

Assessing the diversity of phytotelmic frogs along Madagascar's east coast: Redefinition of *Guibemantis bicalcaratus* (BOETTGER, 1913) and revalidation of *Guibemantis methueni* (ANGEL, 1929)

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Abstract

Guibemantis bicalcaratus (BOETTGER, 1913) has been considered as a widespread member of the subgenus *Pandanusicola* of largely phytotelmic-breeding Malagasy frogs. Based on new collections from its type locality, the Malagasy island Nosy Boraha, and several other localities on Madagascar's east coast, we revise the status of *G. bicalcaratus* using molecular and morphological data. Mitochondrial DNA sequences of the 16S rRNA gene demonstrate the presence of two deep genealogical lineages of *Pandanusicola* on Nosy Boraha. Of these, one so far has only been found on Nosy Boraha whereas the second is also present at several other localities along the central east coast of Madagascar. Specimens assigned to the two mtDNA lineages did not share alleles in sequences of the nuclear RAG1 gene except for one specimen, suggesting reduction or absence of gene flow despite sympatry, with occasional hybridization. No differences between the two lineages were found in external morphology but faint differences in color allowed us to discriminate the majority of specimens, and we therefore conclude that they should be considered as belonging to two distinct species. Based on these chromatic differences and comparison with type specimens, we assign the species present only on Nosy Boraha to *Guibemantis bicalcaratus* whereas the second species also occurs at Ambila, the type locality of *Gephyromantis methueni* ANGEL, 1929. We consequently resurrect this nomen as *Guibemantis methueni* for this species. It is likely that the two species are restricted to relatively small ranges on Madagascar's central east coast, but due to the rudimentary knowledge on their distribution and habitat requirements we suggest a threat status of Data Deficient for both of them.

Key words

Mantellidae; *Guibemantis*; *Pandanusicola*; *Guibemantis bicalcaratus*; *Guibemantis methueni*; Nosy Boraha; Madagascar; Taxonomy.

Introduction

The anuran family Mantellidae, endemic to Madagascar and the Comoran island of Mayotte, at present contains around 200 nominal species arranged in 12 genera (GLAW & VENCES, 2006). The taxonomy of some of these genera has been intensively studied in recent years. For instance, in the genus *Boophis*, many new species have been described and these are typically diagnosed easily by their loud and prominent advertisement calls, and often also by the morphology of their tadpoles which can be collected in large quantities in rainforest streams (e.g., GLAW *et al.*, 2010; RANDRIANIAINA *et al.*, 2012). Other genera, however, remain taxonomically understudied although it is clear that they comprise a large proportion of undescribed species (VIEITES *et al.*, 2009). Such is the case of the genus *Guibemantis*, a clade currently containing 13 nominal species of arboreal frogs of which four are included in the subgenus *Guibemantis* and the remaining nine in the subgenus *Pandanusicola* (LEHTINEN *et al.*, 2007, 2011, 2012). The nine species in the subgenus *Pandanusicola* are small-sized frogs of snout-vent lengths of 20–35 mm, and as far as known, all but two of them reproduce in the leaf axils of *Pandanus* plants (BLOMMERS-SCHLÖSSER, 1975, 1979; LEHTINEN, 2002, 2003, 2004, 2009; LEHTINEN *et al.*, 2012).

Molecular data (LEHTINEN *et al.*, 2007; VIEITES *et al.*, 2009; WOLLENBERG *et al.*, 2011) have provided evidence for the presence of numerous deep genetic lineages among *Pandanusicola*. The high genetic diversity among these frogs is poorly understood and not easy to interpret taxonomically. Most *Pandanusicola* are not very vocal, and several species appear to be poorly differentiated bioacoustically (LEHTINEN *et al.*, 2012). Furthermore, these frogs are morphologically very similar to each other, and differences between species often are restricted to differences in coloration which in some cases can be subtle. A comprehensive taxonomic revision is not easy because the genetic diversity of *Pandanusicola* reflects both a large number of undescribed cryptic species (LEHTINEN *et al.*, 2007) and the existence of deep conspecific lineages in some species such as *G. liber* (VIEITES *et al.*, 2009), and taxonomic conclusions thus are to be made only after careful analysis of integrative data sets. In addition, several synonyms exist that need to be considered as earlier names for additional species of *Guibemantis*. Given this complex situation, we have opted for a stepwise strategy to achieve taxonomic resolution of *Guibemantis*, first defining and delimiting all those species for which earlier names exist, and newly describing only those which can be diagnosed in an unambiguous way (LEHTINEN *et al.*, 2011, 2012).

One of the historically earliest described species assigned to the subgenus *Pandanusicola* in *Guibemantis* is *G. bicalcaratus* (BOETTGER, 1913). This species was described from the offshore island Nosy Boraha (= Ile Sainte Marie) on Madagascar's central east coast, and many populations of predominantly brownish colored

Pandanusicola from all over Madagascar have since been assigned to this species (BLOMMERS-SCHLÖSSER 1975; BLOMMERS-SCHLÖSSER & BLANC, 1991; GLAW & VENCES, 1994). However, molecular data have shown that the various populations assigned to *G. bicalcaratus* do not form a monophyletic group (LEHTINEN *et al.*, 2007) and probably contain a large number of unrecognized cryptic species. A redefinition of *G. bicalcaratus* based on topotypical material is therefore a high priority to be able to proceed with the taxonomic revision of this group of frogs. However, this was made more difficult by the fact that during preliminary surveys on Nosy Boraha, two highly different mitochondrial lineages of *Pandanusicola* were found which appeared to be sister to each other (LEHTINEN *et al.*, 2011).

Based on additional material collected on a recent expedition to Nosy Boraha and other east coast sites, we here conclude that the two lineages found on this island are characterized by consistent albeit faint differences in color pattern and should be considered as independent evolutionary lineages, i.e., as species. One of these has only been found on Nosy Boraha and agrees with the lectotype of *G. bicalcaratus*, whereas the second is more widespread and also occurs at Ambila, the type locality of a taxon originally described as *Gephyromantis methueni* (ANGEL, 1929), which we resurrect as *Guibemantis methueni*.

Materials and Methods

Specimens were collected by diurnal and nocturnal searches in the leaf axils of *Pandanus* plants. Geographical coordinates were recorded with GPS receivers and transformed into decimal degrees (WGS84). Specimens were deposited in the collections of Université d'Antananarivo, Département de Biologie Animale, Antananarivo (UADBA), Zoological Museum Amsterdam (ZMA), Zoologisches Forschungsmuseum Alexander Koenig, Bonn (ZFMK), and the Zoologische Staatssammlung München (ZSM). RALC, RAX, and ZCMV refer to field numbers. Additional material was examined from the Museum National d'Histoire Naturelle, Paris (MNHN) and the Senckenberg Museum Frankfurt (SMF). All RALC specimens have been deposited and will be catalogued in UADBA; final UADBA catalogue numbers are so far available for 17 RALC specimens.

