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An additional level of cryptic diversity: a new green-coloured Malagasy treefrog of the *Boophis luteus* species group

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Abstract. New genetic, bioacoustic and morphological data on green-coloured *Boophis* treefrogs from eastern Madagascar reveal an additional level of cryptic diversity in these frogs. Two candidate species, *Boophis* sp. Ca36 and Ca37, are closely related to each other and to *B. sandrae,* with uncorrected pairwise distances in the mitochondrial 16S rRNA gene as low as 2.2% between some individuals. However, the three lineages show full concordance between differentiation in the 16S and the nuclear-encoded SACS gene, despite confirmed syntopy of *B. sandrae* and *B.* sp. Ca37 in the Ranomafana region, and probable syntopy of *B.* sp. Ca36 and *B.* sp. Ca37 in the Andasibe region. Most likely, these lineages are also divergent in advertisement calls, but the available recordings cannot be reliably assigned to either of them. Based on new material collected from various new sites, we here formally name *B.* sp. Ca36 as new species *B. asquithi* sp. n., and suggest targeted fieldwork on calls and larval stages to allow for a complete and fully conclusive taxonomic revision of this species complex. The example of these frogs illustrates how continued underestimation of cryptic diversity in anurans can lead to incorrect assignment of specimens, and leads us to emphasize the importance of designating as name-bearing types (holotypes) of anurans only individuals whose identity is unambiguous by genetic data or, at least, call recordings reliably assignable to the type specimen.

Key words. Amphibia, Anura, Mantellidae, *Boophis luteus* species group, *Boophis elenae, Boophis sandrae, Boophis asquithi* sp. n., Madagascar, cryptic species.

Introduction

Within the species-rich anuran family Mantellidae, endemic to Madagascar and Mayotte Island (Comoros archipelago), *Boophis* treefrogs make up the most species-rich genus, with currently 79 species (Glaw et al. 2019, AmphibiaWeb 2021). *Boophis* have been among the first anuran groups from Madagascar in which a remarkable amount of cryptic diversity was observed, starting with the pioneering works of Blommers-Schlösser (1979).

Because many *Boophis* are rather vocal species that in most cases differ distinctly in their advertisement calls, early studies regularly identified – and scientifically named – new species that differed bioacoustically but were morphologically almost indistinguishable (e.g., Andreone 1993, Glaw & Vences 1992, 1994). This trend has been particularly obvious in the *Boophis luteus* species group, a clade of predominantly green-coloured species differing in calls and often in iris coloration, and whose colour rapidly fades into a homogeneous beige-yellow in preservative. While Blommers-Schlösser & Blanc (1991) recognized only one single species, *B. luteus*, the *B. luteus* group plus its split sister clade, the *B. albipunctatus* group (Glaw & Vences 2006, Wollenberg et al. 2011, Hutter et al. 2018), today contain 18 species. Apart from their size differences all these species are almost impossible to distinguish morphologically after some time of preservation in ethanol. The *B. luteus* species group as currently understood

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(Glaw & Vences 2006) contains ten species: *B. andohahela, B. andreonei, B. anjanaharibeensis, B. elenae, B. englaenderi, B. jaegeri, B. luteus, B. sandrae, B. septentrionalis,* and *B. tampoka.*

After the description of *Boophis elenae* by Andreone (1993) from Vohiparara, a village near Ranomafana National Park in the Southern Central East of Madagascar, it soon became clear that the assemblage of green-coloured and relatively large-sized *Boophis* in this region required further revision. In fact, at least one specimen depicted in Andreone (1993), the female paratype MRSN A71.2 from Ambatolahy, showed chromatic characters, in particular a non-striped iris coloration, that led Glaw et al. (2010) to assume that it belongs to a species different from *B. elenae.* Subsequently, Rakotoarivelo et al. (2006) described tadpoles from Andasibe (in the Northern Central East) assigned to this species complex, Vences et al. (2006) published call recordings and Glaw & Vences (2007) photographs of frogs from Ranomafana that differed from those of *B. elenae*. Vieites et al. (2009), based on substantial divergences in the mitochondrial 16S rRNA gene, defined three candidate species. One of these candidate species, *Boophis* sp. 22 (sensu Vieites et al. 2009) from Ranomafana, was later scientifically named and described as *Boophis sandrae* by Glaw et al. (2010). The other two lineages identified by Vieites et al. (2009) were defined as unconfirmed candidate species *B.* sp. 36 from Andasibe, and *B.* sp. 37 from Andasibe and Ranomafana, respectively. These lineages will be named *B.* sp. Ca36 and *B.* sp. Ca37 according to the convention of Perl et al. (2014), in the following.

