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High mitochondrial sequence divergence meets morphological and bioacoustic conservatism: *Boophis quasiboehmei* sp. n., a new cryptic treefrog species from south-eastern Madagascar

Miguel Vences [1], Jörn Köhler [2], Angelica Crottini [1,3] & Frank Glaw [4]

[1] Division of Evolutionary Biology, Zoological Institute, Technical University of Braunschweig, Spielmannstr. 8, D-38106 Braunschweig, Germany; E-mail: m.vences@tu-bs.de

[2] Department of Natural History – Zoology, Hessisches Landesmuseum Darmstadt, Friedensplatz 1, D-64283 Darmstadt, Germany

[3] Sezione di Zoologia e Citologia, Dipartimento di Biologia, Universitá degli Studi di Milano, Via Celoria 26, I-20133 Milano, Italy

[4] Zoologische Staatssammlung München, Münchhausenstr. 21, D-81247 München, Germany

Abstract. We describe a new species of treefrog from Madagascar that is highly similar in external adult morphology, bioacoustics and colouration to *Boophis boehmei* but differs from this species by a remarkable differentiation in a fragment of the mitochondrial 16S rRNA gene. A more detailed analysis revealed that this differentiation is concordant with the pattern in two nuclear genes (Rag1 and POMC) which show no haplotype sharing of the new species with *B. boehmei,* and with a consistent difference in tadpole morphology (third lower row of labial keratodonts reduced in length in the new species). We conclude that concordance between these independent characters indicates two independent evolutionary lineages that should best be considered as separate species, despite their similar adult morphology. The new species, *Boophis quasiboehmei* sp. n., is so far known only from an area in the southern central east and south-east of Madagascar, south of the Mangoro river, while *B. boehmei* is known only from the area around Andasibe north of the river Mangoro. Preliminary data indicate that this group of treefrogs contains several more cryptic species, and a simple explanation assuming the Mangoro river as a barrier being responsible for divergence between them is likely no longer tenable.

Key words. Amphibia, Anura, Mantellidae, *Boophis boehmei, Boophis quasiboehmei* sp. n., Madagascar.

INTRODUCTION

Treefrogs of the genus *Boophis* have long been among Madagascar's less studied amphibians, but intensified fieldwork and application of integrative taxonomy protocols have led to a steep increase of knowledge (Blommers-Schlösser 1979; Cadle 2003; Glaw & Vences 2007; Glaw et al. 2010). Many *Boophis* species call from high positions in the vegetation and intensive nocturnal searches for calling males are needed to find them. Consequently, many species have been described on the basis of only small series or even single individuals, and females are often unknown. Furthermore, many species of *Boophis* are known to be morphologically very similar and a diagnosis based on external morphology alone is often unreliable (Glaw et al. 2001; Vences et al. 2008). However, because the advertisement calls of these species are usually loud and species-specific (Vences et al. 2006), the integration of bioacoustics into their taxonomy has led to an improved understanding of *Boophis* species diversity. Together with an initial screening of molecular diversity, this has led to the description of many new species of *Boophis* (e.g., Andreone 1993, 1996; Andreone et al. 1995; Cadle 1995; Glaw & Thiesmeier 1993; Glaw & Vences 1992, 1994, 1997b, 2002; Glaw et al. 2001, 2010; Köhler et al. 2007, 2008; Vallan et al. 2003, 2010; Vences & Glaw 2002, 2005; Vences et al. 2010; Wollenberg et al. 2008) and the identification of a large number of additional, yet undescribed candidate species (Vieites et al. 2009). Furthermore, tadpoles of *Boophis* are among the most commonly encountered anuran larvae in Malagasy rainforest streams (Vences et al. 2008), and a large number of them have recently been described (e.g., Raharivololoniaina et al. 2006; Randrianiaina et al. 2009a, b).

Taking the latest species descriptions into account, the genus *Boophis*, classified in the endemic Malagasy-Comoroan family Mantellidae, currently comprises 71 described species. The genus is monophyletic and composed of two main clades that correspond to mainly stream-breeding (subgenus *Boophis*) and pond-breeding species (subgenus *Sahona*), respectively (Glaw & Vences 2006, 2007). The stream breeders are further divided into eight phenetic species groups. Most of these species groups probably are monophyletic units although some are not (particularly the *Boophis majori* group).

The *Boophis goudoti* species group contains 13 small to large species of largely arboreal frogs that are mainly distributed in the rainforests and highlands of Madagascar. A subgroup of small-sized species is characterized by colourful eyes, usually with red iris colour and a bluish iris periphery (Glaw & Vences 1997a, b). Several of these species such as *Boophis boehmei, B. burgeri, B. reticulatus,* and *B. rufioculis* are known to occur at the same locality in the Andasibe region in the northern central east of Madagascar and *B. reticulatus, B.* sp. aff. *rufioculis* and *B.* sp. aff. *boehmei* (= *B.* sp. 8 and *B.* sp. 16 of Vieites et al. 2009) in Ranomafana National Park in the southern central east. Of the various confirmed candidate species in the *B. goudoti* group (Glaw & Vences 2007; Vieites et al. 2009), four have recently been described (or older names were resurrected for them) on the basis of molecular, morphological, and/or bioacoustic differences (Glaw et al. 2010). However, no taxonomic conclusions have so far been drawn for the two candidate species from the Ranomafana region mentioned above (*B.* sp. 8 and *B.* sp. 16), mainly because of their high morphological similarity to *Boophis rufioculis* and to *B. boehmei,* respectively.

Boophis boehmei is the smallest species in the *B. goudoti* group and has been originally described from Andasibe, where it is rather common (Glaw & Vences 1992). Populations from more southern localities, initially allocated to this species (Ranomafana region and Andohahela) turned out to be genetically highly divergent (Vieites et al. 2009) and have therefore been considered as *Boophis* sp. aff. *boehmei* (Glaw & Vences 2007) or *B.* sp. 16 (Vieites et al. 2009), although no reliable morphological or bioacoustic difference between them had been observed. The recent discovery of differences in the tadpole labial tooth row arrangements of *Boophis boehmei* and *Boophis* sp. 16 (Randrianiaina et al. 2009b) prompted us to undertake a more detailed comparison. On the basis of high mitochondrial divergences, consistent differences in two nuclear genes, constant differences in tadpole morphology, and subtle differences in iris colour, we conclude that the central south-eastern populations indeed constitute a distinct species which we describe herein as *Boophis quasiboehmei.* It is however worth to note that *B. boehmei* and the newly described species are indeed among the morphologically and bioacoustically most cryptic species pairs so far discovered in Madagascar.

MATERIALS AND METHODS

Frogs were collected at night by opportunistic searching, using torches and head lamps. Specimens were euthanized in a chlorobutanol solution, fixed in 95% ethanol, and preserved in 70% ethanol. Locality information was recorded with GPS receivers. Specimens were deposited in the collection of Université d'Antananarivo, Département de Biologie Animale, Antananarivo (UADBA), Zoologisches Forschungsmuseum Alexander Koenig, Bonn (ZFMK), and the Zoologische Staatssammlung München (ZSM). FGMV, FGZC and ZCMV refer to F. Glaw and M. Vences field numbers. Terminology for biogeographic regions of Madagascar follows Boumans et al. (2007).

Morphological measurements (in millimetres) were all done by M. Vences with a digital caliper (precision 0.01 mm) to the nearest 0.1 mm. Used abbreviations are: SVL (snout–vent length), HW (greatest head width), HL (head length), ED (horizontal eye diameter), END (eye–nostril distance), NSD (nostril–snout tip distance), 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), and RHL (relative hindlimb length). Terminology and description scheme follow Glaw et al. (2010). Webbing formulae follow Blommers-Schlösser (1979). Statistical analyses were performed with Statistica software (Statsoft Corp., Tulsa, USA).

Vocalizations were recorded in the field using different types of tape recorders (Sony WM-D6C, Tensai RCR-3222) and external microphones (Sennheiser Me-80, Vivanco EM 238), and an Edirol R-09 digital recorder with internal microphones and saved as uncompressed files. Recordings were sampled (or re-sampled) at 22.05 kHz and 16-bit resolution and computer-analysed using the software CoolEdit 98. Frequency information was obtained through Fast Fourier Transformation (FFT; width 1024 points). Spectrograms were obtained at Hanning window function with 256 bands resolution. Temporal measurements are given as range, with mean ± standard deviation in parentheses. Terminology in call descriptions follows Köhler (2000).

Two different molecular data sets were studied:

First, we analyzed sequences of the mitochondrial 16S rRNA gene of around 500 bp from all *Boophis goudoti*

Table 1. Primer sequences and PCR conditions used in the present study. PCR conditions start with temperature (in °C) of each step followed by the time in seconds.