Morphological measurements (in millimetres) were all done by AR and MV with a digital caliper (precision 0.01 mm) to the nearest 0.1 mm. The following abbreviations were used: SVL (snout-vent length), HW (greatest head width), HL (head length), ED (horizontal eye diameter), END (eye-nostril distance, between anterior corner of eye to center of nostril), NSD (nostril-snout tip distance, from center of nostril to the most anteriorly projecting point of snout tip), NND (nostril-nostril distance), TD (horizontal tympanum diameter), TL (tibia

length), HAL (hand length), HIL (hindlimb length), FOL (foot length), FOTL (foot length including tarsus), FORL (forelimb length), RHL (relative hindlimb length). Specimens were sexed by either presence of distinct femoral glands in males, or by gonad inspection. Webbing formulas are given according to the system used by BLOMMERS-SCHLÖSSER (1979).

Statistical analysis of morphometric data was carried out in Statistica vers. 7.1 (Statsoft). We performed a Principal Component Analysis with normalized Varimax rotation and excluded Factor 1 which was mainly influenced by different body size of the specimens measured.

DNA was extracted from ethanol preserved tissue. We sequenced part of the mitochondrial 16S rRNA gene using primers specifically designed to amplify in mantellid frogs a short fragment (about 340 bp) containing a highly polymorphic loop region (PCR primers 16SFrogL1 – CAT AAT CAC TTG TTC TTT AAA, and 16SFrogH1 – GAT CCA ACA TCG AGG TCG). The sequence of this fragment of the 16S gene is sufficiently variable for reliable identification of Malagasy frogs (VENCES *et al.*, 2005a, 2005b). PCR conditions comprised initial denaturation at 94°C (90 sec.) and then 36 cycles of denaturation at 94°C (45 sec.), primer annealing at 51–55°C (45 sec.) and elongation at 72°C (90 sec.), followed by a final extension step at 72°C (5 min.). We furthermore amplified a fragment of the coding region of the nuclear RAG1 gene using primers RAG1 FIII (TGG CAC AGG GTA TGA TGA RA) and RAG1 RIII (TCA ATG ATC TCT GGG ACG TG) (primers developed by A. CROTTINI; see PABIJAN *et al.*, 2011). PCR conditions were as follows: initial denaturation at 94°C (120 sec.) and then 38 cycles of denaturation at 94°C (20 sec.), primer annealing at 53°C (45 sec.) and elongation at 72°C (120 sec.), followed by a final extension step at 72°C (10 min.). Purification of the PCR products was performed by Exonuclease I and Shrimp Alkaline Phosphatase digestion. The 16S amplicons were sequenced with the 16SFrogL1 primer (VENCES *et al.*, 2005a), and the RAG1 amplicons with both the F and R primers, using BigDye v3.1 cycle sequencing chemistry. Sequencing products were run on a 3130xl genetic analyzer (Applied Biosystems).

Sequences were edited in CodonCode Aligner v3.0.3 and deposited in GenBank under accession numbers JX535537–JX535562 (16S) and JX535523–JX535536 (RAG1). The 16S sequences were aligned using the Clustal algorithm in the program MEGA vs. 5 (TAMURA *et al.*, 2011). Because heterozygous nucleotide sites were observed in the RAG1 sequences we used the Phase v2.1.1 algorithm (STEPHENS *et al.*, 2001; STEPHENS & SCHEET, 2005) as implemented in the software DnaSP5 (LIBRADO & ROZAS, 2005) to infer haplotypes for this gene. We used the inferred haplotypes with the highest score for each individual in further analyses irrespective of the probability of correct inference. Wrongly inferred haplotypes would only influence the structure of the resulting network but not the general conclusions regarding haplotype sharing or absence thereof between the two target species of our study which is the essential informa-

tion needed for taxonomic conclusions (see also PABIJAN *et al.*, 2011).

Phylogenetic analyses of the 16S data set consisted of Bayesian inference (BI) in MrBayes (HUELSENBECK & RONQUIST, 2001) after substitution model selection in MrModelTest v2.3 (NYLANDER, 2004). Akaike's information criterion (AIC) implemented in MrModelTest selected GTR + I + G as the best fit model of nucleotide substitution. The Bayesian analysis consisted of two runs with four chains for a total of 10 million generations, sampled every 1000th tree. The two runs had achieved stationarity after the first 1 million generations (discarded as burn in) as judged by plotting the generation numbers against their log-likelihood. A majority rule consensus tree was produced from the retained trees with posterior probabilities calculated as the frequency of samples recovering each clade (HUELSENBECK & RONQUIST, 2001).

The RAG1 sequences after phasing were used to construct a haplotype network using the statistical parsimony approach with a 95% cut-off in the software TCS v1.21 (CLEMENS *et al.*, 2000).

Results

Molecular differentiation

The final 16S alignment comprised a maximum of 515 nucleotides, but for most of the newly sequenced specimens only considerably shorter DNA fragments were obtained. The analysis thus has little value in recovering phylogenetic relationships among species but it served to allocate all of the newly determined sequences to two major lineages (Fig. 1). According to the more extensive DNA sequence set of LEHTINEN *et al.* (2007) these two lineages may be sister to each other although they did not resolve as such in our analysis. To facilitate a consistent description of the results and anticipating our taxonomic conclusions below, we consider these as corresponding to *G. bicalcaratus* and *G. methueni*. These two mitochondrial lineages were clearly distinct from each other, and in the short fragment analysed, the uncorrected p-distance between them was 12.8–15.4%. The maximum divergence among sequences of *G. bicalcaratus* was 3.6% and among sequences of *G. methueni* at most 0.5% for sequences on Nosy Boraha and Ile aux Nattes and up to 3.1% among specimens from these sites and those from Ambila, Besariaka, and Antetazana. However, these values are inflated compared to those reported e.g. in VIETES *et al.* (2009) for a longer 16S fragment including more conserved sites; for those specimens where the entire 515 nucleotides were available, the differences between *G. methueni* and *G. bicalcaratus* were 8.3–9.5% and between *G. methueni* from Nosy Boraha vs. Besariaka 1.5%.

