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Two new bright-eyed treefrogs of the genus *Boophis* from Madagascar

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Abstract. We describe two new species of treefrogs of the *Boophis goudoti* species group mainly based on analyses of morphology and molecular genetics. *Boophis popi* sp. n. is characterized by a red iris and distinctly elevated reticulations on the dorsum. It is distributed in submontane rainforest between 1000–1500 m above sea level. *Boophis fayi* sp. n. is unique among the species in the *B. goudoti* group by exhibiting a green outer iris in life and males lacking a distinct supratympanic fold. It occurs in lowland rainforests of northeastern Madagascar. Both species differ by substantial genetic differentiation in the 16S mRNA gene fragment from known members in the species group: > 8% and > 6% pairwise divergence, respectively, whereas the minimum pairwise divergence between the two new species is 5.3%. The advertisement calls of both species are rather similar to those of other small to medium-sized members of the *B. goudoti* group.

Key words. Amphibia, Anura, Mantellidae, *Boophis popi*, *Boophis fayi*, new species, eye colouration, genetic divergence.

Introduction

Due to their large and often colourful eyes, several species of the mantellid genus *Boophis* are considered to be among the world's most beautiful frogs. The most splendid part of the eye usually is the iris, which can be greyish, brownish, silvery or golden as in most anuran species, but in many bright-eyed *Boophis* species includes red, yellow, white, turquoise or blue colours that may cover the whole iris or are combined, being arranged in distinct rings around the pupil. Additionally, horizontal or vertical markings and vessel-like reticulations, as well as a distinctly coloured posterior iris periphery, which may be black, green, blue, pink or red, often complement the striking appearance of these frogs' eyes (Glaw & Vences 2007).

Although *Boophis* are almost strictly nocturnal in their habits, the colourful parts of the eyes emerged as one of the most important characters for field identification at specific level (Glaw & Vences 1997a). Bioacoustic and genetic data confirmed the validity of species-specific eye colour traits in most cases (Vieites et al. 2009, Glaw et al. 2010, Vences et al. 2010b). In addition to the colour of the eye, other conspicuous markings are often present on the head, e.g., distinct white spots below the eyes (e.g., *B. rufioculis*) or white markings above the eye (e.g., *B. solomaso*), which may either be explained as an antipredator trait, or suggest that visual communication might play a more important role in this genus than currently assumed.

With more than 70 described species, *Boophis* represents the most speciose lineage of amphibians in Madagascar. Although numerous new species have been described recently (Köhler et al. 2007, 2008, Wollenberg et al. 2008, Glaw et al. 2010, Vallan et al. 2010, Vences et al. 2010a, b), many known candidate species still await analysis and description (Vieites et al. 2009) and expeditions to Madagascar's rainforests regularly reveal additional new species.

The genus *Boophis* is classified into one pond-breeding lineage (subgenus *Sahona*), usually without colourful eyes, and a far more speciose radiation (subgenus *Boophis*) that generally breeds in streams (Glaw & Vences 2006). The latter lineage is divided into several species groups the phylogenetic relationships of which are not yet fully resolved (Glaw et al. 2010). One of these, apparently monophyletic, is the *Boophis goudoti* species group, characterized by a distinct and sharp canthus rostralis, predominantly brownish dorsal colours and dermal appendices on elbows and heels (Glaw & Vences 2007). Many of the small to medium-sized species furthermore exhibit colourful eyes, mostly with red pigmentation, making them part of the group of Malagasy bright-eyed frogs.

In the present paper, we describe two new bright-eyed treefrog species of the *Boophis goudoti* group, which are characterized by red and green eyes, respectively. We base the species delimitation on morphology and their phylogenetic position is derived from analyses of mitochondrial DNA sequences.

Material and methods

Frog specimens were collected at night by opportunistic searching with torchlights and headlamps. Specimens were euthanized by immersion in a solution of MS222 or chlorobutanol, fixed in 95% ethanol, and preserved in 70% ethanol. Locality information was recorded with GPS receivers. Specimens were deposited in the collections of the Museo Regionale di Scienze Naturali di Torino (MRSN), Université d'Antananarivo, Département de Biologie Animale, Antananarivo (UADBA), Zoologisches Forschungsmuseum Alexander Koenig, Bonn (ZFMK), Zoological Museum Amsterdam (ZMA) and the Zoologische Staatssammlung München (ZSM). FGMV, FGZC, MV, and ZCMV refer to F. Glaw and M. Vences field numbers; FAZC refers to F. Andreone field numbers. PSG refers to P.-S. Gehring field numbers. Terminology for biogeographic regions of Madagascar follows Boumans et al. (2007).

Morphological measurements (in millimetres) were all taken using a digital calliper (precision 0.01 mm) to the nearest 0.1 mm by MV, except those of three Betampona specimens that were taken by GMR. To avoid any artificial bias, the latter three specimens were excluded from the Principal Component Analysis. 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), TIBL (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 a Tensai RCR-3222 recorder with an EMC-81 external microphone and a Marantz PMD 660 digital recorder, accessorised with a semi-directional microphone, respectively. Recordings were sampled at 22.05 kHz and 16-bit resolution and computer-analysed using the software Adobe Audition. Frequency information was obtained through Fast Fourier Transformation (FFT; width 1024 points). Recordings of *B. popi* were bandpass-filtered at 1700–3000 Hz. The spectrogram was obtained through the Hanning window function with 256 bands resolution. Temporal measurements are given as ranges, with mean ± standard deviations in parentheses. Terminology in call descriptions follows Köhler (2000).

Toe clips or muscle tissue samples (preserved in 99% 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). 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). A fragment of the mitochondrial 16S rRNA gene was amplified using standard protocols. Primers used were 16SFrogL1 (CAT AAT CAC TTG TTC TTT AAA) and 16SFrogH1 (GAT CCA ACA TCG AGG TCG). Purified PCR templates were sequenced using dye-labelled dideoxy terminator cycle sequencing 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 identified sequences have been deposited in GenBank (accession numbers JN679870–JN679895). Additional sequences of the 16S rRNA gene were taken from published data sets as available from GenBank (e.g., Randrianiaina et al. 2009, Vieites et al. 2009, Strauß et al. 2010, Vences et al. 2010b, Rosa et al. submitted).

After alignment and removal of incomplete sections at 5' and 3' ends, the dataset had a length of 520 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 three 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 1500 trees were retained post burn-in and summarized to generate the majority rule consensus tree.

Our molecular analysis focuses on the small and medium-sized species in the *Boophis goudoti* group. Previous molecular data (Vieites et al. 2009) suggested the existence of various candidate species in this group. To better assess genetic variation, we included representatives of all species of the *B. goudoti* group in our analysis, with the small and medium-sized species represented, where possible, by several sequences from different localities. For the two focal taxa, we included all available sequences, while for some others (e.g., *B. boehmei, B. quasiboehmei* and *B. reticulatus*), only a representative subset was used from the datasets of Strauß et al. (2010) and Vences et al. (2010b).

Results

Molecular and morphological differentiation of focal taxa

During fieldwork at various localities in eastern Madagascar, we collected specimens of two bright-eyed species of the *B. goudoti* group that morphologically appeared to represent new taxa. In the following, we will already refer to these under the new scientific names coined herein; formal descriptions are provided below. The first of these species, *Boophis popi* sp. n., is a red-eyed frog that has been known since 1991 from the Andringitra mountain massif and was later also collected at other highland sites (see Fig. 1 for localities). It has either been considered to represent a deviant population of *B. boehmei* (Glaw & Vences 1992, 1994), or a candidate species related to *B. rufioculis* or *B. boehmei*

(Vences et al. 2006, Andreone et al. 2007, Vieites et al. 2009). However, the available data were so far insufficient to fully understand the delimitation and distribution of this form. The second species, *Boophis fayi* sp. n., was first found in 2007 at the Betampona Reserve and was recently collected by us in a lowland forest near the Makira Reserve. It has a remarkable greenish rather than red iris colour and a barely recognizable supratympanic fold in males. It was considered a candidate species related to *B. boehmei* by Rosa et al. (submitted). In the following, we will analyse the molecular and morphological differentiation among and within these forms and other species of the *B. goudoti* group, in order to provide evidence of their identity as independent evolutionary lineages, and thus as support for their species status.