Gene	Primer name	Sequence (5' → 3')	Source	PCR conditions
BDNF	BDNF DRV 1	ACCATCCTTTTCCTKACTATGG	Vieites et al. (2007)	94(120), [94(20), 57(45),
BDNF	BDNF DRV 1	CTATCTTCCCCTTTTAATGGTC	Vieites et al. (2007)	72(120) 39], 72(600)
Rag1	Amp F2	ACNGGNMGICARATCTTYCARCC	s. Chiari et al. (2004)	94(120), [94(20), 50(50),
Rag1	Amp R2	GGTGYTTYAACACATCTTCCATYTCRTA	s. Chiari et al. (2004)	72(180) x 45], 72 (600)
POMC	POMC DRV F1	ATATGTCATGASCCAYTTYCGCTGGAA	Vieites et al. (2007)	95(120), [95(60), 58(60),
POMC	POMC DRV R1	GGCRTTYTTGAAWAGAGTCATTAGWGG	Vieites et al. (2007)	72(90) x 35], 72(600)

group species and candidate species with reddish iris colour as obtained by Vieites et al. (2009), Randrianiaina et al. (2009b) and Strauß et al. (2010). After alignment and removal of incomplete sections at its beginning and end the data set for analysis had a length of 479 bp. Unpartitioned Bayesian inference searches were performed. The best model of evolution (GTR+G) was determined by AIC in MrModeltest (Nylander 2002). Bayesian analyses were performed with MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). Two runs of 10 million generations (started on random trees) and four incrementally heated Markov chains (using default heating values) each, sampling the Markov chains at intervals of 1000 generations were used. The last 5001 trees were retained post burn-in and summarized to generate the majority rule consensus tree.

Second, we used tissue samples of four and one *Boophis boehmei* from Andasibe and An'Ala, respectively, and four and two tissue samples of *B. quasiboehmei* from Sahamalaotra (=Samalaotra) and Ambohitsara (Tsitolaka forest) for newly determining DNA sequences of various nuclear genes. Toe clips or leg muscle tissue samples (preserved in 95% ethanol) were used for DNA extraction. Total genomic DNA was extracted from the tissue samples using proteinase K digestion (10 mg/ml concentration) followed by a standard salt extraction protocol (Bruford et al. 1992). We amplified fragments of three genes from the nuclear DNA (nuDNA): brain-derived neurotrophic factor (BDNF), recombination activating gene 1 (Rag1), and pro-opiomelanocortin (POMC). Standard Polymerase chain reactions were performed in a final volume of 11 µl and using 0.3 µl each of 10 pmol primer, 0.25 µl of total dNTP 10 mM (Promega), 0.08 µl of 5 U/ml GoTaq, and 2.5 µl 5X Green GoTaq Reaction Buffer (Promega). Primers and detailed PCR conditions are provided in Table 1. PCR products were then purified through QIAquick purification kit (Qiagen) according to the manufacturer's instruction. Purified PCR templates were sequenced on an automated DNA sequencer (Applied Biosystems ABI 3130XL). Chromatographs were checked and sequences were edited using CodonCode Aligner (v. 2.0.6, Codon Code Corporation). All newly determined sequences have been deposited in GenBank (HQ380132-HQ380172). Haplotypes of POMC data were inferred using the PHASE algorithm (Stephens et al. 2001) implemented in DnaSP software (Version 5.10.3; Librado & Rozas 2009). Haplotype network reconstruction of phased sequences of the POMC (Fig. 2A) and Rag1 (Fig. 2B) fragments were performed using the software TCS, version 1.21 (Clement et al. 2000). This software employs the method of Templeton et al. (1992) and it calculates the number of mutational steps by which pairwise haplotypes differ, computing the probability of parsimony for pairwise differences until the probability exceeds 0.95 (no manual adjustment of threshold was necessary).

RESULTS

A detailed analysis of all available 16S rRNA sequences of adults and tadpoles assigned to *B. boehmei* (GenBank accession numbers GQ904739–GQ904746, DQ792470–DQ792471, AY341717, AY848560–AY848562) and the candidate species *B.* sp. 16 (sensu Vieites et al. 2009) (accession numbers GQ904717–GQ904738, AY848529–AY848536) confirmed that these two forms are genetically highly divergent. Depending on the length of the sequence available, the uncorrected pairwise distances were between 8.8% and 11.0% (note that these values are higher than the 6.8% reported by Vieites et al. (2009) because of different lengths of the sequences, with a different proportion of hypervariable sites included in the analysis). Next to single substitutions we also detected one major insertion of seven nucleotides in the candidate species which in this extent was not present in any of the related species of *Boophis* (Fig. 1). Pairwise divergences were 0.0–0.9% within *B. boehmei,* 0.0–0.5% within specimens of *B.* sp. 16 from the Ranomafana region, and 3.6–4.9% between the single available sequence of *B.* sp. 16 from Andohahela

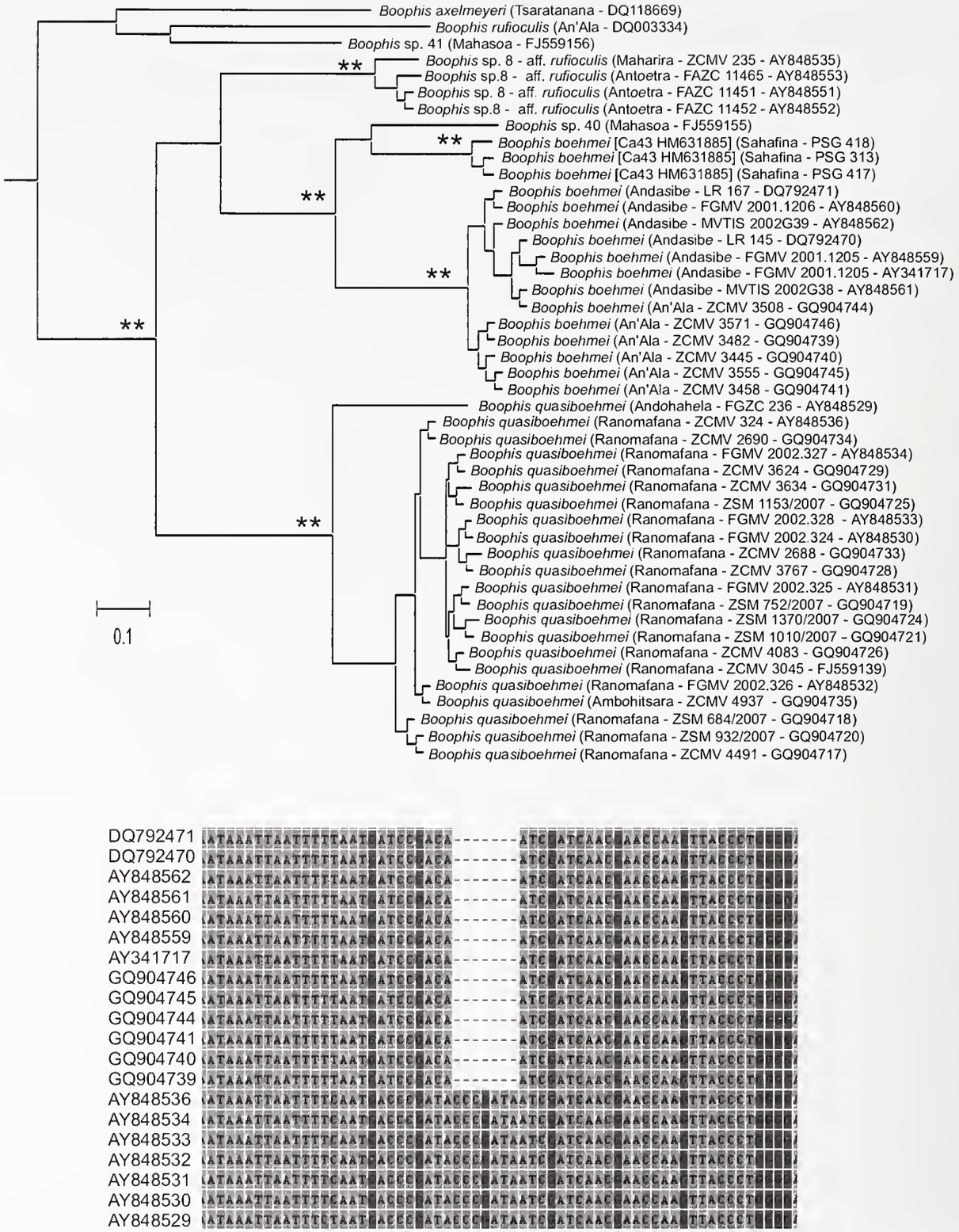

Fig. 1. Phylogenetie tree of speeies and eandidate speeies of the *Boophis goudoti* group with red iris eolour, obtained using Bayesian inferenee based on DNA sequenees of the mitoehondrial 16S rRNA gene (alignment length 479 bp). Bayesian posterior values >0.95 symbolized by a single asterisk, of 0.99-1.00 by two asterisks. For eaeh sequenee, loeality, voueher number and Genbank number are given in parentheses. *Boophis goudoti* was used as outgroup (not shown). Note that the deeper phylogenetie relationships shown are not reliable due to the limited amount of sequenee information used in the analysis, and aeeording to an unpublished multi-gene data set of K. C. Wollenberg, *B. boehmei* and *B. quasiboehmei* are probably sister groups. The alignment in the lower part of the figure shows a seetion of the 16S alignment, with sequenees of *Boophis boehmei* (upper 13 sequenees; numbers to the left are Genbank aeeession numbers) and *Boophis quasiboehmei* (lower seven sequenees). The insertion of seven nueleotides is a synapomorphy of all *B. quasiboehmei* specimens for whieh a sequenee was obtained, and in this extent is laeking also in all other species of the *B. goudoti* group.