On Nosy Boraha we found specimens of both lineages in sympatry, while on the Ile aux Nattes, Besariaka,

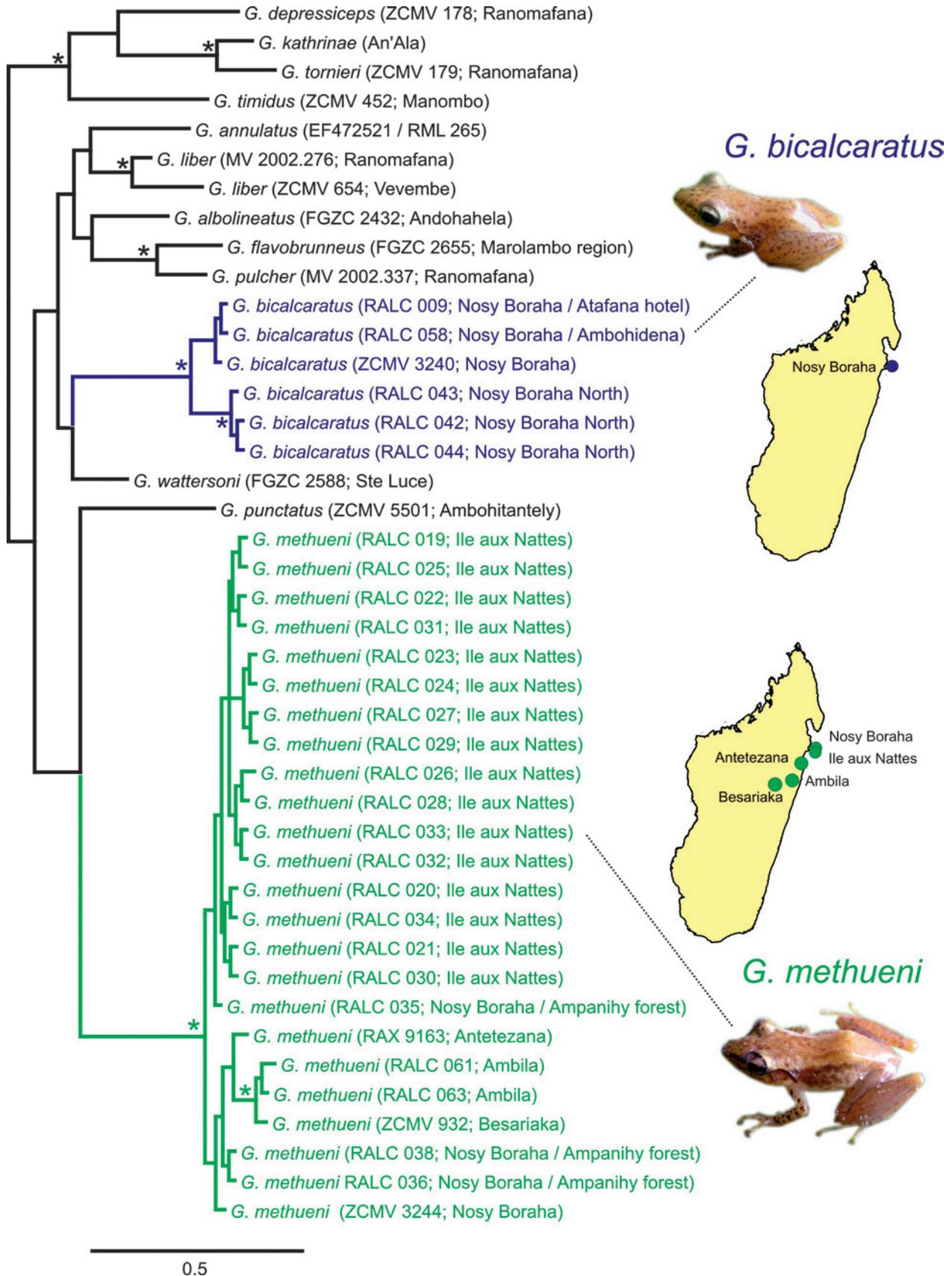


Fig. 1. Majority-rule consensus tree from Bayesian Inference based on a fragment of the 16S rRNA gene (alignment length 515 bp). The known distribution of *G. bicalcaratus* and *G. methueni* according to the molecular data is shown in the inset maps. Note that the specimen RALC 59 is not included in the tree due to the poor quality and short length of the DNA sequence obtained for this specimen; however, the sequence read contained various nucleotide character states diagnostic for *G. bicalcaratus*. Asterisks mark nodes with posterior probabilities > 0.95.

Table 1. List of RAG1 haplotypes in specimens of *Guibemantis bicalcaratus* and *G. methueni*. Note that data refer to haplotypes after phasing of the original sequences representing the diploid genotype, i.e., two separate sequences were included for each specimen. Numbers of haplotypes refer to those used in the haplotype network (Fig. 2). Numbers 1 or 2 after specimen numbers indicate whether a specimen was heterozygous (1) or homozygous (2) for that haplotype.

Haplotype	Specimens
B1	<i>G. bicalcaratus</i> : RALC 42 (1), RALC 44 (1), RALC 57 (2)
B2	<i>G. bicalcaratus</i> : RALC 42 (1), RALC 43 (2)
B3	<i>G. bicalcaratus</i> : RALC 09 (2), RALC 44 (1), RALC 58 (2); <i>G. methueni</i> : RALC 36 (1)
M1	<i>G. methueni</i> : RALC 31 (2), RALC 33 (2), RALC 34 (2), RALC 35 (2), RALC 61 (1)
M2	<i>G. methueni</i> : RALC 32 (1), RALC 63 (2)
M3	<i>G. methueni</i> : RALC 61 (1)
M4	<i>G. methueni</i> : RALC 36 (1)
M5	<i>G. methueni</i> : RALC 32 (1)

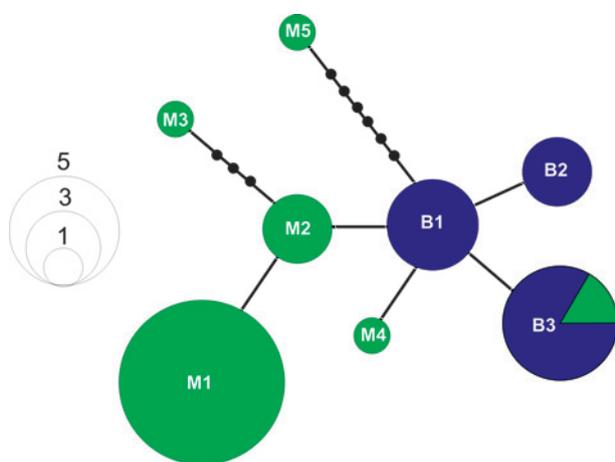


Fig. 2. Statistical parsimony haplotype network of phased RAG1 sequences of 14 specimens of *Guibemantis bicalcaratus* (blue, haplotypes marked B1–B3) and *G. methueni* (green, haplotypes M1–M5 and sharing of B3). Black dots indicate additional mutational steps between haplotypes. See Table 1 for exact list of haplotypes and specimens. Size of circles is proportional to number of sequences found to each haplotype as indicated by empty circles on left.

Antetezana and at Ambila, only *G. methueni* was present. At other localities along the east coast, we found specimens belonging to additional deep mitochondrial lineages, apparently without close relationships to *G. bicalcaratus* and *G. methueni*, and probably corresponding to additional undescribed species. We have excluded these from the phylogenetic tree and will deal with them, as well as with the remaining unconfirmed candidate species of LEHTINEN *et al.* (2007) and VIEITES *et al.* (2009), in forthcoming studies.