The phylogenetic tree (Fig. 2) does not aim at fully resolving the phylogenetic relationships among most included groups (for a more reliable multi-gene phylogenetic analysis of most mantellid species and candidate species, see Wollenberg et al. 2011). Instead, the 16S tree reconstructed herein is used as a tool for species delimitation by confirming monophyly and depicting divergence among lineages regarding their mitochondrial genealogy. Given these restrictions, the tree confirms high genetic divergences among most species and candidate species in the *B. goudoti* group as has already been assessed by Vieites et al. (2009) and provides new information on the identity of recently discovered populations. *Boophis popi* and *B. fayi* are differentiated by pairwise sequence divergenc-

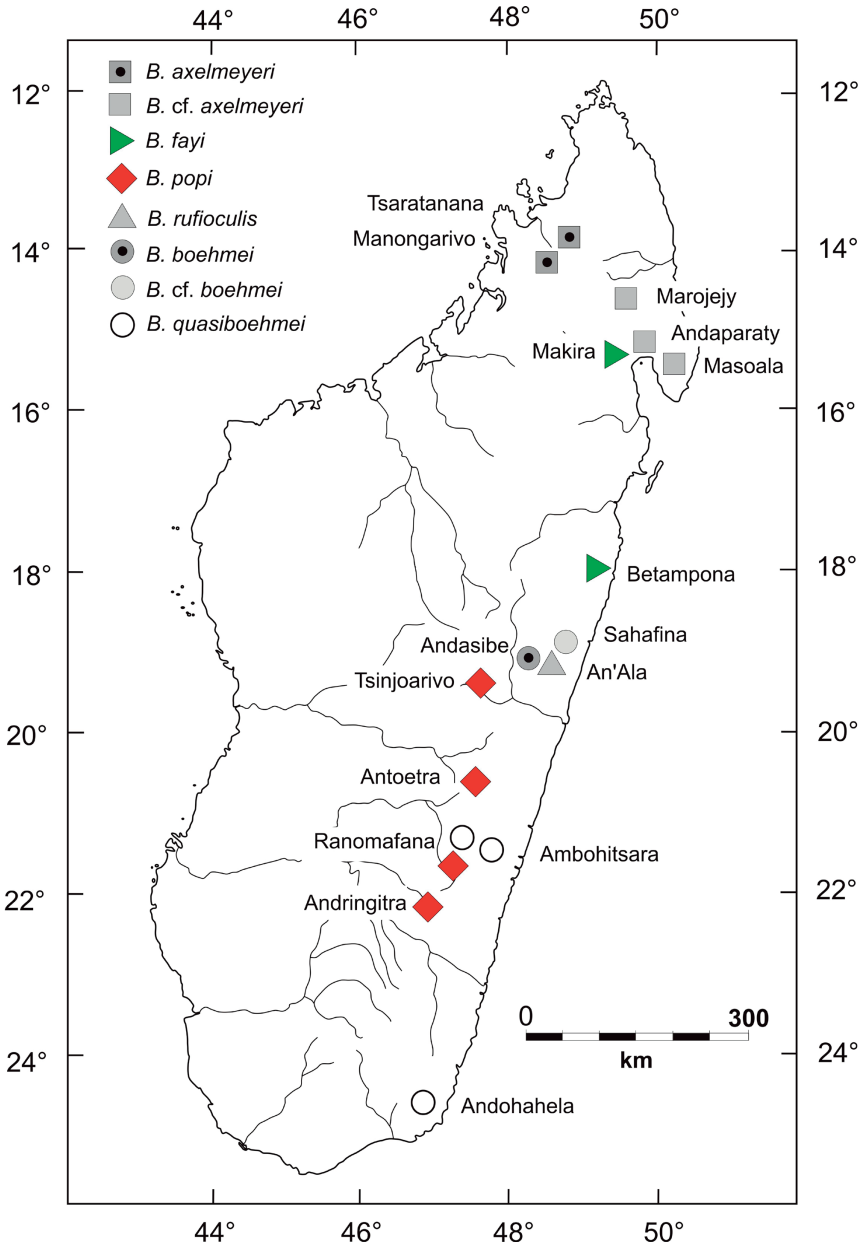

Figure 1. Distribution map of nominal species in the *B. goudoti* group characterized by red or orange iris colouration, and of *B. fayi*. Note that the marking of localities is sometimes slightly imprecise for graphical reasons, especially in areas (Andasibe/An'Ala and Ranomafana/Ambohitsara) where several species occur in sympatry. Ranomafana includes the localities Maharira, Ranomafanakely, Kidonavo (*B. popi*) and numerous collecting sites of *B. quasiboehmei* (see Vences et al. 2010b). Several other candidate species with red irises and occurring north of the Andasibe region have been omitted from the map.

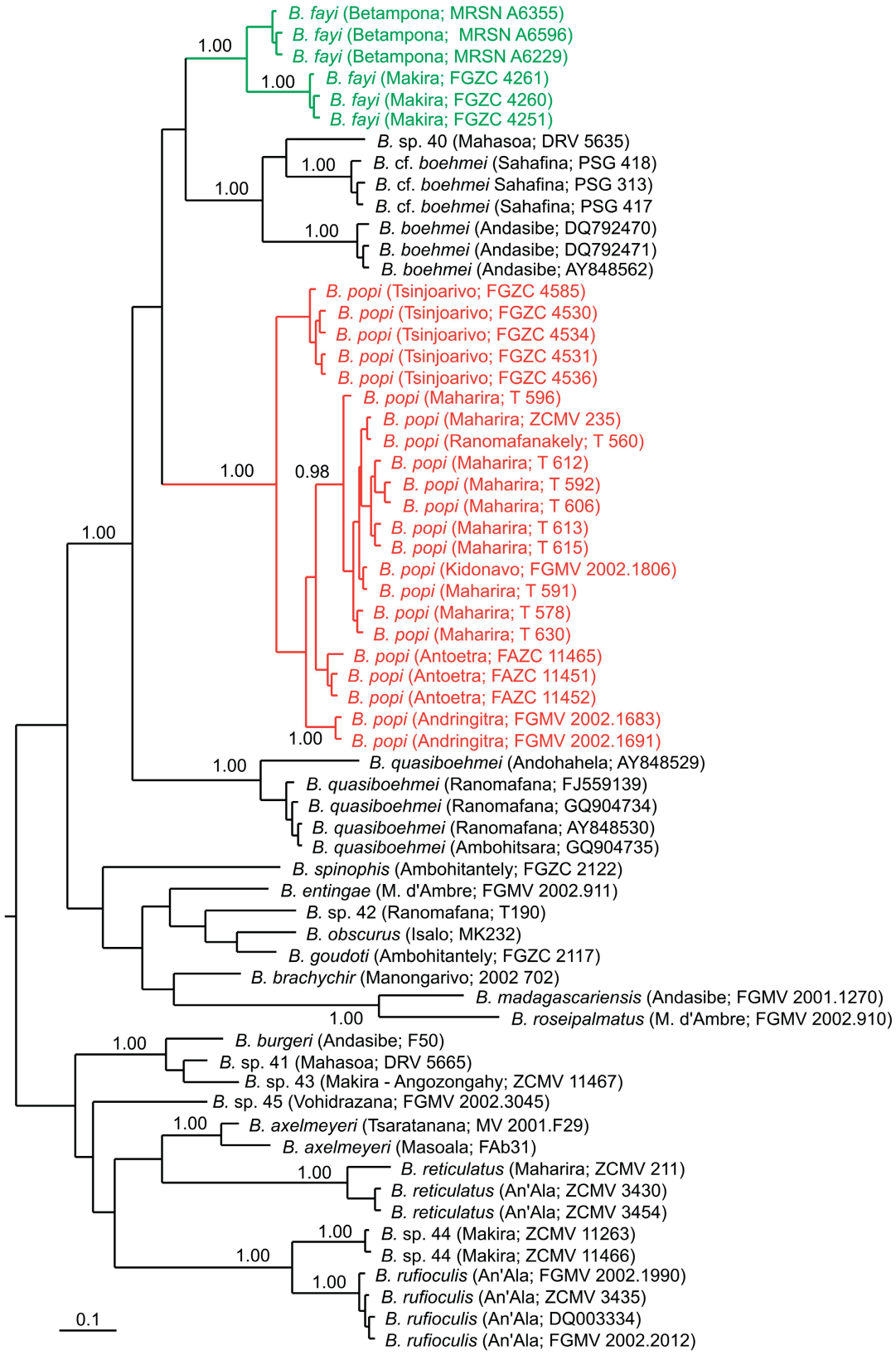

Figure 2. Majority-rule consensus phylogram (all compatible bifurcations included) from a Bayesian analysis of DNA sequences of the mitochondrial 16S rRNA gene in species of the *Boophis goudoti* group. *Boophis tephraeomystax* was used as outgroup; this and the most basal species of the *B. goudoti* group (*B. periegetes*) were removed from the tree for graphical reasons. Numbers at branches are posterior probabilities (only values >0.95 are shown). Numbers provided in parentheses either refer to field numbers, collection numbers or GenBank accession numbers. Note that the purpose of this tree is not to reliably depict phylogenetic relationships among species for which more nucleotide characters from additional genes would be necessary, but to indicate the degree of genetic differentiation within and among species, proportional to branch lengths.

es (*p*-distances) of > 8% and > 6%, respectively, from all other nominal species in the *B. goudoti* group. For *B. popi,* the analysis confirms that this species is genetically moderately variable across its range, including populations from the Tsinjoarivo, Antoetra, Ranomafana, and Andringitra regions (maximum intraspecific pairwise sequence divergence 2.5% between populations from Maharira and Tsinjoarivo). For *B. fayi,* the 16S data provide evidence that it is a new, genetically divergent lineage, encompassing specimens from the lowland Makira region and the Betampona Reserve (intraspecific pairwise sequence divergence between *B. fayi* from Makira and the population from Betampona is 2.6%). *Boophis reticulatus* from An'Ala in the northern central east of Madagascar are genetically only slightly divergent from those in Ranomafana in the southern central east; sequences from the northern central east (Vohidrazana) previously considered as a deep conspecific lineage of *B. reticulatus* (Vieites et al. 2009) are here transferred to a new candidate species *Boophis* sp. 45. Two lineages from higher elevations in the Makira Reserve are assigned to new candidate species *B.* sp. 43 and *B.* sp. 44, respectively. The identities of these forms will be the subject of future studies.