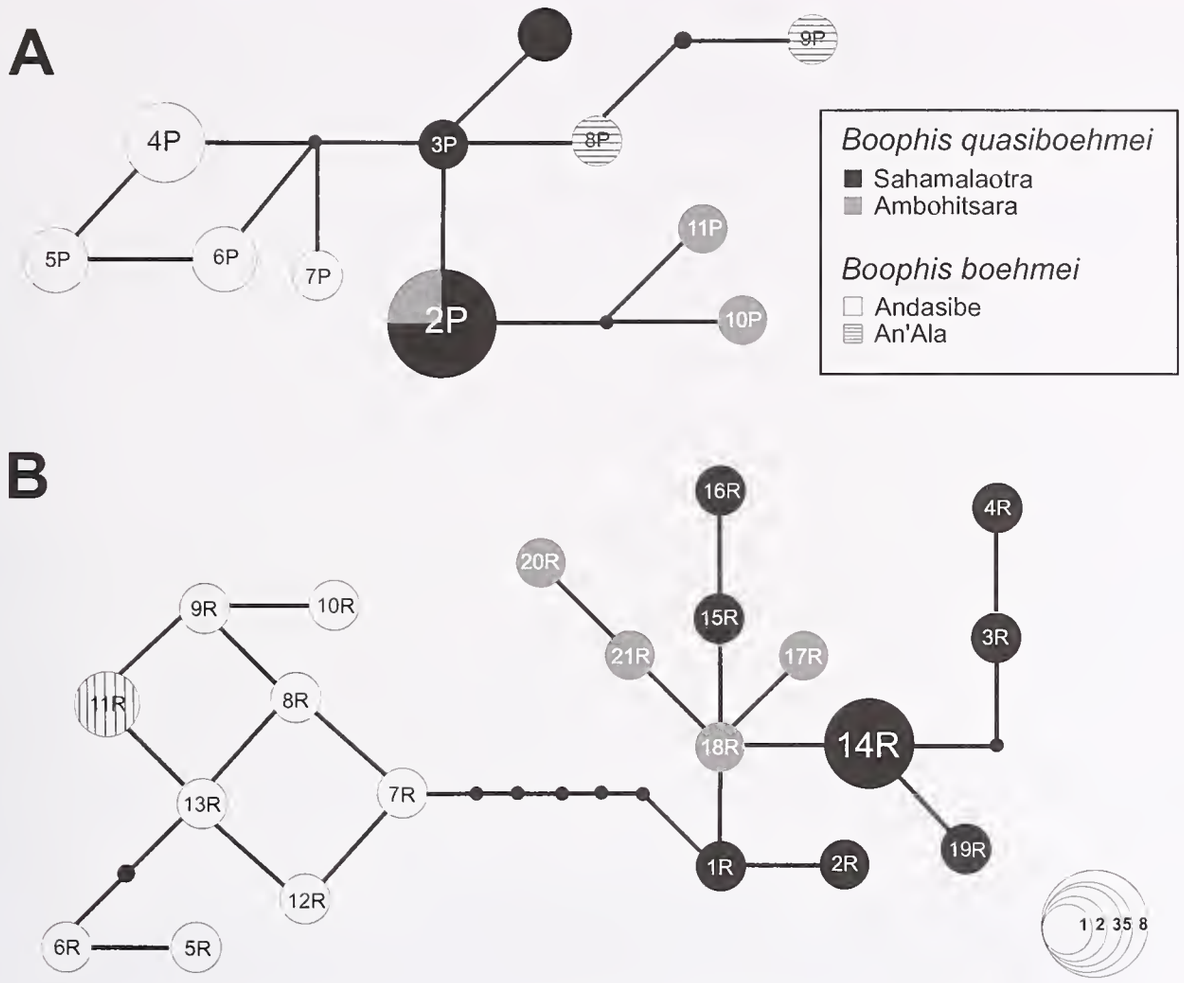

Fig. 2. Haplotype networks of the nuclear POMC (A) and Rag1 (B) genes fragments in *B. boehmei* and *B. quasiboehmei,* each from two different localities. Haplotypes per each individual were inferred using the Phase algorithm. The networks show complete absence of haplotype sharing among the two taxa.

(AY848529) and those from Ranomafana. Genetically identified specimens assigned to *B. boehmei* were from Andasibe and An'Ala. Specimens from Sahafina (Gehring et al. 2010) had quite divergent DNA sequences and their status is unclarified, but they clustered with *B. boehmei* (Fig. 1). Following the scheme suggested by Padial et al. (2010), this population was considered a new unconfirmed candidate species *Boophis boehmei* [Ca43 HM631885] by Gehring et al. (2010). Probably, specimens from Ankeniheny for which no molecular data are available belong to this species as well. Specimens assigned to *B.* sp. 16 were from the Ranomafana area (including Ambatovory, Sahamalaotra, Imaloka, Kidonavo, Vohiparara) and Ambohitsara, as well as from Andohahela.

Besides a simple assessment of molecular divergences between *Boophis* sp. 16 and *B. boehmei* it is also necessary to comment on its phylogenetic position. The analysis of Vieites et al. (2009) placed *B. boehmei* with *B.* sp. 8 from Ranomafana and *B.* sp. 40 from Mahasoa forest, and the clade made up by these species was sister to *B.* sp. 16. Our analysis (Fig. 1) included sequences of all these taxa and confirmed the phylogenetic relationships suggested by Vieites et al. (2009). However, an unpublished analysis based on multiple mitochondrial genes by K.C. Wollenberg instead suggested a probable sister-group relationship between *B. boehmei* and *B.* sp. 16, confirming that the 16S rRNA gene alone as used here is insufficient to clarify the phylogeny among *Boophis* species. Altogether, the phylogenetic relationships among all these species require a much more detailed analysis which however is beyond the scope of the present paper.

The results of the mitochondrial marker indicate no or limited gene flow between *B. boehmei* and *B.* sp. 16. This result was corroborated by the analysis of two nuclear markers (Fig. 2; the conserved BDNF gene showed no variation). While in POMC (Fig. 2A), the single included An'Ala specimen had a different haplotype not clustering with those of Andasibe, in Rag1 the haplotypes belonging to the two species formed two well-defined clusters separated from each other by a minimum of six mutation-

Table 2. Morphometric measurements (all in mm) of examined voucher specimens of *Boophis boehmei* and *B. quasiboehmei*. For abbreviations of morphometric measurements and collection acronyms see Materials and Methods. Additional abbreviations: HT, holotype; PT, paratype; M, male; F, female. RHL (relative hindlimb length) is coded as follows: when hindlimb is adpressed along body, tibiotarsal articulation reaches (1) anterior eye corner, (2) nostril, (3) between nostril and snout tip, (4) snout tip, (5) beyond snout tip.