The RAG1 data confirmed that the *G. bicalcaratus* and *G. methueni* lineages are largely though not completely genetically isolated. The sequences of the 14 specimens studied (of which 6 were assigned by the mtDNA data to *G. bicalcaratus*, and 8 to *G. methueni*) contained 3 haplotypes for *G. bicalcaratus* (B1–B3 in Fig. 2) and 5 haplotypes in *G. methueni* (M1–M5). Haplotype sharing was observed in a single individual only (Table 1): specimen

RALC 36 from Ampanihy forest (Nosy Boraha) was assigned by mtDNA to *G. methueni* but was heterozygous for haplotypes M4 and B3, suggesting a possible hybridization among the two species. To confirm this heterozygous state, we reextracted DNA from this sample and resequenced it with the same results. Unfortunately this specimen is a juvenile and thus its morphology and coloration could not be meaningfully compared with that of other specimens. While the three *G. bicalcaratus* haplotypes differed only by one mutational step from each other, one haplotype of *G. methueni* (M3) differed by four steps from M2, and M5 differed by 7 steps from M1. These divergent haplotypes were found in the specimens RALC 32 from the Ile aux Nattes (M5) and RALC 61 from Ambila (M3).

Morphological and chromatic differentiation

No reliable call recordings could be assigned to the frogs collected by us (see species accounts below) which prevents an assessment of possible bioacoustic differences between the two lineages (*bicalcaratus* and *methueni*). We also found no discrete differences in external morphology among them.

Morphometric comparison was carried out in a multivariate approach. Because of the relatively low number of reliably sexed adult specimens available (Table 2) and the absence of obvious sexual dimorphism, we merged data of all specimens with more than 20 mm SVL for multivariate analysis. No obvious morphometric differences were observed among the specimens studied, and this was confirmed by the multivariate analysis. The first PCA factor explained 76% of the total variance and had positive loadings for all variables; it was therefore mainly influenced by body size. Factors 2 and 3 with Eigenvalues of 1.1 and 0.5 explained 7.6% and 3.6% of the total variance. Main influences (loadings > 0.5) on factor 2 were TD, NSD and NND, main influence on factor 3 was END. A scatterplot of factors 2 and 3 (Fig. 4) did not reveal any clustering of the specimens that had been assigned by molecular data to *G. bicalcara-*

Table 2. Morphometric data (in mm) of *G. bicalcaratus* and *G. methueni*. LT = lectotype; PLT = paralectotype; M = male; F = female. See Materials and Methods for other abbreviations.

Species / Field number	Locality	Sex	SVL	HW	HL	TD	ED	END	NSD	NND	FORL	HAL	HIL	FOTL	FOL	TIL
<i>G. bicalcaratus</i>																
SMF 6811 (LT)	Nosy Boraha	F	27.3	9.4	11.1	1.5	3.7	2.9	1.7	2.6	14.7	7.8	41.2	18.2	11.2	13.6
SMF 6812 (PLT)	Nosy Boraha	F	26.7	9.7	11.2	1.8	3.7	2.9	1.8	2.5	16.2	7.4	42.3	18.8	12.0	13.2
SMF 6813 (PLT)	Nosy Boraha	F	24.1	7.9	9.8	1.8	3.1	2.7	1.6	2.2	13.3	5.7	37.8	16.5	10.3	12.3
ZSM 425/2006 (ZCMV 3240)	Nosy Boraha	F	20.5	7.3	8.5	1.4	3.1	2.1	1.3	2.1	11.1	5.1	32.6	14.5	8.4	10.4
UADBA-A 62197 (RALC 57)	Ambohidena	F	21.8	7.5	9.8	1.6	3.2	2.2	1.7	2.3	11.3	5.6	34.0	14.3	9.0	11.0
UADBA-A 62214 (RALC 58)	Ambohidena	M?	21.7	6.7	8.7	0.9	2.9	1.8	1.2	1.7	11.8	5.3	33.1	14.4	8.4	11.0
UADBA-A 62198 (RALC 59)	Ambohidena	M	20.9	6.9	8.9	1.2	3.0	2.3	1.6	1.9	10.4	5.4	33.3	15.0	9.2	11.0
<i>G. methueni</i>																
MNHN 1929.209 (LT)	Ambila	?	23.2	7.7	9.7	1.4	3.5	2.8	1.7	2.3	12.6	5.8	35.4	15.2	10.4	11.7
ZSM 429/2006 (ZCMV 3244)	Nosy Boraha	M	21.5	7.4	8.9	1.5	3.0	2.9	1.6	2.3	12.0	5.6	33.8	15.1	9.2	10.9
UADBA-A 62211 (RALC 19)	Ile aux Nattes	F	24.4	7.8	10.2	2.0	3.5	2.3	1.8	2.3	14.1	6.6	37.4	16.6	10.7	12.4
UADBA-A 62202 (RALC 21)	Ile aux Nattes	F	25.8	8.1	10.3	1.9	3.3	2.7	1.7	2.7	13.9	6.5	39.2	17.6	10.7	12.5
UADBA-A 62200 (RALC 22)	Ile aux Nattes	?	24.0	8.1	9.6	1.4	3.3	2.4	1.5	2.1	13.6	6.3	36.1	16.4	9.9	11.8
UADBA-A 62208 (RALC 23)	Ile aux Nattes	?	22.5	7.3	9.9	1.3	2.7	2.3	1.5	2.0	13.5	6.5	36.8	15.8	10.3	11.9
UADBA-A 62204 (RALC 24)	Ile aux Nattes	F	24.9	7.9	9.9	1.5	3.5	2.5	1.6	2.4	13.7	6.4	35.9	16.8	9.9	11.9
UADBA-A 62201 (RALC 25)	Ile aux Nattes	F	23.8	7.9	9.6	1.4	3.5	2.6	1.5	2.5	13.4	6.1	35.7	16.7	9.6	11.7
UADBA-A 62206 (RALC 26)	Ile aux Nattes	M	21.9	7.4	9.2	1.1	3.0	2.4	1.5	1.8	11.5	5.8	32.0	14.9	9.4	11.3
UADBA-A 62213 (RALC 27)	Ile aux Nattes	?	22.0	7.2	9.4	1.3	3.3	2.2	1.4	1.9	12.8	6.0	35.7	15.3	9.6	11.5
UADBA-A 62209 (RALC 28)	Ile aux Nattes	F	24.0	8.0	9.5	1.5	3.5	2.6	1.7	2	13.6	6.2	38.4	17.7	10.5	12.7
RALC 32	Ile aux Nattes	?	25.2	8.3	10.1	1.7	3.2	2.8	1.9	2.6	14.2	5.7	39.2	17.6	10.6	12.5
RALC 33	Ile aux Nattes	F	26.6	8.7	10.4	2.1	3.9	2.8	1.8	2.7	14.6	6.8	41.0	17.6	10.5	13.9
RALC 35	Ampanihy	?	24.1	8.2	10.1	1.9	3.6	2.9	1.5	2.2	13.2	6.3	38.1	16.8	10.4	12.8
UADBA-A 62199 (RALC 61)	Ambila	?	21.0	6.6	8.1	1.1	2.8	1.9	1.1	1.9	12.5	5.7	35.2	16.0	9.2	11.3
UADBA-A 62210 (RALC 63)	Ambila	?	23.4	7.6	9.8	1.7	3.6	2.3	1.7	2.5	14.3	6.2	38.2	17.7	10.7	12.4

tus or *G. methueni*. The type specimens of *bicalcaratus* and *methueni* clustered in between the newly collected specimens, suggesting that they are all morphometrically similar. Males and females, in both species, did not form separate clusters (not shown).