Comparative morphological analyses of *B. popi* and *B. fayi* revealed several differences to other species that at a first glance appear to be morphologically similar. *Boophis popi* shares a red iris colour with *B. boehmei, B. quasiboehmei, B. rufioculis* and *B. axelmeyeri.* Based on a Principal Component Analysis of male morphometric measurements (Fig. 3a, Tab. 1, 2), *B. popi* is differentiated from *B. axelmeyeri* mainly along the first factor (PC1) which essentially reflects size differences. In fact, males of *B. axel-*

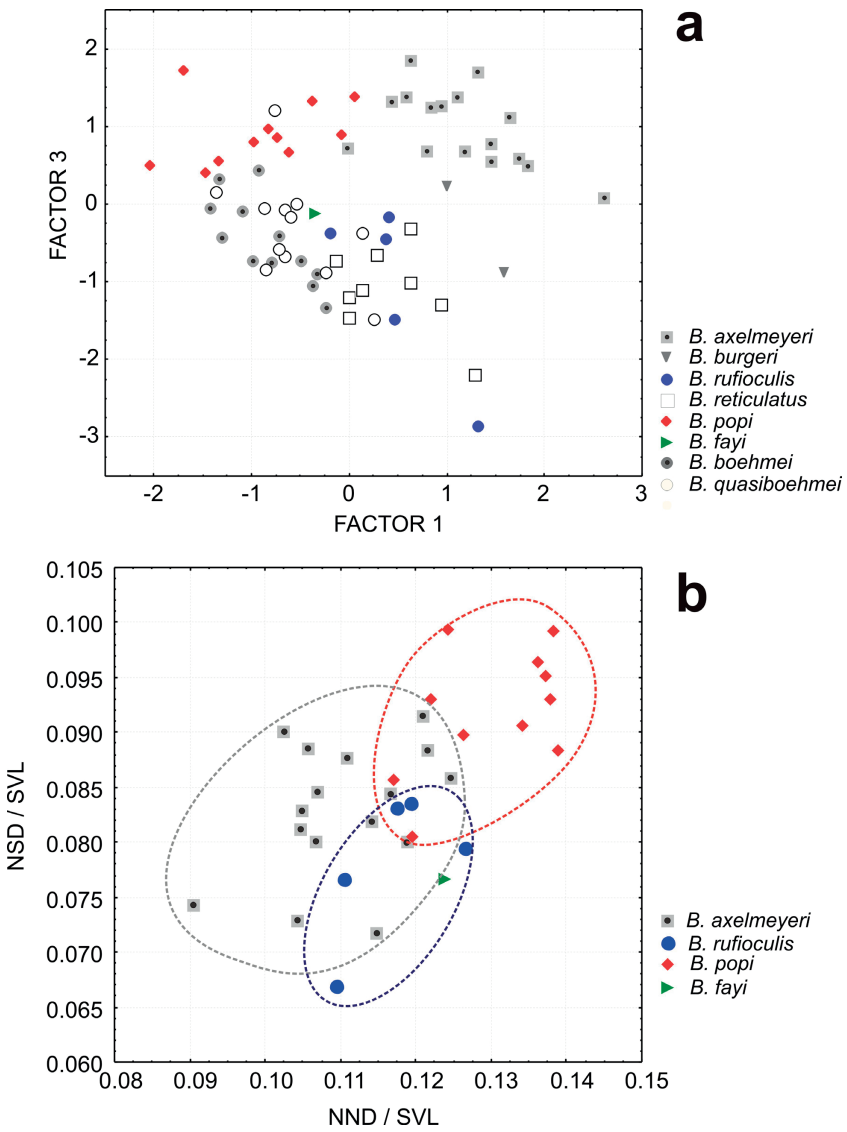

Figure 3. Morphometric differentiation among nominal species in the *B. goudoti* group characterized by red or orange iris colouration, and of *B. fayi and B. reticulatus* (males only). Morphometric data from Table 1, in addition to previously published data from Vences et al. (2005, 2010b). (a) Results from a PCA showing differentiation along the first and third factors (corresponding mainly to body size, and to head proportions, respectively). (b) Univariate scatterplot of relative nostril–snout tip (NSD) and internarial distances (NND) for selected species, showing the differentiation of *B. popi* from *B. axelmeyeri*, from *B. rufioculis,* and from *B. fayi* in proportions of the anterior region of the head.

Table 1. Morphological measurements (all in mm) of specimens of *Boophis popi, B. fayi,* and *B. rufioculis*. Abbreviations as in Material and methods; additional abbreviations: M, male; F, female; HT, holotype; PT, paratype; RHL, relative hindlimb length, given as the point reached by the tibiotarsal articulation when the hindlimb is adpressed along the body: 1, between eye and nostril; 2, nostril; 3, between nostril and snout tip; 4, snout tip. All measurements were taken by MV, except those for specimens marked with an asterisk, which were taken by GMR and consequently were excluded from the Principal Component Analysis to avoid any possible artificial bias.

Locality	Catalogue Number	Field number	Type status	Sex	SVL	HW	HL	ED	TD	END	NSD	NND	HAL	FORL	HIL	FOTL	FOL	TIBL	RHL
B. popi																			
Tsinjoarivo	ZSM 248/2010	FGZC 4530	HT	M	35.0	13.2	13.1	5.1	2.5	2.5	3.0	4.1	11.7	21.0	59.6	25.3	15.2	19.1	2
Tsinjoarivo	ZSM 249/2010	FGZC 4531	PT	M	34.4	13.6	15.2	5.1	2.4	2.6	3.2	4.2	11.5	20.8	58.7	26.7	16.0	18.2	–
Tsinjoarivo	ZSM 250/2010	FGZC 4585	PT	F	47.2	17.7	16.6	5.8	2.4	3.1	4.0	5.6	15.8	30.7	82.6	38.5	23.5	25.7	3
Andringitra	ZSM 736/2001	MV 2001-559	PT	M	31.2	13.1	12.8	4.2	2.4	2.6	2.9	4.3	10.6	21.6	52.8	23.2	14.1	16.4	2
Andringitra	ZSM 737/2001	MV 2001-579	PT	F	40.1	14.9	15.3	5.0	2.8	3.2	3.8	4.8	14.1	27.5	70.6	32.2	19.4	22.5	3
Andringitra	ZFMK 59824	–	PT	M	28.2	11.3	11.2	3.7	2.2	2.4	2.8	3.9	9.8	18.4	47.9	21.3	12.1	15.7	4
Andringitra	ZFMK 59825	–	PT	M	29.8	11.6	11.3	4.2	2.4	2.5	2.7	4.0	9.7	18.4	49.4	21.5	12.7	15.8	2
Andringitra	ZFMK 57407	–	PT	M	30.1	12.0	11.9	4.1	2.4	2.2	2.9	4.1	10.4	20.0	53.7	24.3	14.5	17.1	4
Antoetra	MRSN A2240	FAZC 11465	PT	M	30.1	12.0	11.6	4.5	2.1	2.2	2.7	3.8	9.1	18.2	50.8	22.7	13.5	16.2	1
Antoetra	MRSN A2313	FAZC 11451	PT	M	33.6	12.5	12.0	4.9	2.4	2.3	2.7	4.0	10.1	20.6	55.3	24.3	14.2	17.1	2
Antoetra	MRSN A2314	FAZC 11453	PT	M	31.7	12.1	12.2	4.8	2.4	2.2	2.8	4.4	10.0	18.2	50.9	21.8	13.8	15.8	1
Antoetra	MRSN A2315	FAZC 11452	PT	M	32.2	12.1	11.3	4.4	2.1	2.4	3.2	4.0	10.9	19.8	54.8	24.6	14.8	17.2	2
Maharira	ZMA 20193	ZCMV 235	PT	M	28.4	11.5	11.5	4.3	2.5	2.5	2.7	3.9	8.8	18.0	47.0	21.0	12.2	14.6	–
B. fayi																			
Makira	ZSM 280/2010	FGZC 4261	HT	M	33.9	12.8	13.5	5.0	2.4	2.9	2.6	4.2	10.2	20.7	53.7	24.0	13.8	17.3	1
Makira	ZSM 281/2010	FGZC 4304	PT	F	42.0	15.5	17.2	6.0	2.5	4.1	3.5	4.8	12.9	25.2	70.0	31.6	18.7	22.5	2
Betampona	MRSN A6229*	FAZC 13726	PT	M	31.3	12.2	12.7	4.7	2.3	3.0	2.4	3.5	9.4	19.7	50.4	21.6	13.6	16.9	1
Betampona	MRSN A6355*	FAZC 13834	PT	M	30.7	12.7	12.1	4.4	2.4	3.1	2.5	3.4	9.9	20.5	56.2	24.4	15.4	18.3	2
Betampona	MRSN A6596*	FAZC 13971	PT	M	30.7	12.2	13.1	5.0	2.5	2.7	2.7	4.1	9.6	19.6	53.6	23.7	13.6	17.9	2
B. rufioculis																			
An'Ala	ZSM 568/1999	(ZFMK 60081)	PT	M	33.5	12.1	12.8	4.9	2.5	3.0	2.8	4.0	11.0	20.3	53.9	24.8	14.4	18.3	1
An'Ala	ZSM 292/2006	ZCMV 2508		M	34.0	12.2	13.1	4.5	2.1	2.9	2.7	4.3	11.2	20.0	57.2	24.9	14.4	18.1	2

Table 2. Factor loadings, Eigenvalues, and percent explained variance for the first three factors derived from a PCA of morphometric data in Table 1, in addition to previously published data from Vences et al. (2005, 2010b) (males only). For abbreviations, see Material and methods.