Number	Field number	Type	Locality	Sex	SVL	HW	HL	TD	ED	END	NSD	NND	FORL	HAL	HIL	FOTL	FOL	TIBL	RHL
B. boehmei	–																		
ZFMK 53642	–	HT	Andasibe	M	29.4	11.3	11.9	2.0	3.9	2.4	2.3	3.2	17.9	9.0	48.4	21.2	12.4	16.4	2
ZSM 563/1999	(ZFMK 53643)	PT	Andasibe	M	28.4	10.9	11.4	2.2	3.9	2.3	2.4	3.4	17.2	8.5	46.5	20.9	11.8	15.2	2
ZFMK 52637	–	PT	Andasibe	M	27.8	10.8	11.5	2.0	4.0	2.2	2.3	3.0	17.1	8.5	46.6	19.9	11.2	15.3	2
ZFMK 52638	–	PT	Andasibe	M	28.7	11.4	11.7	2.1	4.2	2.3	2.4	3.6	18.2	8.9	49.2	20.6	12.3	14.9	2
ZFMK 52639	–	PT	Andasibe	M	28.5	10.7	11.6	2.0	3.6	2.2	2.2	3.0	18.4	9.3	49.3	21.3	12.5	15.6	2
ZSM 304/2000	–	–	Andasibe	M	29.0	11.5	11.9	2.1	4.4	2.5	2.4	3.4	19.0	9.2	50.2	22.1	12.7	16.1	4
ZSM 22/2002	FGMV 2001.1191	–	Andasibe	M	30.8	10.7	11.4	2.0	4.0	2.3	2.4	3.0	18.4	9.6	48.5	21.6	12.8	15.2	2
ZSM 23/2002	FGMV 2001.1193	–	Andasibe	M	30.6	11.5	12.0	2.2	4.0	2.5	2.5	3.6	19.3	9.4	50.3	21.6	12.5	15.6	2
ZSM 24/2002	FGMV 2001.1195	–	Andasibe	M	28.5	11.0	11.4	2.0	4.2	2.4	2.3	3.3	19.0	8.7	47.6	20.3	12.0	14.9	2
ZFMK 50649	–	–	Andasibe	M	28.8	10.6	10.9	2.2	4.1	2.3	2.6	3.4	16.4	8.6	48.8	20.8	12.3	15.4	4
ZFMK 50650	–	–	Andasibe	M	30.3	11.1	11.4	2.1	4.4	2.4	2.7	3.9	19.2	9.3	50.3	22.4	12.7	16.3	2
ZFMK 60084	–	–	An'Ala	M	27.6	10.6	11.1	2.0	4.2	2.2	2.7	3.3	17.3	8.2	47.8	20.6	12.1	15.2	5
ZSM 25/2002	FGMV 2001.1206	–	Andasibe	F	37.7	14.0	15.2	2.3	5.0	3.1	3.0	4.6	24.9	11.9	62.5	27.9	15.9	20.2	5
ZSM 225/2006	ZCMV 2400	–	An'Ala	F	41.5	14.7	14.9	3.3	5.0	3.3	3.3	4.4	25.1	12.3	63.7	28.7	16.8	20.0	1
ZFMK 60028	–	–	Andasibe	F	40.6	14.8	15.4	2.9	4.3	3.7	3.5	4.2	24.0	11.8	65.5	28.8	17.3	21.1	2
ZFMK 60029	–	–	Andasibe	F	41.6	14.6	14.9	2.4	5.0	3.2	3.4	3.9	24.2	12.0	62.8	28.8	17.3	19.8	1
B. quasiboehmei																			
ZSM 227/2006	ZCMV 3045	HT	Ambatovory	M	26.7	10.6	11.2	2.0	3.7	2.3	2.3	3.3	17.7	9.1	48.1	20.8	11.1	15.2	5
ZSM 715/2003	FG/MV 2002-0363	PT	Vohiparara	M	28.8	10.6	11.0	1.9	3.7	2.3	2.5	3.7	18.4	9.0	48.0	20.8	11.9	15.5	2
ZSM 224/2006	ZCMV 2988	PT	Sahamalaotra	M	27.5	10.7	11.6	1.9	4.1	2.5	2.5	3.3	17.8	8.8	48.2	21.1	12.0	15.1	4
ZSM 226/2006	ZCMV 2951	PT	Imaloka	M	28.6	10.7	11.3	1.8	3.9	2.2	2.0	3.3	18.8	9.6	50.9	22.8	12.7	16.2	5
ZSM 228/2006	ZCMV 3051	PT	Ambatovory	M	29.3	11.2	11.6	1.8	4.1	2.4	2.6	3.4	20.5	9.9	50.9	22.9	13.2	16.2	2
ZSM 229/2006	ZCMV 3069	PT	Ambatovory	M	28.6	11.1	11.1	2.0	3.9	2.3	2.4	3.2	19.0	9.6	51.3	22.6	12.5	15.8	5
ZSM 230/2006	ZCMV 3070	PT	Ambatovory	M	28.4	10.7	11.5	2.1	3.9	2.6	2.7	3.2	17.9	9.4	49.3	21.8	12.8	15.4	2
ZSM 231/2006	ZCMV 3360	PT	Ranomena	M	27.8	10.5	11.0	2.0	3.9	2.3	2.7	3.3	17.5	8.4	45.9	20.4	12.0	14.3	2
ZSM 232/2006	ZCMV 3374	PT	Ranoma -fanakely river (?)	M	28.9	10.7	11.2	1.9	4.0	2.3	2.7	3.2	18.3	9.1	48.5	21.3	12.6	15.5	3
ZFMK 59881	–	PT	Ranomafana region	M	29.2	11.5	11.8	2.0	4.1	2.6	2.8	3.6	18.0	9.6	49.5	22.5	13.3	16.6	3
ZFMK 59882	–	PT	Ranomafana region	M	29.3	11.1	11.3	1.9	4.0	2.3	2.4	3.7	18.2	9.4	48.3	21.6	13.1	15.4	2
ZSM 178/2006	BOR 1079?	–	Befotaka-Midongy	M	30.8	11.5	11.9	1.9	4.2	2.3	3.0	3.8	19.1	9.8	50.4	22.1	12.8	16.0	3

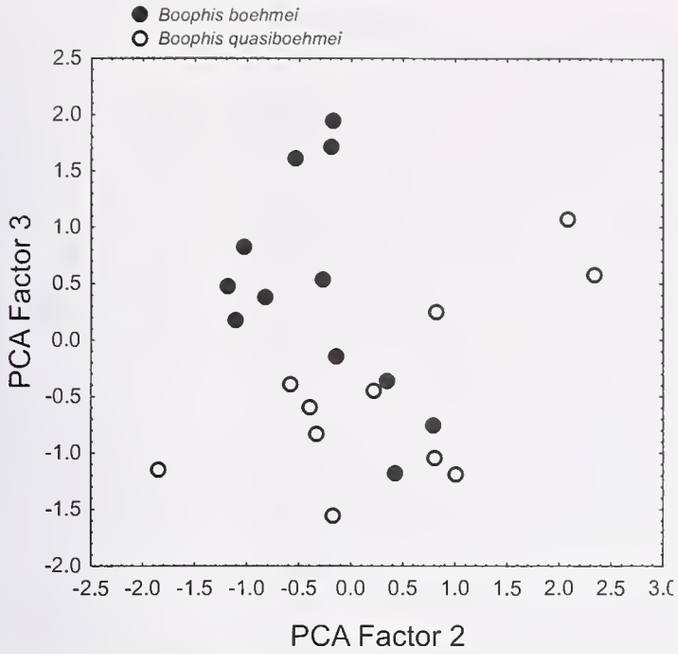

Fig. 3. Scatterplot of individual males of *Boophis boehmei* (filled circles) and *B. quasiboehmei* (open circles) along the second and third factor of a Principal Component Analysis (Varimax normalized rotation). The PCA was based on measurements in Table 1. The specimen from Midongy was excluded from analysis because the species identity of this population is not fully clarified.

al steps. Although the nuclear data set refers to only a limited number of specimens, the fact that there is no haplotype sharing between the two forms suggests that they represent independent evolutionary lineages.

Nevertheless, these pronounced genetic divergences were contrasted by no or low divergences in adult morphology and bioacoustics. The calls of the two forms were similar, with no detectable differences (see call descriptions below). In both forms, notes may be combined to short regular series, and intervals between notes are otherwise highly variable and mostly irregular. The temporal and spectral parameters in calls of both forms are somewhat variable among populations and individuals, but broadly overlap at inter- and intra-populational level. Even the pulse rate within notes, a character shown to be evolutionary highly dynamic among closely related species (e.g. Padial et al. 2008), is identical in both forms (see analysis below). Inter-note intervals outside of regular note series furthermore seem to depend on calling motivation of the individual male.

A close examination of adult morphology yielded no discrete characters that would allow a diagnosis between the two forms. One subtle difference was detected in adult life

