibemantis pulcher</i>	<i>Pandanusicola</i>	Besariaka [D]	ZMA 20317	EF468030, EF472529
<i>Guibemantis pulcher</i>	<i>Pandanusicola</i>	Ranomafana, Kidonavo bridge [F]	ZMA 20242	EF468032, EF472531
<i>Guibemantis pulcher</i>	<i>Pandanusicola</i>	Vohiparara bridge [F]	ZMA 19415	EF468033, EF472532
<i>Guibemantis</i> cf. <i>pulcher</i>	<i>Pandanusicola</i>	Sainte Luce [E]	RML 262	EF468019, EF472518
<i>Guibemantis</i> cf. <i>pulcher</i>	<i>Pandanusicola</i>	Sainte Luce [E]	RML 132	EF468020, EF472519
<i>Guibemantis pulcher</i>	<i>Pandanusicola</i>	Andasibe [A]	ZSM 58/2002	EF468031, EF472530
<i>Guibemantis punctatus</i>	<i>Pandanusicola</i>	Sainte Luce [E]	RML 290	EF468021, EF472520
<i>Guibemantis punctatus</i>	<i>Pandanusicola</i>	Sainte Luce [E]	RML 265	EF468022, EF472521
<i>Guibemantis tornieri</i>	<i>Guibemantis</i>	Zahamena Reserve	UMMZ 213353	AY454369

Letters correspond to locations in Fig. 1.

Collection abbreviations are as follows: FGZC, Frank Glaw Zoological Collection; FMNH, Field Museum of Natural History; RML, uncatalogued tissue samples of R. Lehtinen at UMMZ; UMMZ, University of Michigan Museum of Zoology; ZCMV, Zoological collection of Miguel Vences; ZMA, Zoological Museum Amsterdam; ZSM, Zoologische Staatssammlung München.

GTR + I + G substitution model, as determined by hierarchical likelihood ratio tests in Modeltest version 3.7 (Posada and Crandall, 1998). Empirical base frequencies were: freq A = 0.3499; freq C = 0.2110; freq G = 0.1716; freq T = 0.2675. The proportion of invariable sites (I) was 0.2733 and the gamma distribution shape parameter (G) was 0.6913. Because of computational limitations, we used only 100 bootstrap replicates to assess clade support in the ML analyses. For both MP and ML analyses, nodes with 70% bootstrap support were regarded as sufficiently resolved (Hillis and Bull, 1993).

The Bayesian analysis was conducted in MrBayes 3.1.1 (Huelsenbeck and Ronquist, 2001) using the substitution model indicated by Modeltest (above). Priors were set using the default setting in MrBayes, and the model parameters were estimated in the analysis. Two independent analyses with different random starting trees were run

simultaneously with three heated chains and one cold chain, as per the program defaults. The run was conducted for 1.4 million generations, with the first 400,000 being discarded as burn-in. This produced 1 million post burn-in generations. The analysis was terminated when stationarity was reached (the standard deviation of split frequencies was less than 0.01). Majority rule consensus trees were used to display the results of all analyses. For the Bayesian analysis, nodes with posterior probability values over 95% were deemed well supported.

Sequences from three of the four members of the subgenus *Guibemantis* (*G.* cf. *depressiceps*, *G. kathrinae*, and *G. tornieri*) were used as outgroups in the ML and MP analyses. Since MrBayes allows only a single outgroup, *G.* cf. *depressiceps* was arbitrarily chosen as the outgroup for the Bayesian analysis. *Guibemantis* is the sister taxon to *Pandanusicola* (Richards et al., 2000; Lehtinen et al., 2004).

### 3. Results

After excluding 219 hypervariable or ambiguous characters, 1748 characters were available for analysis. The results of the ILD test were significant ( $p = 0.03$ ), so we conducted partitioned analyses of the 12S and 16S genes separately as well as a total evidence analysis of the concatenated dataset. The total evidence parsimony reconstruction (1748 unordered characters, 514 parsimony informative) produced two equally parsimonious trees of 1945 steps (CI = 0.453; RI = 0.657). The likelihood and Bayesian analyses of these data generally produced similar topologies, and for simplicity we present and discuss the consensus tree from the Bayesian analysis. The separate analyses of the 12S and 16S genes also yielded very similar topologies, differing primarily in the level of support at basal nodes. Table 2 summarizes support for major branches recovered from our phylogenetic analyses.