	Factor 1	Factor 2	Factor 3
SVL	0.823014	0.250473	0.460888
HW	0.741555	0.321275	0.540005
HL	0.789585	0.312898	0.447911
ED	0.552799	0.450054	0.477241
TD	0.243928	0.889739	0.263662
END	0.667286	0.566829	0.016260
NSD	0.411169	0.147796	0.844679
NND	0.458484	0.426924	0.678784
HAL	0.854846	0.227507	0.389808
FORL	0.860935	0.218221	0.349933
HIL	0.886157	0.277179	0.318990
FOTL	0.877319	0.229628	0.298141
FOL	0.852309	0.269119	0.362851
TIL	0.878451	0.290372	0.309319
Eigenvalue	11.19383	0.76192	0.64753
% Total variance	79.95596	5.44232	4.62524

meyeri are consistently larger than those of *B. popi* (see Diagnosis below). PC_3, which is chiefly influenced by head proportions (Tab. 2), provides with some overlap a separation from all other included species. Only one individual of *B. quasiboehmei* has a PC_3 value deeply within the range typical for *B. popi*. This specimen belongs to the Midongy du Sud population and has only been tentatively assigned to *B. quasiboehmei* (Vences et al. 2010b); consequently, we consider the Midongy population in need of further study and excluded it from the distribution map (Fig. 1). *Boophis quasiboehmei* occurs sympatrically with *B. popi* in the Ranomafana area, but here is differentiated by tadpole morphology (Randrianiaina et al. 2009, Vences et al. 2010b). An univariate analysis of relative values (division by SVL) of nostril–snout tip and inter-nostril distances confirms that the combination of these two values serves to distinguish almost all individuals of *B. popi* from *B. axelmeyeri* and *B. rufioculis*.

Because measurements of only a single male of *B. fayi* were included in the morphological analyses, no reliable conclusions can be drawn regarding its morphometric differentiation. However, as highlighted in the diagnosis of *B. fayi* below, this species is morphologically mainly characterized by its weakly developed supratympanic fold in males and by its unique iris colour. These differences were ascertained in various additional specimens in the field.

Description of new species

***Boophis popi* sp. n.**

Remarks: This species was referred to as *Boophis boehmei* from Andringitra by Glaw & Vences (1992, 1994), *Boophis* sp. aff. *boehmei* by Vences et al. (2006), *Boophis* sp. 2 (aff. *boehmei*) by Andreone et al. (2007), *Boophis* sp. aff. *rufioculis* by Glaw & Vences (2007) and Glaw et al. (2010), and *Boophis* sp. 8 by Vieites et al. (2009) and Vences et al. (2010b).

Holotype: ZSM 248/2010 (field number FGZC 4530), adult male (Fig. 4a, b), from east of Tsinjoarivo (Camp 2), 19°42'59.4" S, 47°49'17.9" E, 1300 m above sea level, Vakinankaratra Region, central eastern Madagascar, collected on 20 April 2010 by J. L. Brown, P.-S. Gehring, F. Glaw, J. Köhler and E. Rajeriarison.

Paratypes: ZSM 249/2010 (field number FGZC 4531), UADBA uncatalogued (FGZC 4534, 4536), two adult males, ZSM 250/2010 (field number FGZC 4585), adult female (Fig. 4c, d), same data as holotype except for the collection date being 22 April 2010; ZSM 736/2001 (MV 2001-559), adult male, ZSM 737/2001 (MV 2001-579), adult female, from Imaitso forest, 22°08'25" S, 46°56'49" E, 1509 m a.s.l., Andringitra Massif, Ihorombe Region, southern central Madagascar, collected on 14 March 2001 by M. Vences, D. R. Vieites, L. Raharivololoniaina and D. Rakotomalala; ZFMK 57407, ZFMK 59824–59825, three adult males, from near Ambalamarina, Andringitra Massif, Ihorombe Region, southern central Madagascar, collected on 15 January 1994 by F. Glaw and M. Vences; ZMA 20193 (ZCMV 235), possibly subadult male, from Maharira Camp, 21°9.547' S, 47°24.147' E, 1248 m a.s.l., Ranomafana National Park, Haute Matsiatra Region, southern central Madagascar, collected on 24 January 2004 by M. Vences and I. de la Riva; MRSN A2240, A2313–A2315 (FAZC 11465, 11451, 11453, 11452), four adult males, from Farihimazava Forest, 20°50.10' S, 47°19.95' E, 1400 m a.s.l., Antoetra, Amoron'i Region, southern-central Madagascar, on 30 January 2003 and 31 March 2003 by F. Andreone and J. E. Randrianirina; MRSN A2993–A2994 (FAZC 11565, 11580), two adult males, from Antratrabe, 19°31.05' S, 47°48.94' E, 1600 m a.s.l., Vakinankaratra Region, central eastern Madagascar, collected on 2 February 2003 by F. Andreone and J. E. Randrianirina.

Diagnosis: Assigned to the genus *Boophis* based on the presence of an intercalary element between the 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 based on its brownish dorsal ground colour, presence of dermal appendages on heels and elbows, presence of white tubercles ventrally to the cloacal opening, absence of red skin colour, and molecular phylogenetic relationships.

A relatively small species (male SVL 28–35 mm, female SVL 40–47.2 mm) of the *Boophis goudoti* group, characterized by red colour in the outer iris area, a blue iris periphery, comparatively small white tubercles in the cloacal region, and distinct reticulations on the dorsum. *Boophis popi* differs from all described species in the *B. goudoti* group by substantial genetic differentiation (> 8% 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. en-*