Clarifying the taxonomy of this assemblage of frogs has proven to be particularly challenging. Despite Andasibe and Ranomafana being among the best-studied rainforest sites in Madagascar (Vieites et al. 2009), and despite many nights of targeted searches, only few individuals of *B.* sp. Ca36 and *B.* sp. Ca37 could be collected. Furthermore, because these frogs typically call from high perches on trees, to date no advertisement call recordings became available for these two lineages, or for *B. sandrae,* that could be unambiguously assigned to a voucher specimen.

In an effort to clarify the taxonomy of these frogs, we assembled all available information, and assessed the genetic diversity of these frogs in mitochondrial and nuclear-encoded genes more comprehensively, based on sequences of all previously available voucher specimens, plus several newly collected individuals from additional sites. Our data provide evidence for the species status of at least one, possibly two additional species of the *B. luteus* group in Southern and Northern Central Madagascar, one of which we here formally name as new species.

Materials and methods

Frogs were captured mostly at night and located by opportunistic searching during multiple field campaigns between 1994 and 2015, using torches and head lamps. Representative specimens were photographed in life, either in the field or the next morning after capture. Vouchers were euthanized using MS222 or chlorobutanol overdose, and tissue samples were collected for genetic analyses and preserved in 96–100% ethanol. Vouchers were fixed in 96% ethanol, preserved in 70% ethanol, and deposited in the collections of the Université d'Antananarivo, Département de Biologie Animale, Antananarivo, Madagascar (UADBA; now called Mention Zoologie et Biodiversité Animale), Zoological Museum Amsterdam, Netherlands (ZMA; now part of Naturalis, Leiden), Zoologisches Forschungsmuseum A. Koenig, Bonn, Germany (ZFMK), and the Zoologische Staatssammlung München, Germany (ZSM). ACZC, FAZC, FGMV, FGZC, ZCMV, MVTIS, LR, and PSG refer to field numbers of A. Crottini, F. Andreone, F. Glaw, M. Vences, L. Rakotoarivelo, and P.-S. Gehring, respectively. Morphological measurements (in millimetres) were taken by MV with a calliper to the nearest 0.1 mm as follows: snout–vent length (SVL), maximum head width (HW), head length from posterior maxillary commissure to snout tip (HL), horizontal eye diameter (ED), horizontal tympanum diameter (TD), distance from eye to nostril (END), distance from nostril to snout tip (NSD), distance between nostrils (NND), foot length (FOL), foot length including tarsus (FOTL), tibia length (TIBL), hindlimb length from cloaca to tip of longest toe (HIL), forelimb length from axilla to tip of longest finger (FORL), hand length (HAL). The definition of measurements, terminology and the description scheme follow Glaw et al. (2010) and Vences et al. (2010a, b), and Glaw & Vences (1997) for eye coloration. Webbing formulae follow Blommers-Schlösser (1979). Geographical regions in Madagascar (Northern Central East, Southern Central East) are named following the suggestions of Boumans et al. (2007).

Advertisement calls were recorded in the field using different analogue devices such as: Sony WM-D6C and Tensai RCR-3222 tape recorders with external microphones (Sennheiser Me-80, Vivanco EM 238). We sampled recordings at 22.05 kHz and 32-bit resolution and computer-analysed using the software CoolEdit Pro 2.0 (Syntrillium Software Corp.). Frequency information was obtained through Fast Fourier Transformation (FFT, width 1024 points) at Hanning window function; the audiospectrograms were obtained at Blackman window function with 256 bands resolution. Temporal measurements are given in milliseconds (ms) or seconds (s), as range, with mean ± standard deviation in parentheses. Terminology in call descriptions generally follows the call-centred scheme of Köhler et al. (2017).