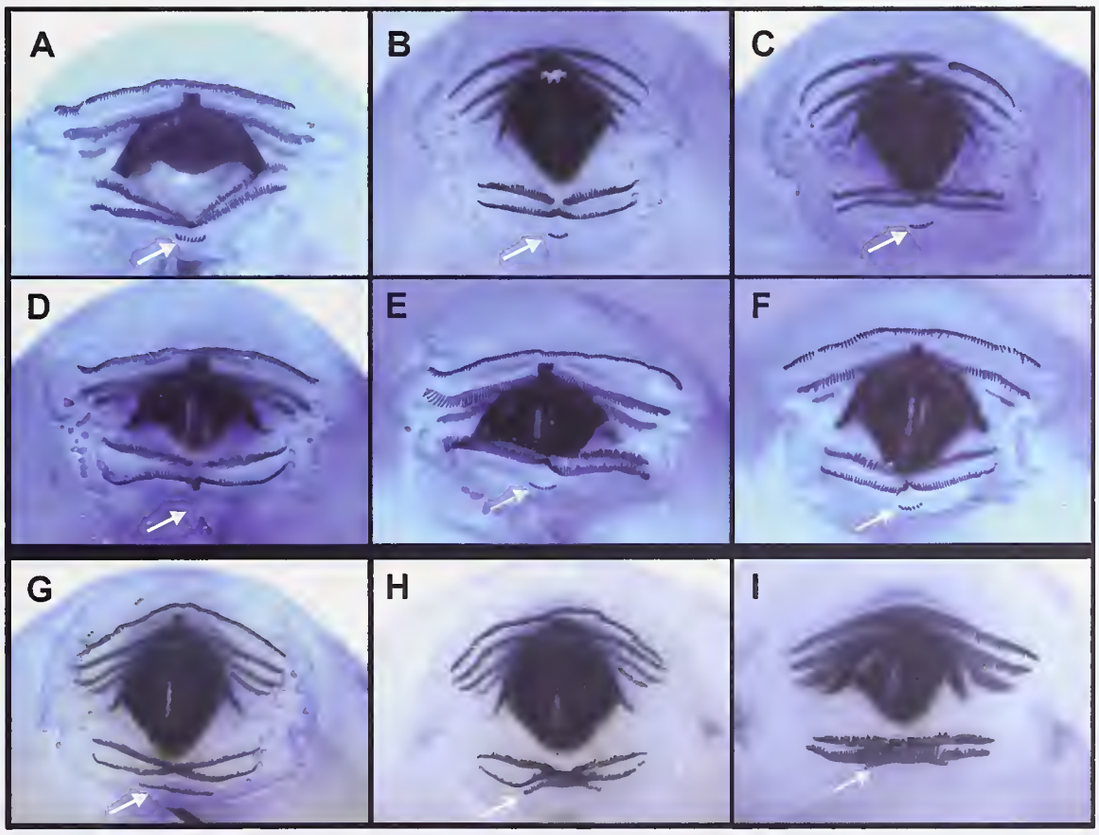

Fig. 4. Comparative photographs of oral discs of preserved tadpoles of *Boophis quasiboehmei* sp. n. (top, A–F) and *Boophis boehmei* (bottom, G–I): (A) ZSM 442/2008, Imaloka; (B–C) ZSM 83–84/2008, Ambohitsara; (D) ZSM 509/2008, Ambatolahy; (E) ZSM 1682/2007, Sahalamaotra; (F) ZSM 443/2008, Imaloka; (G–I) ZSM 1738/2007, 1750/2007, 1779/2007, An'Ala. Note the short third lower (= posterior) keratodont row of *Boophis quasiboehmei* sp. n. with only few (or even misssing [D]) keratodonts, and the less reduced legth of this row in *B. boehmei* (indicated by white arrows). In the tadpoles of other species of the *Boophis goudoti* group, the third lower keratodont row is more extended than in the two species shown (see Randrianiaina et al. 2009b).

Table 3. Factor loadings, Eigenvalues and percent explained variation from a Principal Component Analysis of morphometric data in Table 2. Factor loadings >0.5 are shown in bold.

	Factor 1	Factor 2	Factor 3	Factor 4
SVL	**0.535637**	0.024396	0.278024	0.432915
HW	0.401178	-0.052815	**0.722325**	0.434988
HL	0.283729	-0.059521	**0.901536**	-0.000783
TD	-0.451112	0.171742	**0.508941**	0.315946
ED	0.042109	0.184460	0.248786	**0.727413**
END	0.263940	**0.645782**	**0.593063**	0.078554
NSD	-0.058373	**0.886713**	-0.111786	0.323847
NND	0.169004	0.159007	-0.021443	**0.796064**
FORL	**0.776970**	-0.189957	0.113991	0.209375
HAL	**0.936216**	0.015471	0.113066	-0.107870
HIL	**0.852890**	-0.051712	0.150553	0.154111
FOTL	**0.932469**	0.147455	0.044485	0.068645
FOL	**0.784388**	0.333820	0.095038	0.209488
TIBL	**0.743402**	0.023610	0.266693	0.084073
Eigenvalue	5.977193	2.369640	1.412833	0.905600
% Variance	42.69424	16.92600	10.09166	6.46857

colouration. All specimens of *B. boehmei* had a bright red outer iris area and a brownish inner iris area, whereas *B.* sp. 16 had no such bright red colour but orange, either as a more or less uniformly orange iris or as an orange outer iris area.

In a search for a possible morphometric differentiation, we carried out a Principal Component Analysis on the basis of measurements in Table 2 (males only). The analysis resulted in three factors with Eigenvalues greater than 1 (Table 3) which together explained 70% of the total variation. Because size of specimens was similar, the first factor was not representative mainly of body size, but of relative limb length; the highest factor loadings were for variables associated with limb length (Table 3). Factors 2 and 3 were associated with the shape of the head: Factor 2 with END and NSD, and Factor 3 with mainly HW and HL. While Factor 1 resulted only marginally in a trend of separation of the two species (not shown), Factors 2 and especially 3 separated most specimens of *B. boehmei* vs. *B.* sp. 16 (Fig. 3). However, univariate analyses on the basis of the variables with highest factor loadings did not result in a convincing separation (not shown), indicating that morphometric data cannot serve as diagnostic characters to separate these two forms.

The most convincing diagnostic character comes from tadpole morphology and has been described in detail by Randrianiaina et al. (2009a, b): all tadpoles of *Boophis* sp. 16 (from Ranomafana and Ambohitsara; N = 75) examined had a short (or completely absent) third posterior row of labial keratodonts (P3), whereas in *B. boehmei* (from localities Andasibe and An'Ala) this row was slightly shorter than in other species of the *B. goudoti* group, but still much longer than in *B.* sp. 16, with no overlap in numbers of labial keratodonts in P3 and almost no overlap in relative length of P3 (Fig. 4).

Given this constant difference in tadpole morphology which fully correlates with high mitochondrial divergences (among the highest observed between closely related mantellid frog species), and with fully separated haplotypes in two nuclear genes, we conclude that *B. boehmei* and *B.* sp. 16 constitute two separate and independent evolutionary lineages. Therefore, they should best be considered as distinct species, although cryptic in adult morphology and advertisement calls. In the following we thus describe *B.* sp. 16 as a new species.

Boophis quasiboehmei sp. n.

(Figs 5–6)

Holotype. ZSM 227/2006 (field number ZCMV 3045), adult male (Fig. 5), collected at Ambatovory, at the edge of Ranomafana National Park, south-eastern Madagascar, 21°14,279' S, 47°25,487' E, 966 m a.s.l., on 26 February 2006 by M. Vences, Y. Chiari, T. Rajoafiarison, E. Rajeriarison, P. Bora and T. Razafindrabe.

Paratypes. ZFMK 59881–59882, two adult males, collected in the Ranomafana region, south-eastern Madagascar, in December 1994 by M. Burger; ZSM 715/2003 (FG/MV 2002-0363), one adult male, collected at Vohiparara (close to the Kidonavo bridge), Ranomafana National Park, 21°13' S, 47°22' E, ca. 1000 m a.s.l., on 20 January 2003, by F. Glaw, M. Puente, L. Raharivololoniaina, M. Thomas and D. R. Vieites; ZSM 228/2006 (ZCMV 3051), ZSM 229/2006 (ZCMV 3069), and ZSM 230/2006 (ZCMV 3070), three adult males, from same locality and with same collectors and collection date; ZSM 224/2006 (ZCMV 2988), male, collected at Sahamalaotra, Ranomafana National Park, south-eastern Madagascar, 21°14.113' S, 47°23.767' E, south-eastern Madagascar, on 25 February 2006 by M. Vences, Y. Chiari, T. Rajoafiarison, E. Rajeriarison, P. Bora and T. Razafindrabe; ZSM 226/2006 (ZCMV 2951), male, collected at Imaloka, Ranomafana National Park, south-eastern Madagascar, 21°14,527' S, 47°27,909' E; 1020 m a.s.l., on 23 February 2006 by Y. Chiari, P. Bora, T. Rajoafiarison, E. Rajeriarison, and T. Razafindrabe; ZSM 231/2006 (ZCMV