In the absence of obvious differentiation in discrete external characters and in quantitative morphometric characters, possible differences in color pattern remain as the sole external means to differentiate among the forms. We here only refer to material collected by us and assigned to either lineage by the molecular data; for assignment of type material to the lineages, see Identity sections in species accounts below. Not considering juveniles, four adult specimens of the lineage here considered as *G. bicalcaratus* were available. Two of these were uniformly-colored (RALC 57 and 59; Fig. 5a), having a reddish brown dorsum without any pattern except some very fine dark dotting on the hindlimbs, and a dark-colored tympanum in RALC 59. These specimens also lacked a black line along the canthus rostralis running from the nostril to the eye. The other two specimens (ZSM 425/2006 and RALC 58), although collected in different years from different sites, were very similar to each other in having a light brown dorsal ground color, with a relatively regular pattern of small dark brown spots (largely absent only in the area of the indistinct beige dorsolateral bands). Especially on the

hindlimbs, the fine dark spotting was very regular and not forming dark transversal bands. The supratympanic fold was dark colored. Along the flanks, especially in ZSM 425/2006, there was a series of dark spots forming a distinct narrow dark band along the first third of the flanks.

Specimens of the lineage here assigned to *G. methueni* were highly variable as well. Some were largely uniformly colored while others were rather densely covered with spots and other markings. The supratympanic fold was dark colored in all but one individual. Traces of light dorsolateral bands were visible in many individuals. Distinct dark spots and other, larger markings in the central part of the dorsum (between the dorsolateral bands) were present in only 2 out of 18 specimens from Nosy Boraha, and in the specimen from Ambila. Of the remaining 16 Nosy Boraha specimens, three were very light uniform colored, and seven had rather distinct dark flanks, this dark color typically formed by diffuse dark patches. The hindlimbs in most specimens either had transversal dark bands or poorly marked and irregular dark dots and markings. Only three out of 18 specimens (RALC 19, 23 and 36) had a regular pattern of small dark dots covering the whole dorsal part of hindlimbs. A black line along the canthus rostralis, running from nostril to eye, was present in all specimens, including the ones with otherwise uniform color.

As a conclusion, there is no clear character in morphology or color pattern that allows an unambiguous diagnosis of the two lineages. However, the combination of various characters allows to distinguish them: (i) a regular spotting on the central dorsum in combination with regular fine dots on the entire hindlimbs and only a narrow line of dark dots along the flanks is only rarely observed in *G. methueni* but characterizes 2 of 4 specimens of *G. bicalcaratus*; (ii) a uniform reddish brown dorsum without a dark line along the canthus rostralis is found only in specimens of *G. bicalcaratus*.

Given the concordance between mitochondrial and nuclear genes, and subtle differences in coloration, we conclude that these two lineages are best seen as separate species, *Guibemantis bicalcaratus* and *G. methueni*.

Guibemantis bicalcaratus (BOETTGER, 1913)

Original name: *Rhacophorus bicalcaratus* BOETTGER, 1913

Identity. Because the differentiation between the two encountered mitochondrial lineages using morphological and chromatic characters is highly difficult, it also is not straightforward to apply any described name to either of them. Based on a comparison with the lectotype and two paralectotypes (Fig. 3), we here propose to apply the name *G. bicalcaratus* to the lineage found exclusively on Nosy Boraha, based on the following arguments: (i) this is one of 2 lineages of *Guibemantis* identified from Nosy Boraha, the type locality of *G. bicalcaratus*; (ii) all three types are characterized by a regular fine dark dotting on the entire hindlimbs including the dorsal surface of the feet as it is typical for the spotted morph of this lineage; (iii) one of the paralectotypes (SMF 6813) is characterized by regular dark spots in the central part of the dorsum as it is found in the spotted morph of this lineage but only very rarely in the second lineage; (iv) None of the types has an extended diffuse dark color on the flanks as it is typical for a large number of specimens of the second lineage. We are aware that this decision is ambiguous but consider it as the most taxonomically parsimonious solution to solving the identity of *G. bicalcaratus*.

Material examined. SMF 6811 (lectotype) and SMF 6812–6813 (paralectotypes), three adult females from “Insel Ste Marie” (= Nosy Boraha), collected by A. VOELTZKOW in 1903–1905. ZSM 425/2006 (ZCMV 3240), adult male, collected by J. E. RANDRIANIRINA and M. VENCES on 7–8 March 2006 in a forest on Nosy Boraha (no precise coordinates taken). Seven specimens, all collected by A. RAKOTOARISON, A. MALALAN^{NY} AINA RAKOTONDRAZAFY, F. M. RATSOAVINA, and R. D. RANDRIANIAINA in 2011, with the following details: RALC 9, juvenile, found close to a stream around Atafana Hotel, Nosy Boraha, on 9 May 2011, –16.91057, 49.86827, 31 m a.s.l.; RALC 42–44, three juvenile individuals a site in the extreme North of Nosy Boraha locally known as “Piscine naturelle”

(–16.727617, 50.014774); UADBA-A 62197, 62214 and 62198 (RALC 57–59), one female and two males, from Ambohidena forest on Nosy Boraha, collected on 12 May 2011, –16.83622, 49.96157, 1 m a.s.l.

Redescription. Based on the female specimen ZSM 425/2006. Specimen in good state of preservation, tissue removed from left thigh for molecular characterization. SVL 20.5 mm. For full morphometric measurements see Table 2. Head slightly longer than wide and somewhat wider than body; snout pointed in dorsal, ventral and lateral views; canthus rostralis distinct and concave; loreal region concave; nostrils nearer to tip of snout than to eye and protruding, tympanum distinct, 45% of horizontal eye diameter; supratympanic fold present from posterior edge of eye around the tympanum nearly to the arm insertion, dark in color (integrated in a broader dark band), rounded; small oblong patches of vomerine odontophores medial and posterior to choanae; maxillary teeth present; tongue stout, strongly bifid at tip and free posteriorly. Arms thin, length of lower arm is 86% of hand length; relative finger length $1 < 2 < 4 < 3$ ($1 < 2 = 4 < 3$ on right hand), finger discs moderately enlarged and circular in shape, no webbing between fingers, subarticular tubercles single, round and large. Hindlimbs long and slender, tibiotarsal articulation reaches center of eye when hindlimbs are adpressed against the body; femur length 79% of tibia length, foot length 81% of tibia length; lateral metatarsalia connected; inner metatarsal tubercle oblong (0.8 mm in length, 0.4 mm in width); outer metatarsal tubercle round and flat (0.3 mm in diameter); webbing formula between toes 1(–) 2i(–) [no webbing between toes 1 and 2] 2e(1) 3i(2.5) 3e(1.5) 4i(3) 4e(3.25) 5(2); relative length of toes $1 < 2 < 5 < 3 < 4$; toe discs moderately enlarged, width of fourth toe disc similar to width of third finger disc. Skin smooth dorsally and ventrally except granular ventral surface of thighs. Femoral glands absent.