In all reconstructions, *G. liber* is strongly supported as being nested deep within a clade containing only members of *Pandanusicola* (Fig. 1) and does not group with members of the outgroup (subgenus *Guibemantis*). However, while sequences of typical *G. liber* (i.e., as defined by Blommers-Schlösser (1979) and subsequent authors who referred to non-phytotelm-breeding populations) strongly group together (clade 2 in Fig. 1), other specimens attributed to this species by morphological similarity are strongly genetically differentiated and occur in relatively distant portions of the tree. Specimens that we attributed to typical *G. liber* from clade 2 based on

external morphology come mostly from the northern part of the island (Manongarivo, Tsaratanana, Montagne d'Ambre) but also include one specimen from the south (Vevebe; Fig. 2). Two other specimens attributed to *G. liber* (clade 3) are genetically very divergent from the *G. liber* in clade 2 (average pairwise sequence divergence >9.0%; Table 3). These occur in a relatively small area of northeastern Madagascar (Marojeje and the Ankavana River area; Fig. 2). Lastly, one morphologically similar lineage from Vevembe and Andringitra in south and central eastern Madagascar is also very divergent (clade 6 in Figs. 1 and 2) and, at least in the Bayesian analysis, is the sister taxon to all other members of *Pandanusicola* (Table 2).

Similar genetic differentiation and non-monophyly is also seen in *G. pulcher* (Fig. 1). There is strong support for the monophyly of the highland populations of *G. pulcher* (clade 1 in Figs. 1 and 2, Table 2); however, a lowland form from extreme southeastern Madagascar is only distantly related to highland forms (average pairwise sequence divergence >8.0%; Table 3) and groups in yet another strongly divergent clade (clade 4, Figs. 1 and 2, Table 2).

Specimens attributed to *G. flavobrunneus* are found in three different portions of our tree (Fig. 1). Two specimens (one from the Ankaratra Mountains and one from the Mangoro River region) are the sister taxon to the highland forms of *G. pulcher* (clade 1). Two other specimens attributed to *G. flavobrunneus* (from the area in and around Andringitra) are the sister taxon to the lowland *G. pulcher*

Table 2  
Summary of partitioned and total evidence phylogenetic analyses

Branch supported	MP (total)	ML (total)	B (total)	MP (12S)	ML (12S)	B (12S)	MP (16S)	ML (16S)	B (16S)
1. <i>G. (?) bicalcaratus</i> from Maharira and Vohiparara	+(96)	+(86)	+(1.00)	+(97)	+(90)	+(1.00)	+(74)	+(73)	+(0.78)
2. <i>Pandanusicola</i> + <i>G. liber</i>	+(100)	+(91)	+(1.00)	+(100)	+(89)	+(1.00)	+(75)	+(63)	+(0.80)
3. <i>G. cf. liber</i> from Andringitra, Besariaka and Vevembe	+(100)	+(100)	+(1.00)	+(100)	+(99)	+(1.00)	+(100)	+(100)	+(1.00)
4. <i>Pandanusicola</i> excluding <i>G. cf. liber</i> from Andringitra, Besariaka and Vevembe	(-)	(-)	+(1.00)	(-)	(-)	-	(-)	(-)	(-)
5. <i>G. liber</i> in clade 2 of Fig. 1	+(100)	+(100)	+(1.00)	(-)	(-)	+(0.89)	+(100)	+(99)	+(0.95)
6. <i>G. liber</i> in clade 2 of Fig. 1 + <i>G. punctatus</i> and <i>G. bicalcaratus</i> from Sainte Luce	(-)	(-)	+(0.89)	(-)	(-)	+(0.92)	(-)	(-)	+(0.57)
7. <i>G. bicalcaratus</i> from Sainte Luce + <i>G. punctatus</i>	(-)	(-)	+(0.62)	(-)	(-)	(-)	(-)	(-)	+(0.97)
8. <i>G. pulcher</i> in clade 1 of Fig. 1	+(100)	+(98)	+(1.00)	+(92)	+(76)	+(1.00)	+(100)	+(97)	+(1.00)
9. <i>G. pulcher</i> in clade 1 of Fig. 1 + <i>G. flavobrunneus</i> from Ankaratra and Mangoro River region	+(100)	+(97)	+(1.00)	+(75)	+(78)	+(1.00)	+(97)	+(84)	+(1.00)
10. <i>G. cf. pulcher</i> from Sainte Luce + <i>G. flavobrunneus</i> from Andringitra and Manombo (= clade 4 of Fig. 1)	+(73)	+(68)	+(1.00)	+(89)	+(88)	+(1.00)	+(93)	+(94)	+(1.00)
11. <i>G. cf. liber</i> in clade 3 of Fig. 1	+(100)	+(100)	+(1.00)	+(70)	+(85)	+(1.00)	+(100)	+(100)	+(1.00)
12. <i>G. bicalcaratus</i> in clade 5 of Fig. 1	+(54)	+(59)	+(1.00)	+(68)	(-)	+(1.00)	+(67)	+(54)	+(0.99)

ML, maximum likelihood; MP, maximum parsimony; B, Bayesian; +, topology was supported; -, topology not supported; (-), topology not resolved. Numbers in parentheses are bootstrap support values (MP and ML) and Bayesian posterior probabilities (only given if >50%). Node designations follow Fig. 1.