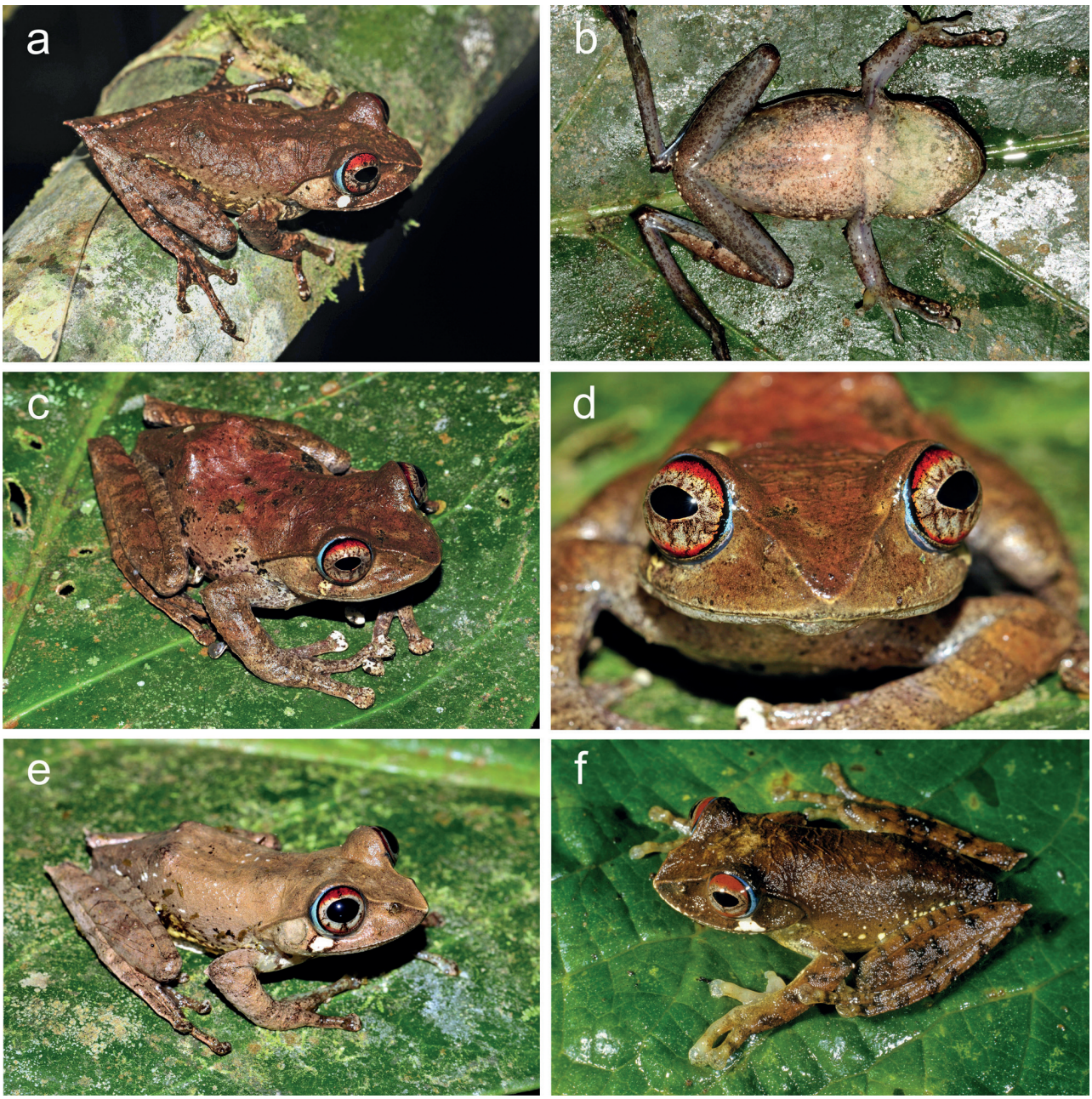

Figure 4. *Boophis popi* sp. n. in life: (a, b) male holotype (ZSM 248/2010) in dorsolateral and ventral views; (c) female paratopotype (ZSM 250/2010); (d) frontal close up of head of the same female paratopotype (note red colour of outer iris); (e) male paratopotype (UADBA); (f) male from Andringitra (probably in UADBA).

tingae, and *B. spinophis* by smaller size (male SVL of adult males up to 35 mm versus > 41 mm), presence of red colour in the outer iris area and blue colour in the iris periphery. It differs from the similarly sized *B. burgeri* and *B. reticulatus* by the presence of red colour in the outer iris (absent in *B. burgeri* and *B. reticulatus*) and furthermore from *B. reticulatus* by the blue iris periphery (greenish in *B. reticulatus*). *B. popi* differs from *B. boehmei* and *B. quasiboehmei* by typically larger male SVL (with overlapping ranges; 28.2–35.0 versus 26.7–30.8 mm) and presence of distinct reticulations on the dorsum (versus absence), and from *B. rufioculis* by comparatively smaller cloacal tubercles and shorter hindlimbs (tibiotarsal articulation reaching snout tip versus beyond tip of snout). By its external morphology, *B. popi* is most similar to *B. axelmeyeri,* but differs from the latter by its smaller size (adult male SVL 28.2–35.0 versus 35.5–43.1 mm) and a less pointed snout in dorsal view.

Description of holotype: Adult male in a good state of preservation, tongue removed as DNA tissue sample. SVL 35.0 mm. Body moderately robust; head virtually as long as wide, slightly wider than body; snout slightly pointed in dorsal view, rounded in lateral view; nostrils directed laterally, nearer to eye than tip of snout; canthus rostralis sharp, straight in dorsal view from eye to nostril, pointing from nostril to tip of snout; loreal region straight; eye large; tym-

panum distinct, rounded, TD 49% of ED; supratympanic fold narrow, prominent; vomerine odontophores distinct, well separated in two slightly elongated patches, positioned medially and posteriorly between choanae; choanae medium-sized, rounded. Tongue removed as tissue sample, but no differences to other *Boophis* tongues were noted during tissue sampling, suggesting that the tongue was distinctly bifid and free posteriorly. Arms moderately slender, lower arms without recognizable lateral fringes in preservative (with recognizable fringes in life, Fig. 4); a very small and indistinct pointed dermal appendage on elbow in preservative (distinct in life; Fig. 4); subarticular tubercles single, round; inner palmar tubercle not unambiguously recognizable; fingers poorly webbed and without lateral dermal fringes; webbing formula 1(--), 2i(--), 2e(1), 3i(2), 3e(1.5), 4(1); relative length of fingers 1<2<4<3 (finger 2 distinctly shorter than finger 4); finger discs enlarged; first finger with unkeratinized nuptial pad. Hind limbs slender; a very small pointed dermal appendage on heel in preservative (distinct in life; Fig. 4); tibiotarsal articulation reaches snout tip when hind limb is adpressed along body; lateral metatarsals separated by webbing; inner metatarsal tubercle small, moderately distinct, elongated; no outer metatarsal tubercle; toes moderately webbed; webbing formula 1(0), 2i(1), 2e(0), 3i(1), 3e(0), 4i(1.5), 4e(1.5), 5(0.5); relative lengths of toes 1<2<3=5<4; toe discs enlarged. Skin on dorsum largely smooth with fine reticulations in preservative (with very distinct reticulations in life; Fig. 4); skin on dorsal faces of thigh, shank and tarsus largely smooth in preservative (with distinct tubercles in life; Fig. 4); skin smooth on throat and chest, slightly granular on belly, smooth on ventral faces of thighs, prominent scattered whitish tubercles around cloaca. For measurements, see Table 1.

After 15 months in preservative, ground colour of upper faces of head, dorsum and limbs dark brown; supratympanic fold dark brown, tympanic region light brown; dorsal faces of thigh, shank, tarsus and toes, as well as lower arm, hand and external fingers with dark brown crossbands; a distinct bright white, rounded spot of ca. 1 mm in diameter in a posteroventral position relative to the eye. Flanks brown with whitish spots and dots, forming a reticulated pattern; several whitish dots below the cloaca; posterior faces of thighs brown, ventral faces of thighs brown marbled with cream; ventral faces of shanks cream with some scattered fine brown dots, ventral faces of tarsus and feet dark brown; throat and chest whitish with brown and grey spots, belly light grey marbled with brown dots and spots.

In life, dorsal colours much lighter than in preservative. Dorsal faces of head and back brown with several blackish spots and dots on lower back and flanks and a few small, poorly delimited beige spots on body; flanks marbled with brown and yellow; crossbands on limbs much more distinct than in preservative; tips of the two outer fingers marbled with white and brown; light spot below the eye as distinct as in preservative (Fig. 4a). Throat and chest beige with grey marbling; belly light brown with scattered small brown spots; ventral faces of limbs grey with dense brown spotting; nuptial pad on first finger yellowish (Fig. 4b). Outer iris area bright orange-red, broadened dorsally; inner iris ring brownish with some vessel-like brown reticulation; iris surrounded by a black ring; posterior iris periphery blue.

Variation: The paratypes were very similar to the holotype in general morphology except that females are distinctly larger than males. For measurements of the type specimens, see Table 1. The dorsal ground colouration in preservative of ZSM 250/2010 and ZSM 249/2010 is lighter than in the holotype, but otherwise similar to it. The white spot below the eye is less distinct in the female ZSM 250/2010, surrounded by black in UADBA uncatalogued (Fig. 4e) and completely absent in ZSM 249/2010. In the female ZSM 250/2010, the blue iris periphery is also visible anteriorly (Fig. 4d).

Vocalization: Calls of *Boophis popi* were recorded at Imaitso Forest, Andringitra, on 16 January 1994 at 19°C air temperature (Vences et al. 2006, CD 1, track 65, clips 1 & 2). Recordings contain two short series of notes, one comprised by three (Fig. 5) and one comprised by two notes only. Notes are strongly pulsatile in character. Note duration varies from 18–29 ms (n = 5) and inter-note intervals range from 97–120 ms (n = 3). Dominant frequency is distributed between 1800 and 3000 Hz with a maximum call energy at 2120–2320 Hz.

In comparison, these parameters are within the range of calls described for *Boophis boehmei* and *B. quasiboehmei* (Vences et al. 2010b) apart from a slightly lower dominant frequency in *B. popi,* which is in agreement with its slightly larger body size. However, as already stated by Glaw & Vences (1997b) and Vences et al. (2010b), the calls of the smaller-sized species in the *B. goudoti* group are all quite similar and not particularly diagnostic.