For genetic analysis, DNA was extracted from tissue samples using a standard salt extraction protocol (Bruford et al. 1992). We amplified and sequenced a fragment of the mitochondrial 16S rRNA gene (16S) using standard protocols (Glaw et al. 2010, Vences et al. 2010a, b), and with primers 16Sar-L and 16Sbr-H of Palumbi et al. (1991), as well as a fragment of the nuclear-encoded sacsin gene (SACS) using primers and the nested PCR conditions de-

scribed in Shen et al. (2012). For phylogenetic analysis of 16S sequences, we carried out Maximum Likelihood inference under a GTR+I+G model as in Vences et al. (2010a, b), in MEGA7 (Kumar et al. 2016), testing robustness of nodes with 500 bootstrap replicates. Sequence divergences were calculated as uncorrected pairwise distances (p-distances) in MEGA7. The SACS sequences were studied separately because our goal was to assess concordant divergence in the two unlinked markers with the purpose of species delimitation under a genealogical concordance criterion (Avise & Ball 1990, Avise & Wollenberg 1997). For this, we pursued two lines of analysis: (i) For those samples for which full-length sequences (960 nucleotides) could be obtained, we separated the sequences into haplotypes using the Phase algorithm (Stephens et al. 2001) as implemented in DNASp 5 (Librado & Rozas 2009) and used the phased sequences to construct a haplotype network following the approach of Salzburger et al. (2011) with the program Haplotype Viewer (http://www.cibiv.at/~greg/haploviewer), based on a ML tree computed with MEGA7 under the Jukes-Cantor model. (ii) We also assembled a further SACS data set, which included sequences that were shorter than the 960 nucleotides, in order to allocate additional samples to lineages based on information from this nuclear-encoded gene. With this data set we calculated a ML tree under the Jukes-Cantor model with 500 bootstrap replicates from the unphased sequences. All new sequences were submitted to GenBank (accession numbers MZ484691–MZ484724 and MZ494675–MZ494695).

Nomenclatural acts

The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature, and hence the new names contained herein are available under that Code from the electronic edition of this article. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN.

The LSID (Life Science Identifier) for this publication is: urn:lsid:zoobank.org:pub: DCCC4C62-EABA-4E20-B589-1D253C412053. The electronic edition of this work was published in a journal with an ISSN and has been archived and is available from the following digital repositories: www.zenodo.org and www.salamandra-journal.com.

Results

Genetics

The molecular analysis confirmed the existence of two mitochondrial lineages in central eastern Madagascar, previously named *Boophis* sp. Ca36 and *B.* sp. Ca37. In the 16S tree (Fig. 1), these were placed sister to each other with 69% bootstrap support. Together they formed the sister group of *B. anjanaharibeensis,* albeit without bootstrap support, and these three lineages were sister to *B. sandrae.* The clade containing *B. anjanaharibeensis, B. sandrae*, *B.* sp. Ca36 and *B.* sp. Ca37 received 86% bootstrap support. Uncorrected pairwise distances in this species complex range from 5.0 to 6.3% between *B. anjanaharibeensis* and the other lineages; 2.8 to 3.2% between *B. sandrae* and *B.* sp. Ca36; 4.2 to 5.4% between *B. sandrae* and *B.* sp. Ca37; and 2.2 to 2.6% between *B.* sp. Ca36 and Ca37.

The analysis of sequences of the nuclear-encoded SACS gene confirmed these same lineages in the *B. sandrae* complex (Fig. 2): no haplotype sharing between any pair of lineages was found in the phased sequences in the haplotype network, and they all formed clades in the phylogenetic analysis of the unphased sequences.

Among the additional findings from our molecular analysis, by the inclusion of 16S sequences of additional samples, is (i) the occurrence of *B. elenae* in Tsinjoarivo, north of the northernmost record known so far (Antoetra; Andreone et al. 2007), and (ii) the occurrence of *B. andreonei* in Ambohitantely Special Reserve, distinctly further south than its previously known localities in northern Madagascar.