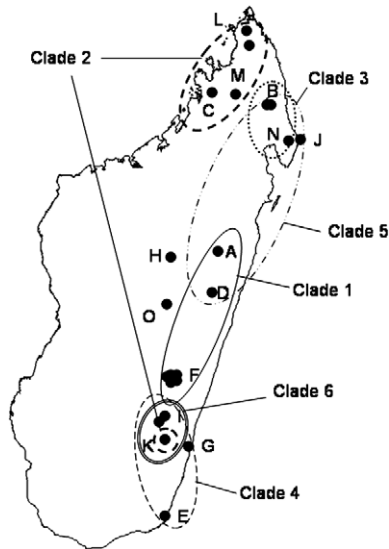


Fig. 2. Map of Madagascar with collection localities indicated. Letters refer to specific place names indicated in Table 1. Clade numbers refer to those presented in the phylogram in Fig. 1.

Generally, the most nested portions of the tree are well resolved in most reconstructions, but many of the more basal nodes in our tree are not well supported (bootstrap values less than 70%; posterior probabilities less than 95%; Table 2).

#### 4. Discussion

A recent analysis of the molecular phylogenetic relationships within the subgenus *Guibemantis* placed *G. liber* as the sister taxon to this clade (Vences and Glaw, 2005). However, this analysis did not include any members of *Pandanusicola*, and the authors suggested that the placement of *G. liber* needs to be confirmed. Our analysis (the first to include multiple members of both subgenera *Guibemantis* and *Pandanusicola*) confirms that *G. liber* is part of *Pandanusicola* and not subgenus *Guibemantis*, as reflected in the most recent classification (Glaw and Vences, 2006).

Natural history studies indicate that *G. liber* is a pond-breeding species, with normal pond-type tadpoles, a relatively large clutch size, and no parental care (Blommers-Schlösser, 1975; Vejarano et al., 2006). As far as it is known, all other members of *Pandanusicola* lay their relatively small clutches of eggs in plant-held water bodies (phytotelmata), have specialized tadpoles for surviving in these small pools (Blommers-Schlösser, 1979; Glaw and Vences, 1994; Lehtinen, 2004), and at least some have parental care (Lehtinen, 2003). Using only a single representative of each putative species in *Pandanusicola* (including *G. liber*), Lehtinen et al. (2004) suggested that these traits in *G. liber* may represent a reversal to the ancestral reproductive mode (see also Lehtinen and Nussbaum, 2003). In the present study, specimens assigned to *G. liber* occur in three distinct portions of the tree with strong support. However, from field observations linked to the

voucher specimens, it is beyond doubt that the taxa in clade 2 (Fig. 1) represent typical non-phytotelm-breeding *G. liber* (sensu Blommers-Schlösser, 1975, 1979; Blommers-Schlösser and Blanc, 1991; Glaw and Vences, 1994). A larger DNA barcoding survey of Malagasy frogs based on a short fragment of the 16S rRNA gene (Vences et al., 2005a) also provided evidence that populations from central eastern Madagascar (Mandraka, Andasibe) studied by Blommers-Schlösser (1975, 1979) have haplotypes belonging to this clade (Genbank Accession Numbers AY684187, AY848079–AY848082). Therefore, based on the nesting of clade 2 within a group of phytotelm-breeders (e.g., clades 1, 5 and *G. bicalcaratus* and *G. punctatus* from Sainte Luce; Fig. 1), we can strongly confirm that non-phytotelm-breeding in *G. liber* constitutes a reversal from specialized reproduction in leaf axils.

Also remarkable is clade 6; these frogs are morphologically similar to *G. liber* but have a greenish color reminiscent of *G. pulcher*. We have previously collected individuals of this form from other localities in the south-east. It has a very different advertisement call and clearly represents a separate species. Field observations indicate that this species breeds in swamps rather than in leaf axils, although this inference is so far based only on calling behavior, as eggs or tadpoles have not yet been identified (M. Vences, personal observation). If confirmed (and if this species is the sister taxon to other *Pandanusicola* as our Bayesian tree indicates; Fig. 2) this would suggest that pond-breeding is the ancestral reproductive mode in this group.