After six years in preservative, dorsally with beige to light brown ground color with a pattern of small brown spots which are regularly spaced especially on hindlimbs where they do not form crossbands. Transversal crossbands present on forelimbs. A rather broad dark band running from snout tip over nostrils and eye to the tympanum and forelimb insertion. Pale and poorly defined broad dorsolateral bands formed largely by the absence of dark spotting in this region. Ventrally unpigmented with very fine black spots on throat and brown pigment on underside of hindlimbs. Dorsal color in life was similar, but more strongly contrasted (Fig. 5c).

Variation. All measured specimens (Table 2) had a similar relative hindlimb length, the tibiotarsal articulation reaching the anterior corner of the eye. The only reliably sexed male measured 20.9 mm, females collected by us measured 20.5–21.8 mm and the three female type specimens measured 24.1–27.3 mm. Females thus appear to be on average larger than males. No information on the shape of the vocal sac is available.

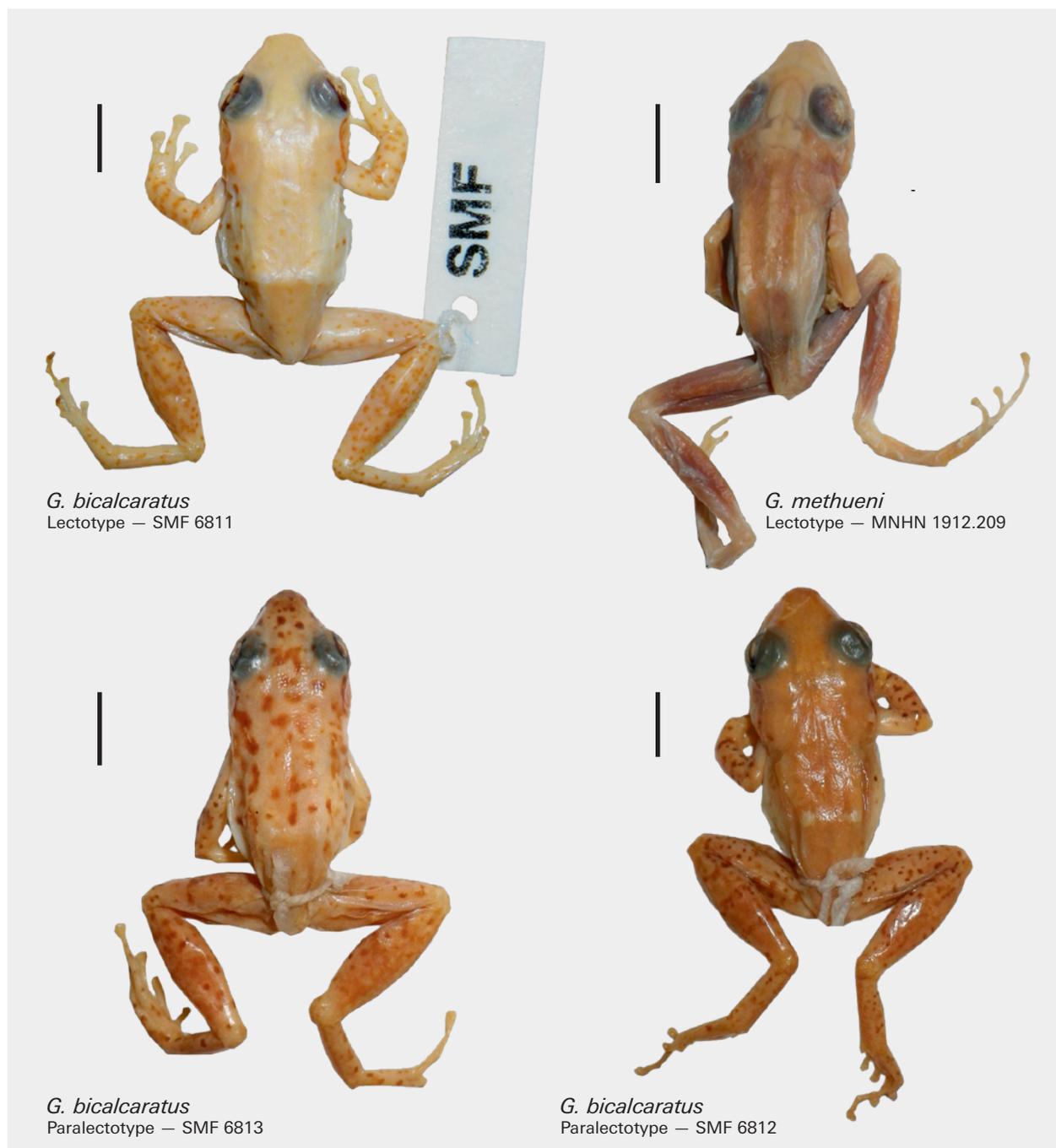


Fig. 3. Preserved type specimens of *Guibemantis bicalcaratus* and *G. methueni*. Scale bar = 5 mm.

Natural history and distribution. The species as redefined herein is only known from Nosy Boraha, although its occurrence at some localities on the adjacent mainland is probable. Almost no information is available on the habits of this species. All specimens were collected in the leaf axils of *Pandanus* plants. Calls and tadpoles are unknown.

Specimen RALC 59, an adult male from Ambohidena was found on dead leaves of *Pandanus* plants with water-filled axils, located in dense *Pandanus* forest, but no tadpoles were seen. 2–4 frog specimens were collected per plant but some plants were apparently unoccupied.