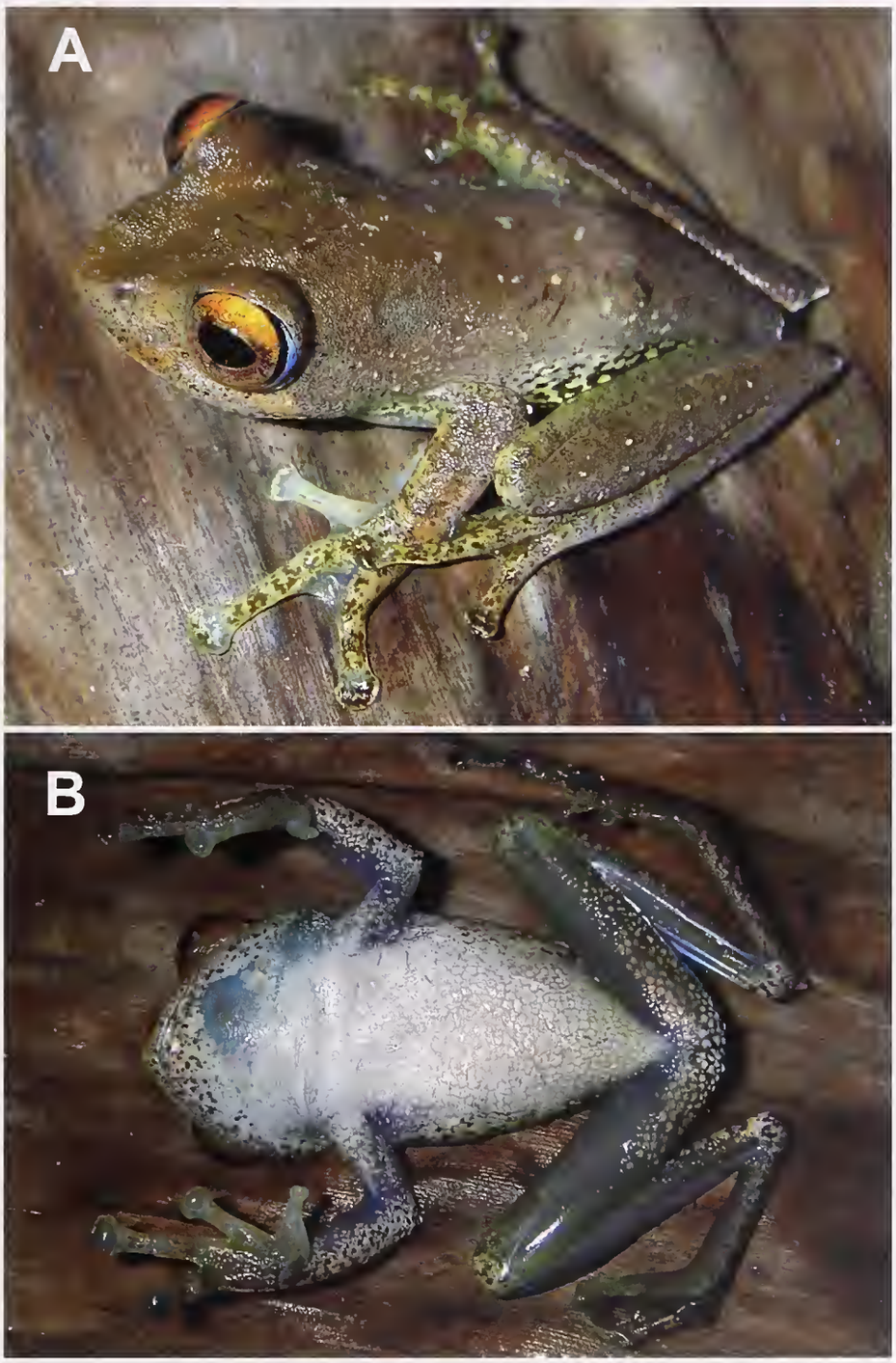

Fig. 5. Dorsolateral (A) and ventral (B) views of the male holotype of *Boophis quasiboehmei* sp. n. (ZSM 227/2006) in life.

3360), male, collected at Ranomena, 21°12,736' S, 47°26,010' E, Ranomafana National Park, south-eastern Madagascar, on 28 February 2006, M. Vences, Y. Chiari, T. Rajoafiarison, and E. Rajeriarison; ZSM 232/2006 (ZCMV 3374) from the Ranomafana region, perhaps collected at Ranomafanakely river but without precise collecting data; ZSM 2322/2007 (ZCMV 5948), male, collected at Sahamalaotra, Ranomafana National Park, south-eastern Madagascar, 21°14.113' S, 47°23.767' E, on 5 March 2007 by M. Vences, A. Strauß, R. D. Randrianiaina, and K. C. Wollenberg.

Diagnosis. Assigned to the genus *Boophis* based on the presence of an intercalary element between ultimate and penultimate phalanges of fingers and toes (verified by external examination), presence of nuptial pads and absence of femoral glands in males, and overall similarity to other *Boophis* species. Assigned to the *Boophis goudoti* group because of its brownish ground colour, presence of dermal flaps or tubercles on heels and elbows, presence of white tubercles ventrally of the cloacal opening, presence of a sharp canthus rostralis, absence of red skin colour, and molecular phylogenetic relationships.

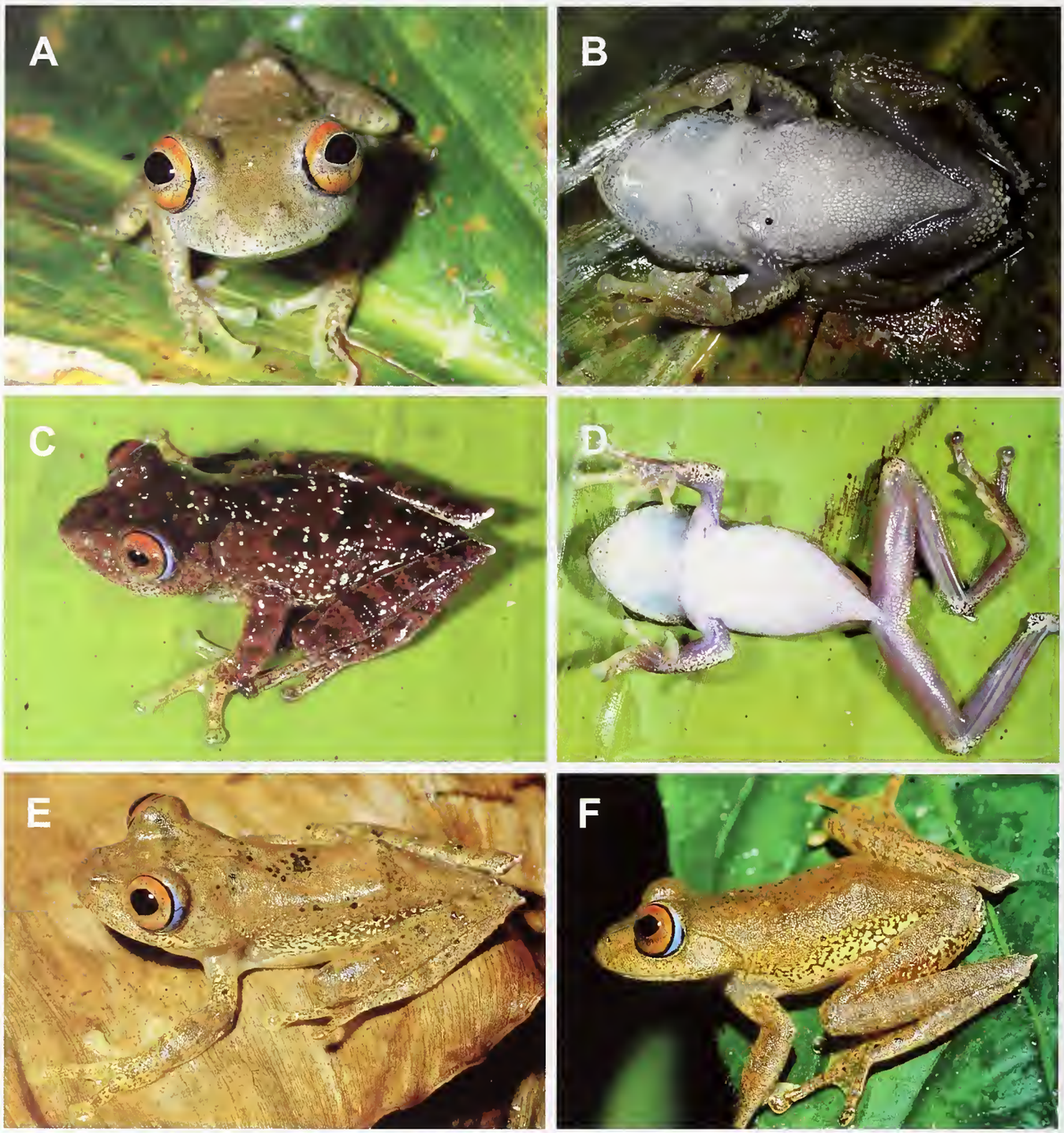

Fig. 6. Specimens of *Boophis quasiboehmei* sp. n. in life: (A) frontal and (B) ventral views of a male from Ambohitsara (field number ZCMV 5867); (C) dorsolateral and (D) ventral views of a male from Andohahela (deposited in UADBA); (E) male from Ranomafana (deposited in UADBA); (F) male paratype ZFMK 59882 from Ranomafana (photo by M. Burger).

Together with *B. boehmei*, the smallest species in the *Boophis goudoti* group characterized by a deviant oral morphology of the tadpole which is unknown from any other *Boophis* species. *Boophis quasiboehmei* sp. n. differs from all described species in the *B. goudoti* group by substantial genetic differentiation (> 6% pairwise divergence in a fragment of the 16S rRNA gene) and furthermore from *B. goudoti, B. obscurus, B. periegetes, B. madagascariensis, B. roseipalmatus, B. brachychir, B. entingae, B. rufioculis*, *B. burgeri*, *B. reticulatus*, *B. axelmeyeri*, and *B. spinophis* by smaller size (SVL of adult males 28–31 mm versus 31–82 mm) and bioacoustic differentiation (see Vences et al. 2006 for details). *B. quasiboehmei* sp. n. is most similar to *B. boehmei* and differs