As with *G. liber*, our tree indicates that *G. bicalcaratus*, *G. flavobrunneus*, and *G. pulcher* are also non-monophyletic (Fig. 1). This again suggests that very probably undescribed, cryptic species masquerade under these names. For example, specimens assigned to *G. pulcher* occur in three distinct parts of the tree with high support. The *G. pulcher* specimens from Sainte Luce (a coastal site in south-eastern Madagascar) are morphologically and acoustically distinct from *G. pulcher* from highland areas (R.M.L., unpublished data), are genetically divergent and likely represent an undescribed species. Similarly, specimens currently assigned to *G. bicalcaratus* and *G. flavobrunneus* appear in a number of areas of the tree and may represent cryptic lineages deserving species status.

Interestingly, the *G. bicalcaratus* from Maharira and Vohiparara group outside *Pandanusicola* altogether. A comprehensive phylogenetic analysis of over 1500 mantelid sequences for a fragment of the 16S rRNA gene (Vences et al., 2005b, unpublished data) indicate that this population may belong to the genus *Spinomantis*. Any labeling error can be excluded since multiple 16S rRNA sequences of this population are available (Genbank Accession Numbers AY848381–AY848386). The population from Maharira consists of small frogs living in *Pandanus*, the males bearing femoral glands typical for *Pandanusicola*. They are rather common in Ranomafana National Park (Maharira being just one locality within the park where the species



occurs). It is unknown if these frogs use *Pandanus* plants for breeding or just as shelter; more fieldwork is necessary to understand the reproductive mode of this species, which has acquired phytotelm-dwelling habits and possibly phytotelm-breeding independently from *Pandanusicola*.

Many of the clades revealed in these analyses are concordant with geography (Fig. 2), suggesting that they diagnose population lineages rather than representing incomplete lineage sorting or introgression of haplotypes. For example, all of the central highland forms assigned to *G. pulcher* group together in clade 1 (Figs. 1 and 2), and clade 4 represents a number of related forms from the southeastern part of the island. Similarly, clade 3 (Fig. 1) tightly clusters geographically in the northeast (Fig. 2). Other clades, however, are less geographically concordant. For example, four of five haplotypes assigned to *G. liber* in clade 2 are from northwestern Madagascar, while the remaining specimen is geographically distant, in the southeast (Figs. 1 and 2), confirming that this taxon (the typical *G. liber*) occurs over a wide range in Madagascar with slight to moderate regional differentiation.

The 12S and 16S mitochondrial genes have been used repeatedly in molecular systematic studies of amphibians. Hertwig et al. (2004) asserted that these genes may not be well suited for assessing deep splitting events. While the younger portions of our tree were well resolved, the more basal portions lack robust support from at least some methods of analysis (Table 1). This may be due to rapid diversification in this lineage or the decreased ability of the 12S and 16S genes to discern deep divergences. Better taxon sampling and additional data, using nuclear genes or other characters, will likely help to resolve the deepest nodes in this group. As others have suggested, multiple sources of data are likely to provide the most robust estimates of evolutionary history (Rubinoff and Holland, 2005).

For intra-familial and intra-generic assessment, however, these genes appear to function well and have even been suggested as bar codes for amphibians (Vences et al., 2005b). However, other recent studies have revealed surprisingly high estimates of intra-specific variation in mitochondrial genes in amphibians from both temperate and tropical areas (e.g., Austin et al., 2002; Camargo et al., 2006; Loughheed et al., 1999). For example, Vences et al. (2005a) found high levels of intra-population nucleotide diversity (up to 14%) in some mantellids. However, the cytochrome oxidase I gene used there is known to evolve faster than the rRNA genes used in the present study. While mitochondrial genes may be particularly well suited to delimiting species, especially when concordance with other data sets is found (Wiens and Penkrot, 2002), many believe that strong mitochondrial sequence divergence should not be used as the sole determinant for species diagnosis (e.g., Funk and Omland, 2003). Nevertheless, mitochondrial divergences can be an excellent starting point to delimit candidate species that may then be the focus of

additional geographic sampling and detailed morphological and acoustic studies (Vences et al., 2005a). There is a possibility that we amplified non-functional nuclear copies (Numts) of these mitochondrial genes (Sorenson and Fleischer, 1996). However, these transposed sequences are apparently less common than initially thought and most often involve the mitochondrial control region and the cytochrome *b* gene (Pereira and Baker, 2004).

Our data support two major conclusions. First, even though it has a different mode of reproduction, *G. liber* is part of the *Pandanusicola* lineage, confirming recent taxonomy and strongly suggesting a reversal of its reproductive mode. Second, most of the described species in *Pandanusicola* consist of two or more genetically well differentiated, but morphologically cryptic, allopatric lineages that may deserve recognition at the species level.

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