Guibemantis methueni (ANGEL, 1929)

Original name: *Gephyromantis methueni* ANGEL, 1929

Identity. After assigning the nomen *Rhacophorus bicalcaratus* BOETTGER, 1913 to the first of the two mitochondrial lineages encountered on Nosy Boraha based on the rationale provided in the Identity section of that species above, the question remains if any previous name is available for

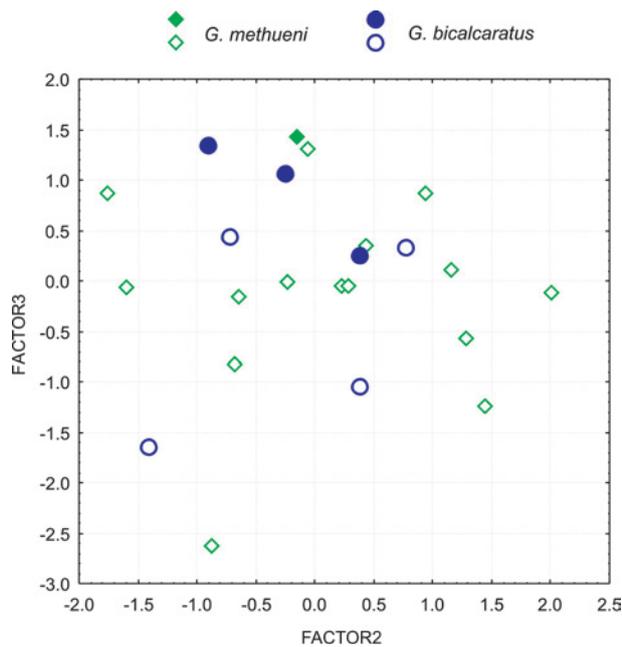


Fig. 4. Morphological differentiation among specimens of *Guibemantis bicalcaratus* and *G. methueni*. The plot shows factors 2 and 3 from a Principal Component Analysis based on morphometric data from Table 2. The filled rhomboid and circles indicate the lectotype of *G. methueni*, and lectotype and 2 paralectotypes of *G. bicalcaratus*. Males and females were merged because numerous individuals could not reliably be sexed (see Table 2). Note that the data are unable to separate the two species.

the second lineage. *Gephyromantis methueni* ANGEL, 1929 from the type locality Ambila is currently considered a junior synonym of *Guibemantis bicalcaratus* (see BLOMMERS-SCHLÖSSER, 1979; BLOMMERS-SCHLÖSSER & BLANC, 1991). The lectotype of *G. methueni* has a completely faded coloration and is in very poor state of preservation (Fig. 3). Morphometric data (Table 2 and Fig. 4) do not contradict this specimen being conspecific with specimens of the second lineage. Because this lineage was the only one that we found at Ambila, we conclude that it is taxonomically most parsimonious to resurrect it as a valid species in the genus *Guibemantis*, as *Guibemantis methueni*.

Material examined. MNHN 1929.209, lectotype (by implication) of *Gephyromantis methueni*, collected at “Ambila (lagune); province de Tamatave”, collected by G. PETIT. ZSM 429/2006 (ZCMV 3244), adult male, collected by J. E. RANDRIANIRINA and M. VENCES on 7–8 March 2006 in a forest on Nosy Boraha (no precise coordinates taken). 31 specimens, all collected by A. RAKOTOARISON, A. MALALAN’NY AINA RAKOTONDRAZAFY, F. M. RATSOAVINA, and R. D. RANDRIANIINA in 2011, with the following details: RALC 19–34 from Ile aux Nattes, far south of Nosy Boraha, E – 17.09960 S 49.80878, 2 m a.s.l.; RALC 35–36 and RALC 38 from Ampanihy forest on Nosy Boraha, –16.87188, 49.94505, at 7 m a.s.l.; and RALC 61 and RALC 63, two specimens from Ambila, –18.83430, 49.15687, collected on May 15th 2011. Of these specimens, 14 have been catalogued in UADBA, with numbers specified in Table 2.

Redescription. Based on the adult male specimen ZSM 429/2006. Specimen in good state of preservation but

hindlimbs somewhat dried (or too strongly fixed), and right hindlimb damaged (femur broken). SVL 21.5 mm. For full morphometric measurements see Table 2. Head slightly longer than wide and distinctly wider than body; snout pointed in dorsal, ventral and lateral views; canthus rostralis moderately developed, slightly concave; loreal region concave; nostrils much nearer to tip of snout than to eye and protruding; tympanum distinct, 50% of horizontal eye diameter; supratympanic fold present from posterior edge of eye around the tympanum nearly to the arm insertion, moderately dark in color, curved; large oblong patches of vomerine odontophores medial and posterior to choanae; maxillary teeth present; tongue stout, bifid at tip and free posteriorly. Arms thin, length of lower arm 61% of hand length; relative finger length $1 < 2 < 4 < 3$, finger discs moderately enlarged and circular in shape, no webbing between fingers, subarticular tubercles single, round and large. Hindlimbs long and slender, tibiotarsal articulation reaches between eye and nostril when hindlimbs are adpressed against the body; femur length 85% of tibia length, foot length 84% of tibia length; lateral metatarsalia largely connected; inner metatarsal tubercle oblong (1.2 mm in length); outer metatarsal tubercle round and flat; webbing formula between toes 1(1) 2i(1) 2e(1) 3i(2.5) 3e(1) 4i(3) 4e(3) 5(2); relative length of toes $1 < 2 < 5 < 3 < 4$; toe discs moderately enlarged. Skin smooth dorsally and ventrally, including the belly. Femoral glands (type 2) distinct and oblong (4.9 mm in length, 2.0 mm in width), sharply delimited, containing ca. 35 granules in internal view (after removal and reflection of skin).

After six years in preservative, dorsally beige with some very faint irregular darker mottling. A dark stripe runs from the snout tip over the nostril, eye, and tympanum to the forelimb insertion. Weakly expressed transversal crossbands on forelimbs, some darker pigment also on hindlimbs. Ventrally uniformly light (unpigmented). In life (Fig. 6f) similar, with a greenish shade and silvery white lateral pigment on throat marking the vocal sac which might thus be paired or bilobed subgular.

Variation. All measured specimens (Table 2) had a similar relative hindlimb length, the tibiotarsal articulation reaching the anterior corner of the eye. Two males measured 21.5–21.9 mm in SVL, six females measured 23.8–26.6 mm and thus were consistently larger. No calling specimens were observed, but the throat had a laterally whitish color in some males (Fig. 6f).

Vocalizations. During our expeditions, no vocalizations could be heard or recorded. However, three recordings exist that might represent *G. methueni* although this remains speculative and in need of confirmation. One of these recordings, from the 1990s, was provided by T. ZEHRER who recorded *Pandanusicola* frogs on the Ile aux Nattes where the sole species found by us was *G. methueni*. The call can be described as a single pulsed note containing 5–9 (mean 7) pulses. Irregular intervals were spaced between notes (855, 2828 und 4722 ms



Fig. 5. Specimens in life of *Guibemantis bicalcaratus*. (a) Uniformly colored female specimen from Ambohidena (Nosy Boraha) (RALC 57); (b) spotted specimen (probably a male) from Ambohidena (RALC 58); (c) dorsolateral view of a female specimen collected in 2006 on Nosy Boraha (ZCMV 3240 - ZSM 425/2006); (d) specimen from Nosy Boraha (not identified by molecular data, tentatively assigned to the species by color pattern only) photographed in 1991 in northern Nosy Boraha.

between the four notes recorded). Call duration (equaling note duration) was 258–482 ms (mean 378 ± 95 ms, $n=4$), pulse duration 10–18 ms (14 ± 2 ms, $n=29$), and interval duration between pulses 34–52 ms (45 ± 4 ms, $n=25$). The pulse repetition rate ranged between 18.7–19.7 pulses per second. Spectral frequency was between 2800–5000 Hz, dominant frequency 3600–4850 Hz.