Distribution: *Boophis popi* occurs in a relatively narrow altitudinal band (ca. 1000–1500 m) in the montane rainforest of central Madagascar and is known from the following localities (Fig. 1): (1) east of Tsinjoarivo, (2) several localities in the Antoetra region (see Andreone et al. 2007), (3) Mt. Maharira in the Ranomafana National Park, and (4) Imaitso forest, Andringitra National Park. In addition, the

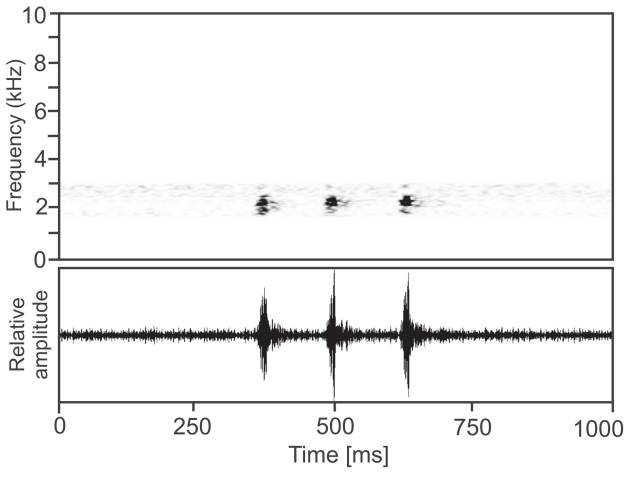

Figure 5. Audiospectrogram and corresponding oscillogram of the advertisement call of *Boophis popi* sp. n. from Imaitso Forest, Andringitra.

species was recorded by DNA barcoding of tadpoles from two additional localities in the Ranomafana region, namely Ranomafanakely, and near the Kidonafo bridge at approximately 1000 m a.s.l.

Natural history and conservation status: *Boophis popi* is a nocturnal treefrog of the submontane rainforest region. Males were usually found perching on low vegetation at night during the rainy season along small or slow-flowing (quiet) parts of streams. At the end of the rainy season in late April, no calling males were heard at Tsinjoarivo, whereas males were found calling in January at Andringitra. In a preliminary barcoding survey of the herpetofauna of the highland forests east of Tsinjoarivo, we found a single tadpole (PSG 2642) of this species in a rainforest stream with a sandy bottom in late April.

Seemingly, *B. popi* is tolerant to some degree of habitat degradation, as we found the species in disturbed and/or rather fragmented rainforests. The comparatively large distribution range of approximately 300 km from Tsinjoarivo in the north to Andringitra in the south and its occurrence in at least two protected areas lead us to propose an IUCN red list status of "Least Concern" for this newly described species (compare Andreone et al. 2005, 2008).

Etymology: The specific name is dedicated to the company "pop-interactive GmbH" (Hamburg, Germany), in recognition of their support of biodiversity research and nature conservation through the BIOPAT initiative. The name is used as an invariable noun in apposition.

***Boophis fayi* sp. n.**

Remark: This species was referred to as *Boophis* sp. aff. *boehmei* [Ca HM364594] from Betampona by Rosa et al. (2011).

Holotype: ZSM 280/2010 (field number FGZC 4261), adult male (Fig. 6a, b), from Ambodivoahangy (Makira Region), 15°17'23.8" S, 49°37'13.0" E, ca. 100 m above sea level, Analanjirofo Region, north-eastern Madagascar, collected on 2 April 2010 by P.-S. Gehring, F. Glaw, J. Köhler, M. Pabijan and F. M. Ratsoavina.

Paratypes: ZSM 281/2010 (FGZC 4304), adult female (Fig. 6c,d), UADBA uncatalogued (FGZC 4251), adult male, UADBA uncatalogued (FGZC 4260), adult male, all with the same data as holotype; MRSN A6229 (FAZC 13726), MRSN A6355 (FAZC 13834), two adult males (calling individual MRSN A6355; Fig. 7d) from a campsite locally known as Sahabefoza, Réserve Naturelle Intégrale de Betampona, 17°54'52.5" S, 49°12'32.1" E, ca. 330 m a.s.l., Commune Rurale de Sahambala, Atsinanana Region, east Madagascar, collected on 7 March 2007 and 31 October 2007, respectively, collected by G. M. Rosa, J. Noël and F. Andreone; MRSN A6596 (FAZC 13971) (Fig. 7a), adult male, from a campsite locally known as Vohitsivalana, 17°53'06.2" S, 49°12'11.7" E, ca. 494 m a.s.l., Réserve Naturelle Intégrale de Betampona, Commune Rurale de Sahambala, Atsinanana Region, east Madagascar, collected on 11 December 2007 by G. M. Rosa and J. Noël.

Diagnosis: Assigned to the genus *Boophis* based on the presence of an intercalary element between the 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, absence of red skin colour and molecular phylogenetic relationships.

A relatively small species (male SVL 30.7–33.9 mm, adult female SVL 42.0 mm) of the *Boophis goudoti* group, characterized by its green colour in the outer iris area, a turquoise iris periphery, comparatively small white tubercles in the cloacal region and a weakly developed supratympanic fold. *Boophis fayi* differs from all described species in the *B. goudoti* group by substantial genetic differentiation (> 5.3% pairwise divergence in a fragment of the 16S rRNA gene) and the poorly developed supratympanic fold (versus well-developed in the other species). Furthermore, it differs from *B. goudoti, B. obscurus, B. periegetes, B. madagascariensis, B. roseipalmatus, B. brachychir, B. entingae*, and *B. spinophis* by its smaller size (SVL of adult males 30.7–33.9 mm versus > 41 mm) and the presence of green colour in the outer iris area. It differs from the similarly sized *B. burgeri, B. popi*, *B. reticulatus, B. rufioculis*, and *B. axelmeyeri* by the presence of green colour in the outer iris (versus differently coloured) and the lack of elevated dorsal reticulation. *Boophis fayi* differs from *B. boehmei* and *B. quasiboehmei* by its slightly larger male SVL (30.7–33.9 versus 26.7–30.8 mm), green outer iris colour (versus red or orange outer iris), shorter hindlimbs (tibiotarsal articulation reaching between eye and nostril versus beyond tip of snout), and advertisement call (see below).

Description of holotype: Adult male in good state of preservation, tongue removed as DNA tissue sample. SVL 33.9 mm. Body slender; head slightly longer than wide, wider than body; snout pointed in dorsal view, rounded in lateral view; nostrils directed laterally, slightly nearer to tip of snout than to eye; canthus rostralis rounded, slightly curved in dorsal view from eye to nostril, nearly straight from nostril to tip of snout; loreal region slightly concave; eye large; tympanum distinct, rounded, TD 48% of ED; supratympanic fold very weak, barely recognizable; vomerine odontophores distinct, well separated in two ovoid patches, positioned slightly posteriorly between choanae; choanae medium-sized, rounded. 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(2), 3e(2), 4(1.5); relative lengths of fingers 1<2<4<3 (finger 2 distinctly shorter than finger 4); finger discs enlarged. Hind limbs slender; a well-developed pointed dermal appendage on heel; tibiotarsal articulation reaching to between eye and nostril when hind limb is adpressed along body; lateral metatarsals separated by webbing; inner metatarsal tubercle small, moderately distinct, elongated; no outer metatarsal tubercle; toes moderately webbed; webbing formula 1(1), 2i(1), 2e(0), 3i(1), 3e(0), 4i(2), 4e(2), 5(1); relative lengths of toes 1<2<3<5<4; toe discs enlarged.

Skin smooth on dorsal faces, smooth on throat and chest, coarsely granular on belly, rather smooth on ventral face of thighs, scattered tubercles around cloaca. Measurements are provided in Table 1.

After 15 months in preservative, ground colour of upper faces of head, dorsum and limbs brown, with few, irregularly scattered, small, cream-coloured spots, and irregular dark brown flecking; supratympanic and tympanic region not distinctly coloured; irregular cream fleck below eye; upper lip creamy white; dorsal faces of thigh, shank, tarsus and outer toe as well as lower arm, hand and outer finger with distinct dark brown crossbands; flanks brown with small pale cream spots and dots, forming a narrow strip of reticulated pattern along the border to belly; several whitish tubercles below the cloaca, thin white line above cloaca; posterior faces of thighs cream with brown mottling on the proximal part; ventral faces creamy white without any flecks or mottling except some fine brown mottling at the anterior edge of throat.