All genetic records of *B.* sp. Ca36 refer to localities in the Northern Central East region of Madagascar, i.e. Andasibe, Anosibe An'Ala, and Tarzanville, whereas *B. sandrae* was only found in the Southern Central East, i.e., Ranomafana and the nearby site Mahakajy (Fig. 3). In the Ranomafana region, *B. sandrae* occurs syntopically with *B.* sp. Ca37, apparently without admixture as can be judged by genealogical concordance of 16S and SACS. While the majority of records of *B.* sp. Ca37 are from the Ranomafana area, two samples are from Andasibe. These sequences refer to tadpoles collected by L. Raharivololoniaina in the period December 2001 to January 2002 (LR214, LR243) in the same stream and in the same period as other tadpoles (LR228, LR230, LR 234, LR249) assigned to *B.* sp. Ca36. We confirmed these sequences by re-extraction of DNA and re-sequencing from the same samples, which were collected in a year when no fieldwork took place in the Ranomafana region (and sample confusion with samples from that area, where all other *B.* sp. Ca37 were recorded, is thus extremely unlikely). Nevertheless, since the record of *B.* sp. Ca37 in the Northern Central East has since not been confirmed by any records of adults or advertisement calls, we consider its occurrence (and thus, syntopy without admixture of *B.* sp. Ca36 and Ca37) as in need of confirmation (albeit likely).

Bioacoustics

Recordings of two advertisement calls are available that differ distinctly from each other in temporal characters (Figs 4–5), leaving no doubt that they were emitted by different species. Both were emitted from high perches in trees, and in both cases, searches revealed individuals of large-sized green *Boophis* at the spots of call emission, which however could not unambiguously be observed emitting the recorded call, or could not be collected or se-