These calls were very similar to those recorded by R. BLOMMERS-SCHLÖSSER in October 1971 from specimen ZMA 6952 at Fenoarivo, on the eastern Malagasy coast close to Nosy Boraha (BLOMMERS-SCHLÖSSER, 1979; calls published by VENCES *et al.*, 2006 under the name *G. bicalcaratus*). Three calls were emitted after intervals of 4511 and 3867 ms. They consisted each of a single pulsed note of a duration of 225, 304 and 305 ms (4, 5 and 5 pulses). Pulse duration was 6–11 ms (8 ± 2 ms, $n=14$), inter-pulse interval duration 60–74 ms (66 ± 4 ms, $n=11$), frequency 3100–5100 Hz, dominant frequency 3800–4600 Hz.

Different calls were recorded by M. VENCES in 2004 from specimens calling during the day in a *Pandanus* swamp in Besariaka, a place where our molecular data also suggest the presence of *G. methueni* (Fig. 1). These calls (referred to by VENCES *et al.*, 2006 as *G. sp. aff. bi-*

calcaratus) consist of regular series of single click notes. *Natural history and distribution.* The species is known from five low-altitude localities in the central portion of Madagascar's east coast: (1) its type locality Ambila, (2) several sites on Nosy Boraha, (3) Ile aux Nattes, (4) Antetezana north of Toamasina, and (5) close to Besariaka (south of Moramanga). Its elevational range is from near sea level (e.g., at Ile aux Nattes) to 980 m (Besariaka). Almost no ecological information is so far available on this species. The adult male RALC 26 from Ile aux Nattes was collected around 4:00 pm on a *Pandanus* plant along a foot path. This site is located on a little patch of *Pandanus* along the road at Ile aux Nattes, close to the village. 2–4 specimens were collected per plant, and only some plants had frogs sitting in their water-filled axils. No tadpoles were observed.

Discussion

The present study exemplifies the difficulties in taxonomically revising species in the subgenus *Guibemantis*



Fig. 6. Specimens in life of *Guibemantis methueni*. (a) specimen from Ambila (RALC 63); (b) Strongly patterned specimen collected in 2006 on Nosy Boraha; (c) specimen from Ile aux Nattes (Nosy Boraha; RALC 34); (d) specimen from Ile aux Nattes (RALC 33); (e - f) dorsolateral and ventral view of almost uniformly colored male specimen collected in 2006 on Nosy Boraha (probably specimen ZSM 429/2006 - ZCMV 3244).

(*Pandanusicola*). Despite three expeditions to Nosy Boraha (in 1991, 2006, and 2011) and although the last of these was specifically targeted to collect information on these frogs, no advertisement calls were heard and only few natural history observations could be made. Mitochondrial data indicate the presence of two deeply divergent mitochondrial lineages on Nosy Boraha but morphology and color pattern do not allow distinguishing them unambiguously. Mitochondrial and nuclear DNA

show concordant differentiation, confirming that gene flow among these two lineages is largely interrupted (only rare allele sharing despite sympatry). Along with additional evidence of faint (though not fully consistent) differences in color, this suggests that these two lineages should be seen as distinct species, despite the indication of possible hybridization. Although there is no doubt on the reality of species within *Pandanusicola*, we speculate that due to their faint differences in call and morphology,

and sharing of a similar breeding habitat, hybridization with introgression is perhaps more common than in many other mantellids.

Advertisement call recordings from three sites exist that might be referable to *G. methueni* or (less likely) to *G. bicalcaratus*, but this remains highly speculative without being able to link the call voucher specimens to either of the two species using molecular data. We here have described these calls tentatively under *G. methueni*, to inform and guide future bioacoustic assessments in *Pandanusicola*. It needs to be confirmed whether the pulsed notes (as recorded from the Ile aux Nattes) indeed refer to *G. methueni*; this island is very small and we could not find any other species of *Pandanusicola* there, which makes it at least plausible to assign these calls to *G. methueni*. Whether the regular series of single-click notes recorded from Besariaka also refer to this species (which then would have two quite distinct call types) or to a different *Pandanusicola* species is important to be clarified as well, in order to understand the value of bioacoustic characters in the taxonomy of these frogs.

Many of the yet unstudied and unnamed lineages of *Pandanusicola* are superficially similar to *G. bicalcaratus* and *G. methueni* but are distributed largely in allopatry (e.g., in the northern mountain massifs Marojejy, Manongarivo, and Montagne d'Ambre; LEHTINEN *et al.*, 2007; VIEITES *et al.*, 2009). Careful taxonomic work will be necessary to understand the taxonomic relevance of the genetic and (faint) chromatic variation observed and the potential value of acoustic signals. The analysis of nuclear multi-gene data sets in Bayesian assignment test frameworks (WEISROCK *et al.*, 2010) could provide an objective means for species delimitation in these frogs.

G. bicalcaratus has long been considered a widespread species in Madagascar's rainforests, ranging from the northern to the southern tips of the island and spanning a considerable elevational range (BLOMMERS-SCHLÖSSER & BLANC, 1991; GLAW & VENCES, 1994). Although widespread species of *Pandanusicola* exist, e.g. *G. pulcher* and especially *G. liber* (LEHTINEN *et al.*, 2012), it seems clear now that the majority of *Pandanus*-dwelling frogs in Madagascar have restricted ranges, similar to other small-sized and microendemic animals on the island (e.g., WILMÉ *et al.*, 2006; PABIJAN *et al.*, 2011; WOLLENBERG *et al.*, 2011; GLAW *et al.*, 2012). This probably also applies to *G. bicalcaratus* which according to its redefinition herein is only known from Nosy Boraha, while *G. methueni* is known from Nosy Boraha and several sites in the adjacent mainland coastal and mid-elevation forests and might have a slightly larger range.

At present the geographic ranges of *G. bicalcaratus* and *G. methueni* remain poorly known, and the same is true for their natural history. All specimens of both species have so far been collected in *Pandanus* leaf axils, and it is probable that eggs are laid and tadpoles develop within these phytotelms, as with the majority of *Pandanusicola*. According to our observations, at least *G. methueni* does not require dense and intact primary forest but can also survive in degraded areas as long as *Pandanus* plants

with water-holding leaf axils are present. In general, many coastal amphibian species in Madagascar seem to tolerate a certain degree of habitat disturbance (GEHRING *et al.*, 2010). Despite their very small ranges and ongoing forest destruction, it is therefore not certain whether *G. bicalcaratus* and *G. methueni* will qualify as threatened according to IUCN criteria. *G. bicalcaratus* has previously been listed as Least Concern due to its presumably vast distribution area and occurrence in many protected areas (ANDREONE *et al.*, 2005), but this is no longer accurate following our redefinition of the species. According to current knowledge, neither *G. bicalcaratus* nor *G. methueni* occur in any formally protected area. However, until more data on their distribution and habitat requirements become available, we suggest listing their conservation status as Data Deficient.

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