In life, general pattern similar to that in preservative, but dorsal faces light brown with large dark brown transversal flecks in interorbital area, scapular region and at urostyle level; dorsum and flanks with irregular dark brown flecking and small, irregularly scattered, green spots; distinct green shading in supratympanal region, on anterior snout and surrounding nostril; edges of dermal appendages on

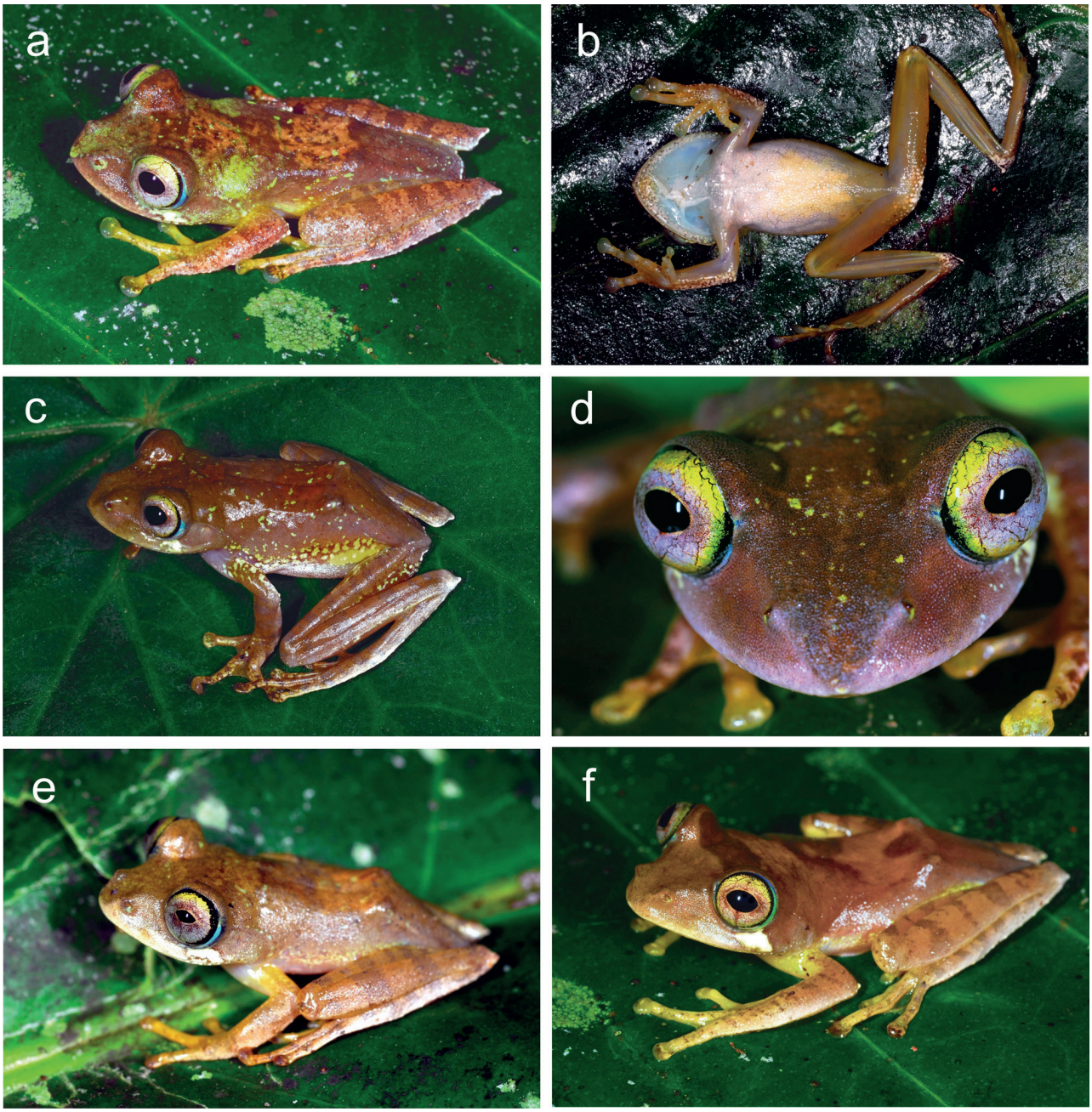

Figure 6. *Boophis fayi* sp. n. from the type locality in life: (a, b) male holotype (ZSM 280/2010) in dorsolateral and ventral views; (c) female paratype (ZSM 281/2010); (d) frontal close up of head of female paratype (note characteristic green iris colour); (e, f) male paratypes deposited at UADBA (FGZC 4251, 4260).

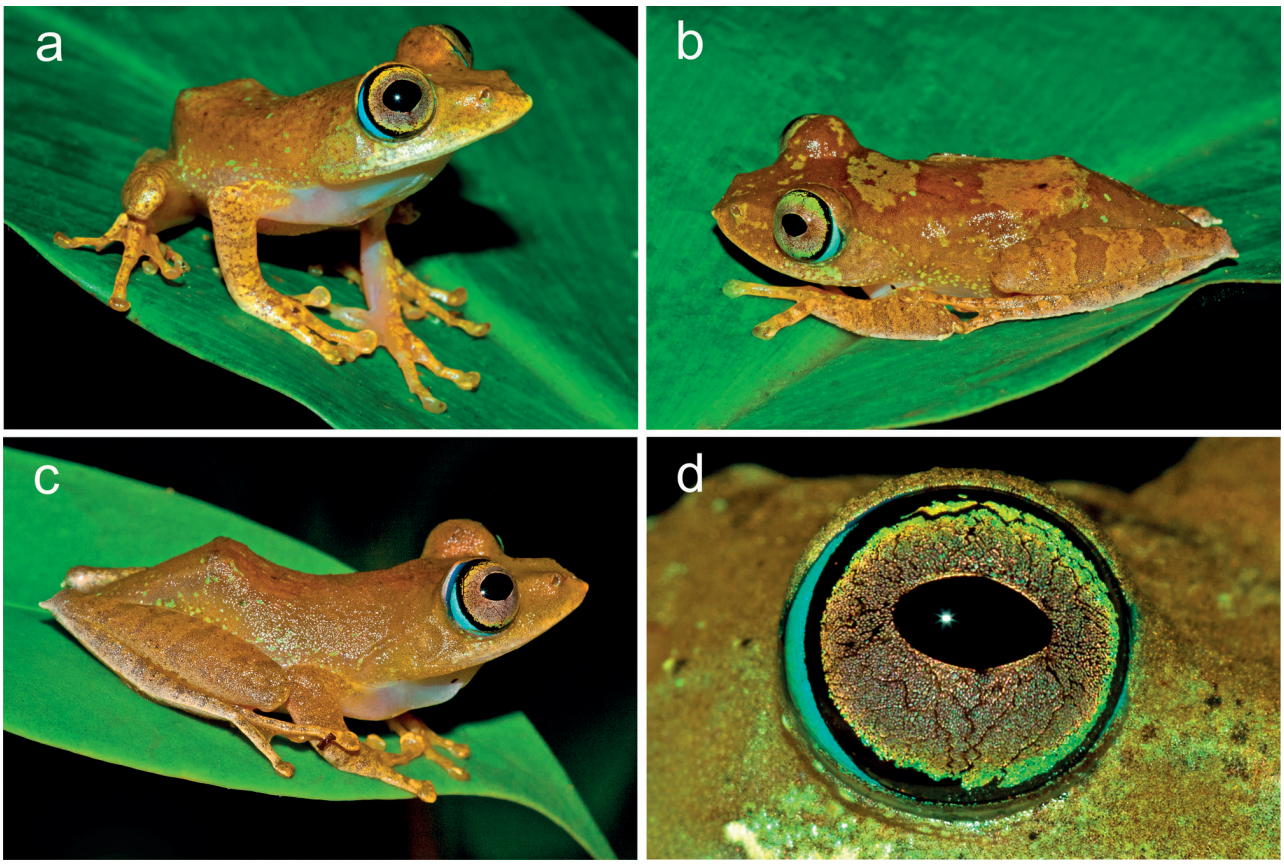

Figure 7. Variation of *Boophis fayi* sp. n. from Betampona in life: (a) adult male (MRSN A6596); (b) adult male (not collected, tissue sample FAZC 13972); (c) adult male (not collected); (d) close-up view of right eye of male (MRSN A6355, call voucher).

heels and ulnar tubercles white; spotting and reticulation on flanks yellowish; dorsal faces of inner toes yellow; dorsal faces of finger discs translucent green (Fig. 6a). Venter medially white with a yellow hue, posteriorly translucent yellow; throat translucent bluish white; ventral faces of shanks translucent yellowish green; bones white (Fig. 6b). Outer iris periphery blue, inner iris periphery turquoise, outer iris ring black; outer iris bright green with fine black reticulation, inner iris silvery with brownish reticulation.

Variation: The female paratype is larger (for variation in measurements, see Table 1). In colouration, the female largely agrees with the holotype, but lacks the green dorsal shading in life and has less distinct dark brown markings on dorsum (Fig. 6c). The yellow reticulation on flanks is more distinctly developed, and the whole dorsum was brown with a distinct reddish violet tint in life. The outer iris is entirely green with black reticulation, the inner iris silvery grey with reddish brown reticulation (Fig. 6d). The two UADBA male paratypes (FGZC 4251, 4260) also generally agree in colouration with the holotype, although one lacks the distinct dark brown transversal markings, and its dorsum is almost uniformly pale brown, whereas the other exhibits distinct dark transversal markings but lacks green dorsal spotting (Fig. 6e, f). Specimens from Betampona generally match this extent of variation in colour pattern (Fig. 7). However, they all exhibit the characteristic green iris colouration and a turquoise iris periphery as well as different degrees of yellow and green markings on dorsum and flanks. The supratympanic fold in males is either completely absent or barely developed and thus hardly discernible, whereas the supratympanic fold in the only known female is weakly developed, but clearly visible.

Vocalization: Two call types were recorded from the same male (MRSN A6355; Fig 7d) at Betampona on 11 December 2007, 21:00 h, at 21°C air temperature (Rosa et al. 2011, track 14, clips 1, 2 & 3).