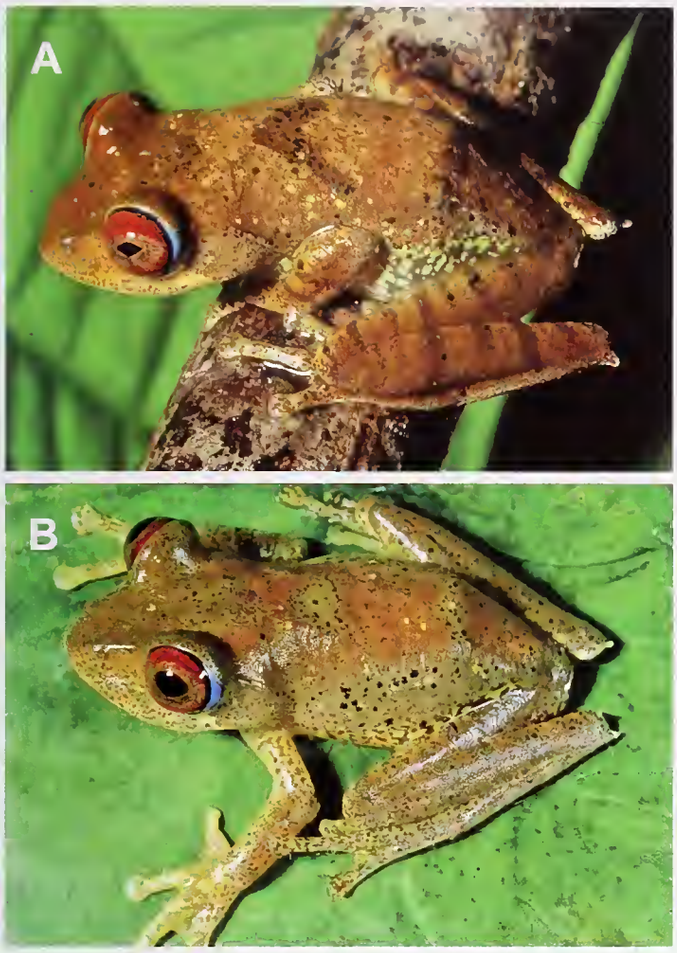

Fig. 7. Male specimens of *Boophis boehmei* from Andasibe in life: (A) paratype ZSM 563/1999 (originally ZFMK 53643); (B) paratype ZFMK 52637.

from this species by an orange (versus red) outer iris ring, by a very short third posterior keratodont row in the tadpole, consisting of only 0–15 keratodonts (versus 23–63 keratodonts in *B. boehmei*, see Randrianiaina et al. 2009b), and substantial genetic differentiation.

Description of holotype. Adult male in excellent state of preservation, muscles of right thigh removed as DNA tissue sample. SVL 26.7 mm. Body moderately slender; head slightly longer than wide, wider than body; snout pointed in dorsal view, obtuse to acuminate in lateral view; nostrils directed laterally, eqidistant to eye and to tip of snout; canthus rostralis sharp, straight in dorsal view from eye to nostril, slightly curved from nostril to tip of snout; loreal region slightly concave; eye large; tympanum distinct, rounded, TD 54% of ED; supratympanic fold narrow, prominent; vomerine odontophores distinct, well separated in two slightly elongated patches, positioned median between choanae; choanae medium-sized, rounded. Tongue distinctly bifid and free posteriorly. Arms moderately slender; a small pointed dermal appendage on elbow; subarticular tubercles single, round; inner palmar tubercle poorly recognizable; fingers poorly webbed and without lateral dermal fringes; webbing formula 1(–), 2i(–), 2e(1), 3i(1.5), 3e(1.5), 4(1); relative length of fingers 1<2<4<3 (finger 2 distinctly shorter than finger 4); finger discs enlarged. Hind limbs slender; a pointed dermal appendage on heel; tibiotarsal articulation reaching widely beyond snout tip when hind limb is adpressed along body; lateral metatarsalia separated by webbing; inner metatarsal tubercle medium-sized, distinct, elongated; no outer metatarsal tubercle; toes moderately webbed; webbing formula 1(0), 2i(1), 2e(0), 3i(1), 3e(0), 4i(2), 4e(2), 5(0.75); relative length of toes 1<2<3=5<4; toe discs enlarged. Skin smooth on dorsal surfaces, smooth on throat and chest, coarsely granular on belly, rather smooth on ventral surface of thighs, prominent scattered tubercles around cloaca. A worm-like parasite (possibly a nematode) apparently tried to escape when the frog was preserved and sticks in the left nostril.

Measurements (in mm): SVL 26.7, HW 10.6, HL 11.2, ED 3.7, END 2.3, NSD 2.3, NND 3.3, TD 2.0, TL 15.2, HAL 9.1, FOL 11.1, FOTL 20.8.

After almost four years in preservative, ground colour of upper surface of head, dorsum and limbs greyish brown, with few irregularly scattered and indistinct darker markings; supratympanic fold and tympanic region not distinctly coloured; upper lip creamy white; dorsal surfaces of thigh, shank, tarsus and external toe, as well as lower arm, hand and external finger with distinct dark brown crossbands; flanks brown with small pale white spots and dots, forming a reticulated pattern; several whitish dots below the cloaca, but no additional single white tubercles in the cloacal region; posterior surfaces of thighs greyish pale brown with beige reticulation on the proximal part, light brown without reticulations in the distal part; ventral surface creamy beige, with some pale greyish mottling along the lower jaw, the lower arms, hands and feet.

In life, ground colour of upper surface of head, dorsum and legs light brown (slightly darker on the head), with few irregularly scattered yellowish spots on the back and scattered dark dots on back and more densely on the lateral parts of the head; flanks with reticulated pattern of brown, yellow and white; upper surfaces of hands and feet mottled with brown and yellowish; outer edge of tarsus with thin white line and white tarsal tubercle, outer edge of lower arm with white tubercle; two irregular rows of white tubercles on shank; dorsal surfaces of limbs with moderately distinct brown crossbands; posterior surfaces of thighs white, numerous white tubercles around the cloaca and uniformly brown posteriorly. Throat, chest and venter creamy white; two irregular bluish spots on throat. Ventral surfaces of limbs only partially with whitish pigment,

largest parts of thighs, shanks, hands and feet without white pigment. Outer iris almost uniformly bright orange, broadened above; inner iris ring brownish with some vessel-like brown reticulation; iris surrounded by a black ring; posterior iris periphery blue.

Variation. All paratypes were similar to the holotype in general morphology. For measurements, see Table 2. Male SVL ranged from 26.7–29.3 in the Ranomafana region, and was 30.8 mm in one specimen from Midongy. No females are known. Colouration was relatively constant in various localities of Ranomafana National Park, and in Ambohitsara (Fig. 6). The rather uniform orange eye colouration in life was typical for most specimens although at Andohahela (Fig. 6C) specimens tentatively assigned to this species had a more reddish eye colour.

Distribution. Besides different sites in (1) the Ranomafana region, the species is also known from (2) Tsitolaka forest near Ambohitsara, about 30 km from Ranomafana, and was tentatively identified from (3) Befotaka-Midongy Reserve (specimen ZSM 178/2006), and from (4) Andohahela National Park (Col Tanatana, 24°44' S, 46°50' E, 750 m a.s.l.). in the extreme southeast of Madagascar (GenBank accession number AY848529; specimen FGZC 236, deposited in UADBA).

Natural history. At Ranomafana National Park, *Boophis quasiboehmei* sp. n. was one of the most common species of frogs and its larvae occurred in 29 out of 30 streams surveyed for tadpoles (Randrianiaina et al. 2009b; Strauß et al. 2010). Adult specimens, however, were less commonly found, and in some areas occurred only in some densely clustered demes along small stretches of the streams. Males were observed calling at night from perch heights of 2–3 m from bushes and trees close to streams in primary as well as degraded rainforest.

Vocalization. Generally, calls of *Boophis quasiboehmei* sp. n. exhibit a characteristic structure, consisting of short to moderately long pulsatile notes. However, the pattern of emission of these notes is highly variable and mostly irregular. Sometimes, notes are combined to regular series (2–6 notes), with the initial note being longer than subsequent secondary notes. The calls emitted by the holotype (Fig. 8) and recorded on 26 February 2006 at Ambatovory have the main frequency distributed between 2100 and 3400 Hz, with additional frequency bands of lower amplitude at 5500–6000 Hz and 8100–8900 Hz. Numerical parameters for the holotype calls are as follows (range followed by mean ± standard deviation): duration of note series, 335–736 ms (519 ± 203; n = 3); number of notes per series, 3–6 (4.3 ± 1.5; n = 3); note duration (including initial notes within series), 66–79 ms (72.1 ± 4.6; n = 8), duration of secondary notes within series,

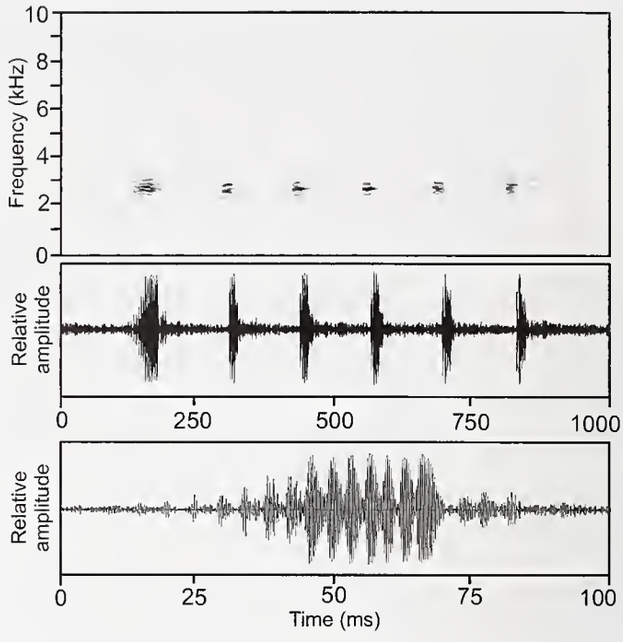

Fig. 8. Spectrogram, corresponding waveform, and expanded waveform (bottom) of the initial note of a regular note series emitted by the holotype of *Boophis quasiboehmei* sp. n. Recording obtained on 26 February 2006 at Ambatovory, Ranomafana National Park.