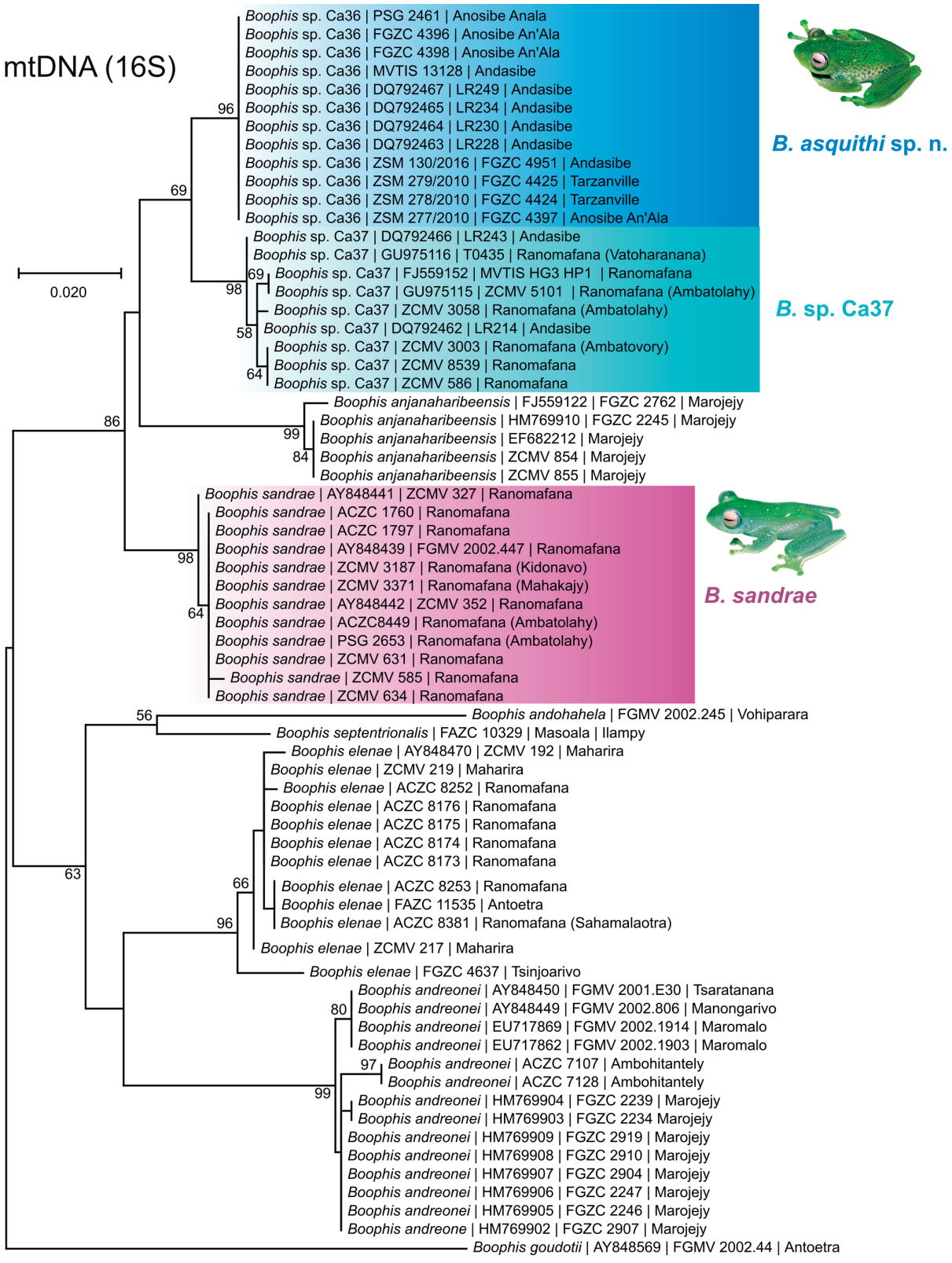

Figure 1. Maximum Likelihood tree of selected taxa of the *Boophis luteus* group (focusing on species characterized by an advertisement call consisting of a long series of fast repeated non-melodious notes), based on a 521 bp alignment of the mitochondrial 16S rRNA gene. Numbers at nodes are bootstrap support values in percent (500 replicates; not shown if < 50%). *Boophis goudotii* (of the *B. goudotii* group) was used as outgroup.

quenced. Because the calls of *B. elenae* and *B. luteus* (the other two large-sized green *Boophis* from the area) have been assigned by observations of unambiguously identified (and DNA barcoded) vouchers (our own unpublished data; see also Vences et al. 2011), the additional calls almost certainly belong to the *B. sandrae* complex. Considering the current knowledge of geographical distribution of lineages, the call recorded from Ranomafana (assigned to *B. sandrae* by Glaw et al. 2010) belongs either to *B. sandrae* (likely) or to the syntopic *B.* sp. Ca37; and the call from Andasibe belongs to *B.* sp. Ca36 (likely) or to *B.* sp. Ca37 (if this species indeed occurs at the site). The two calls are described in the following.

Boophis sandrae or *B.* sp. Ca37. – Advertisement calls recorded on 1 January 1996 at Ranomafana consist of a single pulsed note repeated at regular intervals in very fast succes-