Call type A (Fig. 8a) consists of series of 1–3 harmonious notes (1.4 ± 0.7, n = 9), with note durations of 100–370 ms (247.6 ± 97.3, n = 14). Series usually contain a longer initial note and shorter secondary notes and can last up to one second, depending on the number of notes per series. When the male emits more than one note, they are separated by intervals of 181–266 ms (225.0 ± 40.0, n = 4) duration. Notes are pulsatile in character, but pulse repetition rate is rather fast and single pulses are not recognizable. Note repetition rate is approximately 3.3/s. Frequency is distributed within a broad band of approximately 1000–8000 Hz. Three major frequency bands are recognizable: dominant frequency between 1800 and 3000 Hz and two other bands at 3000–5000 and 5000–7000 Hz. Each band is subdivided into several (8–9) narrow bands. According to the call structure and the context of emission, we consider this call type to represent the advertisement call.

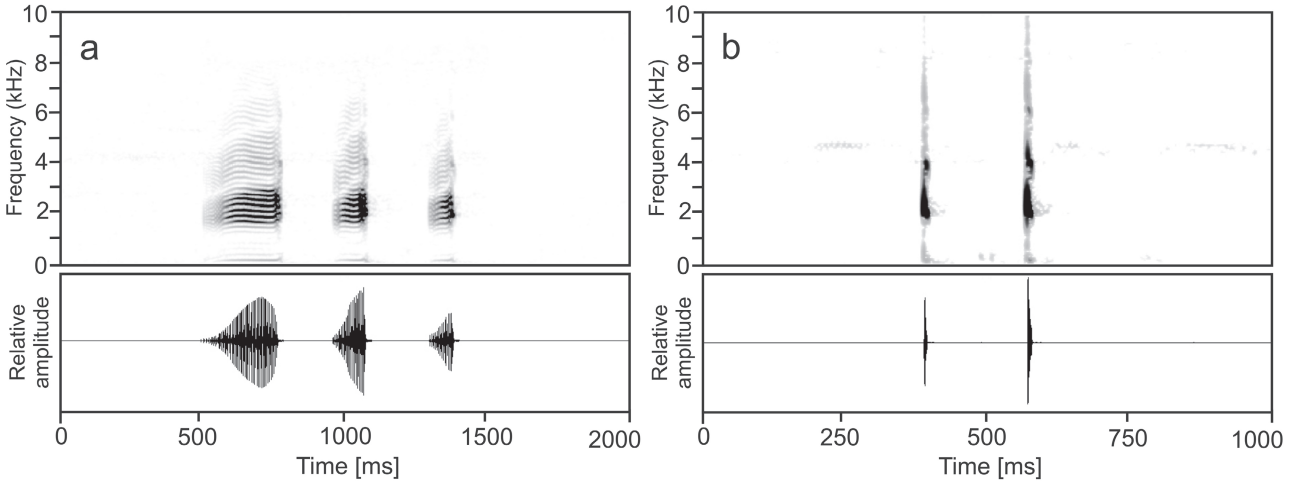

Figure 8. Audiospectrograms and corresponding oscillograms of calls of *Boophis fayi* sp. n. from Betampona (call voucher MRSN A6355): (a) call type A (possible advertisement call), (b) call type B (possible territorial call).

Call type B (Fig. 8b) was recorded from the same individual. It is composed of 1–2 click notes (n = 2) lasting up to 200 ms (n = 1), depending on the number of notes per series. Each note has a duration of 8–11 ms (9.7 ± 1.5, n = 3) and, when the call encompasses more than one note, the duration of the interval between the two notes is about 170 ms (n = 1). Notes are not pulsed and note repetition rate within series is approximately 6–10/s. Calling energy is distributed in a broad frequency band between 1000 and 7000 Hz, with the maximum call energy being present at approximately 2000 Hz. Series are separated by irregular intervals of 1.9–60.5 s (26.1 ± 22.2, n = 6). According to its structure, we suspect this call to have a territorial function, as has also been documented for, e.g., *B. tampoka* (Köhler et al. 2007, Vences et al. 2011).

The call heard at Ambodivoahangy in the Makira region (probably from FGZC 4260) on 2 April 2010 appeared overall similar to the previous description of call type A. Unfortunately, we were not able to record calls from this population because calling activity was rather low at the time of observation and calling occurred very sporadically.

In comparison, the general structure of the putative advertisement call of *B. fayi* is akin to the calls of the morphologically similar species *B. boehmei* and *B. quasiboehmei*, but differs by its longer note duration, longer inter-note intervals, and an apparently much greater pulse repetition rate in notes (compare Vences et al. 2010b).

Distribution: *Boophis fayi* is known from two localities. A small rainforest fragment close to the Makira Reserve along the Antainambalana river basin (see locality data of holotype) and from the Betampona Reserve in a narrow altitudinal range (ca. 300–400 m a.s.l.) at the central east coast 250 km farther south (Fig. 1). The species likely occurs in rainforests along the eastern coast between these two localities.

Natural history and conservation status: *Boophis fayi* is a nocturnal treefrog of the lowland rainforest. Males were usually found perching on vegetation up to ca. 3 m above the ground at night during the rainy season along small streams and swampy areas of secondary vegetation. Calling activity at Betampona was recorded in March and October 2007 (Rosa et al. 2011, track 14, clip 4) and at the beginning of April 2010 at Ambodivoahangy. Because of *B. fayi* and its distributional range being poorly known as yet, we propose an IUCN red list status of "Data Deficient" for this species (compare Andreone et al. 2005, 2008).

Etymology: The specific name is a patronym for Andreas Norbert Fay (Zurich, Switzerland) in recognition of his support of research and nature conservation through the BIOPAT initiative.

Discussion

With the two species described herein, the *Boophis goudoti* species group currently contains 16 species, which may artificially be divided into two groups: large and small species. However, phylogenetic relationships within the group are not fully resolved and so far, analyses of mitochondrial genes have not revealed a consistent grouping with respect to body size (Glaw et al. 2010, Vences et al. 2010b, Vieites et al. 2009). Nonetheless, most small members of the *B. goudoti* group share bright iris colours and rather similar advertisement calls (Glaw & Vences 1997b, Vences et al. 2005, 2010b). As intraspecific variation in external morphology is present to a considerable extent among the small species, the great similarity in vocalization complicates unequivocal species identification in the field. Nevertheless, genetic differentiation in mitochondrial markers is rather pronounced even among the species with the most similar morphology and calls, *B. boehmei* and *B. quasiboehmei* (Vences et al. 2010b). The same is true for *B. popi*. Among the already described species, *B. popi* shares a similar body size, similar advertisement call and reddish iris colours with *B. axelmeyeri*, *B. boehmei, B. rufioculis*, and *B. quasiboehmei*. However, in *B. popi*, pairwise sequence divergence in the 16S rRNA gene fragment as compared

to the mentioned species is 9.0–9.4, 8.1, 9.9 and 9.4%, respectively, and is generally > 8.1% to all other species of the group, with the exception of *B. fayi* (5.3%). Thus, although analyses of advertisement calls did not facilitate species delimitation in these frogs, genetic differentiation and the analysis of certain qualitative morphological characters provide evidence for the existence of distinct evolutionary lineages (see de Queiroz 2007). The situation is slightly different for the second species described herein, *B. fayi*. Its green iris colour and the barely developed supratympanic fold in males are unique features that readily distinguish it from all other described members of the *B. goudoti* group. Its genetic divergence from other species of the group is also pronounced and varies from 5.3% (Betampona population compared to *B. popi* from its type locality) to 10.3% (compared to *B. reticulatus*).

The two new species exhibit different distributional patterns. *Boophis popi* inhabits the submontane rainforests in the central east and southern central east of Madagascar at between 1000 and 1500 m a.s.l. and thus is the species with the highest altitudinal occurrence among the small-sized members of the *B. goudoti* group. At its lower altitudinal limits it could occur in sympatry with *B. boehmei* and *B. quasiboehmei*. *Boophis fayi* occurs in the lowland rainforests of the central east and northeast. The 16S rRNA sequences of individuals from the Betampona population in the east show a 2.6% pairwise divergence to *B. fayi* from the type locality and are thus in the same range of intraspecific differentiation known for other members of this species group (e.g., 2.1% in *B. axelmeyeri*, 2.5% in *B. popi*). The airline distance between both localities is approximately 250 km, however, it seems probable that *B. fayi* inhabits a strip of lowland rainforest along the east coast, whereas *B. popi* occurs in a band of submontane forests along the slopes of the eastern Madagascan mountain range.

Recent fieldwork and genetic analyses have accumulated new datasets for the *B. goudoti* group and we are aware of more strongly divergent lineages, most of which probably qualify for candidate species to be described in the future (Vieites et al. 2009, Glaw et al. 2010). However, these lineages were largely ignored here as detailed studies are still in progress and tentative results indicate that the situation within the group might be rather complex in several cases. As documented for the *B. luteus* group (Vences et al. 2011), the presence of considerable intraspecific variation and deep conspecific lineages in the *B. goudoti* group is rather probable and further studies are needed to clarify the status of various populations and the geographic distributions of species.

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