20–34 ms (24.9 ± 4.6; n = 8); pulses/note, 5–19 (12.6 ± 6.2; n = 15); inter-note intervals, 97–125 ms (109.9 ± 7.5; n = 10); dominant frequency, 2680–2963 Hz (2807 ± 86; n = 10).

A short sequence with three notes recorded on 1 March 1996 at Ranomafana (Vences et al. 2006, CD 1, track 66) has the following parameters: duration of note series, 373 ms, notes/series, 3; note duration, 18–58 ms; pulses/note, 5–12; inter-note intervals, 139–142 ms; dominant frequency, 2550–2637 Hz.

Calls of *B. quasiboehmei* sp. n. from Ambohitsara recorded on 3 March 2007 generally agree in structure with those emitted by the holotype, although they have shorter note duration and more variable, distinctly longer inter-note intervals. Numerical parameters are as follows: duration of note series, 527 ms (n = 1); number of notes per series, 6 (n = 1); note duration (including initial notes within series), 22–47 ms (35.2 ± 7.3; n = 13); pulses/note, 4–12 (7.9 ± 2.5; n = 18); inter-note intervals, 475–942 ms (724.4 ± 138.8; n = 16); dominant frequency, 2293–2572 Hz (2465 ± 90; n = 9).

Comparative call data. The morphologically most similar species, *Boophis boehmei*, has an almost identical call compared to that of *B. quasiboehmei* sp. n. A re-analysis of calls of *B. boehmei* from Andasibe (type locality) recorded on 12 January 1992 at 23°C (Fig. 9) revealed the following parameters: duration of note series, 455–530 ms

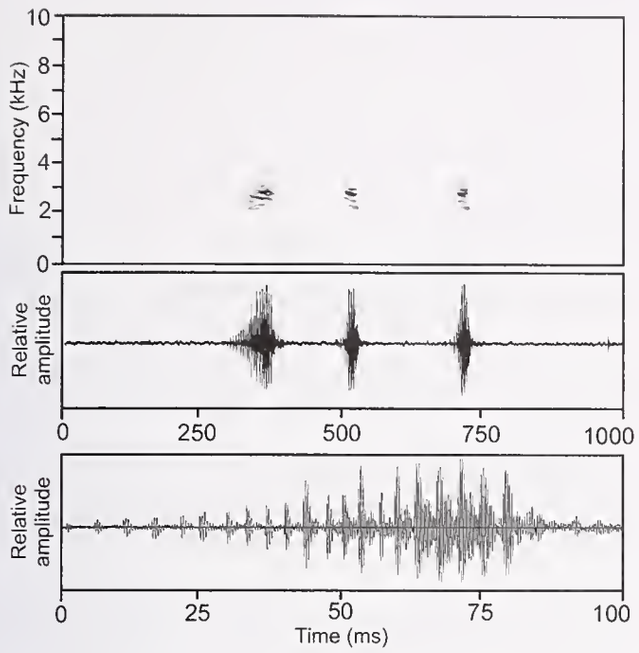

Fig. 9. Spectrogram, corresponding waveform, and expanded waveform (bottom) of the initial note of a regular note series emitted by *Boophis boehmei*. Recording obtained on 12 January 1992 at Andasibe (air temperature 23°C).

(n = 2); number of notes per series, 3–4 (n = 2); note duration, 27–106 ms (62.6 ± 23.0; n = 11); pulses/note, 10–24 (15.7 ± 4.8; n = 9); inter-note intervals, 93–157 ms (125.0 ± 29.6; n =5); dominant frequency, 2640–3177 Hz (2835 ± 165.6; n = 8).

A second recording from the type locality of *B. boehmei* recorded on 7 December 2001 at 24.8°C (Vences et al. 2006, CD 1, track 64) differs from the one described above by longer inter-note intervals. Numerical parameters are as follows: note duration, 34–98 ms (62.7 ± 19.7; n = 18); pulses/note, 13–23 (16.7 ± 3.6; n = 11); inter-note intervals, 591–1070 ms (766.1 ± 210.0; n =7); dominant frequency, 2360–2980 Hz (2760 ± 198; n = 12). In this recording, a single regular series composed of 6 notes is present, exhibiting note durations of 34–44 ms and inter-note intervals within the series of 61–85 ms.

A call recording of *B. boehmei* from Ankeniheny recorded on 20 March 1994 at 22°C air temperature showed note duration of 16–61 ms, inter-note intervals of 162–164 ms and a dominant frequency of 2500–2800 Hz.

In conclusion, there are no temporal or spectral call characters that distinguish *B. boehmei* from *B. quasiboehmei* sp. n. (see above).

Etymology. The specific epithet is a combination of the Latin word 'quasi', meaning 'almost', and a patronym for Wolfgang Böhme (ZFMK). It refers to the impressively cryptic morphological and bioacoustic similarity of the new species to *Boophis boehmei*.

DISCUSSION

The initial detection of a probable species status of *Boophis quasiboehmei* sp. n. was based on its large divergence in a single marker of mitochondrial DNA. Due to the extent of this divergence (>6% to all described species), Vieites et al. (2009) deviated slightly from their usual rationale and listed this species as confirmed candidate species, despite the lack of concordant indications by independent taxonomic characters. Although the work protocol of integrative taxonomy proposed by Padial et al. (2010) would allow for the description of species based on single characters if these are deemed to be sufficiently indicative of the existence of independent evolutionary lineages, we do not recommend this procedure. Instead, we only decided to formally describe *B. quasiboehmei* sp. n. as new species once that independent and congruent evidence of various taxonomic characters had accumulated, even if those were subtle at first view: (1) a weak and not fully constant difference in adult eye colouration, (2) a slight tendency of morphometric differentiation detectable only by multivariate techniques, (3) a constant difference in tadpole morphology, and (4) concordance between three independent molecular markers (two nuclear and one mitochondrial). The molecular concordance alone would be sufficient for species recognition under the genealogical concordance method of phylogenetic species recognition, GCPSR (Avise & Ball 1990), but the further strict concordance with one morphological character (tadpole labial keratodonts) provides a more convincing evidence, especially because it is based on large series of individuals (Randrianiaina et al. 2009a, b). We are therefore convinced that *Boophis quasiboehmei* sp. n. and *B. boehmei* are to be considered as distinct species under an evolutionary or general lineage species concept (de Queiroz 2007).

Among the various mechanisms of species diversification discussed for Madagascar (Vences et al. 2009), two (the watershed and the river barrier mechanism) might apply to the species pair *B. boehmei* and *B. quasiboehmei* sp. n. that occur in two different neighbouring centres of endemism (CE2 and CE3) as defined by Wilmé et al. (2006), and because these two CEs are divided by the Mangoro river that has been invoked as an important river barrier in eastern Madagascar (see Vences et al. 2009). Discerning between these hypotheses is difficult, but both are contradicted by the fact that *B. quasiboehmei* sp. n. also occurs in Andohahela, which is in a different CE (CE5) and separated by a further large river barrier (the Mananara river). Also, the fact that numerous other red-eyed

treefrog species and candidate species have been already identified from eastern Madagascar (see Vieites et al. 2009: *B. axelmeyeri, B. rufioculis, B.* sp. 8, *B.* sp. 40, *B.* sp. 41), several of which appear to be microendemic to small areas while others might be more widespread, indicates a more complex situation. Only a more comprehensive study of this group, with assessments of the status of all candidate species and their phylogenetic relationships, and a more detailed analysis of their distribution, will significantly contribute to the understanding of the diversification mechanisms that may have lead to this surprising morphological cryptic diversity. However, the fact that the phylogenetic position of *B. boehmei* and *B. quasiboehmei* is unclarified should not be interpreted as casting doubts on the species status of *B. quasiboehmei* since this new species is differentiated from topotypical *B. boehmei* by a high genetic differentiation and tadpole mouthparts, and from all other nominal species in the *B. goudoti* group by a high genetic differentiation, tadpole mouthparts, adult morphology, and advertisement calls. However, clarifying the phylogenetic relationships of all species and candidate species will be important to understand the status of the various UCS and CCS in the group and to be able to provide formal descriptions of those for which the data will confirm the status as distinct species. Additional data still missing at this time are on tadpole morphology of the populations from Andohahela, Midongy, Sahafina and Mahasoa that herein we have assigned in a preliminary way to *B. quasiboehmei* (Andohahela, Midongy) or different candidate species (Sahafina, Mahasoa).

At Ranomafana National Park, *Boophis quasiboehmei* sp. n. was commonly encountered at least in its tadpole stage, and its occurrence was confirmed at Andohahela National Park and tentatively in Befotaka-Midongy National Park. Although we never observed the species in secondary vegetation formations, it appears to be tolerant to some degree of rainforest degradation. The relatively large distribution area (from Ranomafana to Andohahela), its occurrence in at least two protected areas and large area of occupancy at least in the Ranomafana area lead us to propose an IUCN red list status of Least Concern for this newly described species (compare Andreone et al. 2005, 2008).

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