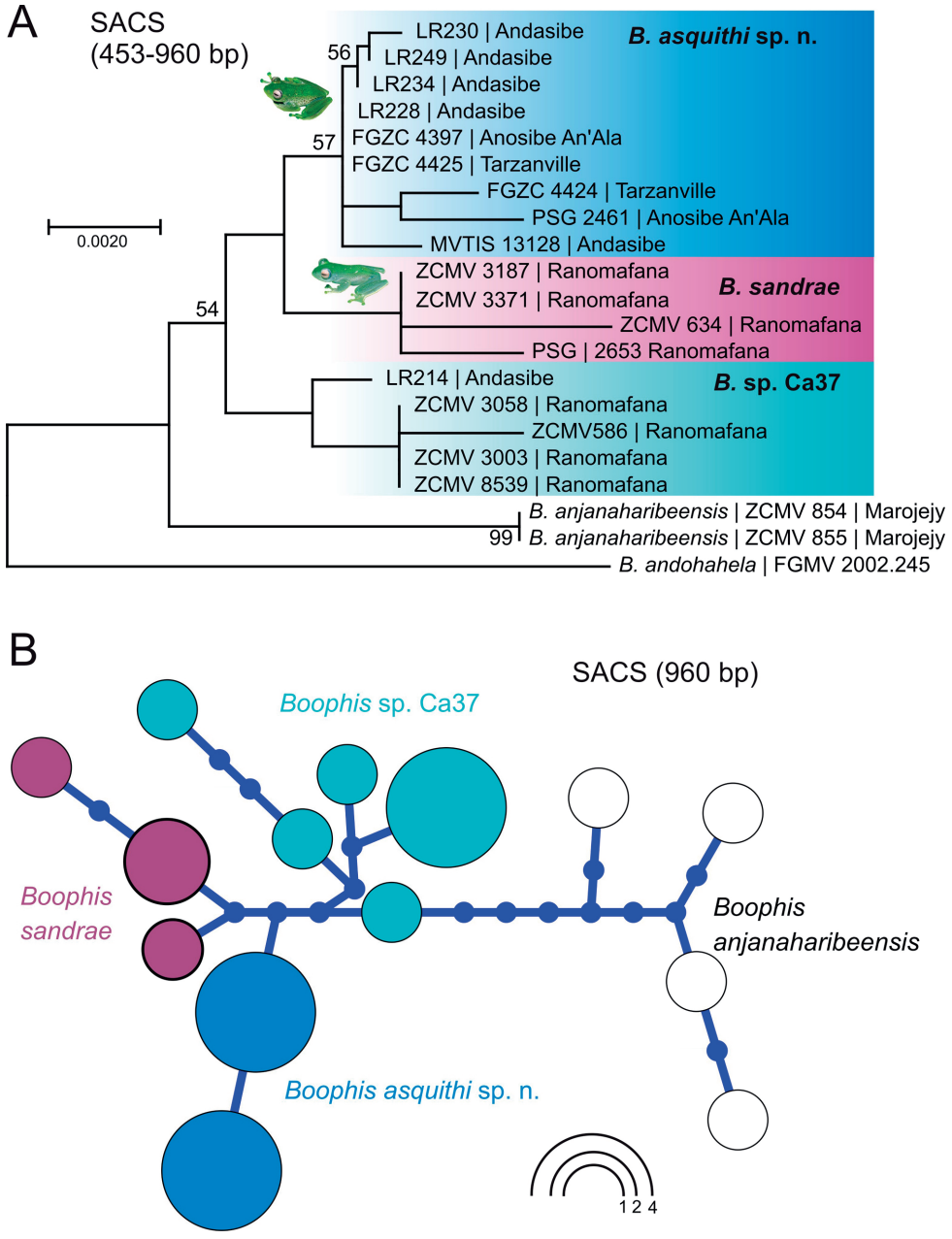

Figure 2. Molecular differentiation in the target taxa (*Boophis asquithi* sp. n., *B.* sp. Ca37, *B. sandrae*, and the related *B. anjanaharibeensis*) in the nuclear-encoded sacsin (SACS) gene. (A) Maximum Likelihood phylogenetic tree of all available sequences based on 453–960 bp unphased sequences (*B. andohahela* was used as outgroup; numbers at nodes are bootstrap proportions in percent and are only shown if > 50%). (B) Haplotype network based on phased sequences, for those samples where full-length sequences (960 bp) were available; each specimen is represented with two haplotypes in this network.

sion and very long series (duration of one recorded call series 33.6 s; Fig. 4). Each note is distinctly pulsed and exhibits pronounced amplitude modulation, with continuously increasing call energy from the beginning towards the end of the note. Numerical call parameters of 34 analyzed calls are as follows: call duration (= note duration) 13–23 ms (18.9 ± 2.4 ms); inter-call intervals (= inter-note intervals) 27–43 ms (32.5 ± 4.7 ms); call repetition rate within regular call series approximately 1300 calls/min; 3–7 pulses/note (4.7 ± 1.0); pulse duration 2–8 ms (4.1 ± 2.1 ms); pulse repetition rate within notes approximately 400 pulses/s; dominant frequency 2832–2970 Hz (2878 ± 46 Hz); prevalent bandwidth 2100–3400 Hz. Another call recording of rather poor quality obtained on 28 January 2004 near Vohiparara, Ranomafana, at 20–21°C air temperature, differs slightly in the measurements of call duration (27–34 ms) and inter-call intervals (14–18 ms), but the general character of both calls recorded is nearly identical and leaves little doubt that both belong to the same species. Differences observed are most probably due to different recording qualities. Both call recordings have already been described by Glaw et al. (2010), but information on locality and date were mixed among the two recordings.

Boophis sp. Ca36 or *B.* sp. Ca37. – Advertisement calls recorded on 15 January 1995 at Andasibe at 24.5°C air temperature, consist of a single, distinctly pulsed note repeated at regular intervals in fast succession and very long series (Fig. 5). Inter-call intervals (= inter-note intervals) are very short. Pulse structure within notes is somewhat irregular, with the initial pulse usually being the shortest and only very narrowly separated from the second pulse of the note. Moreover, notes exhibit some amplitude modulation with

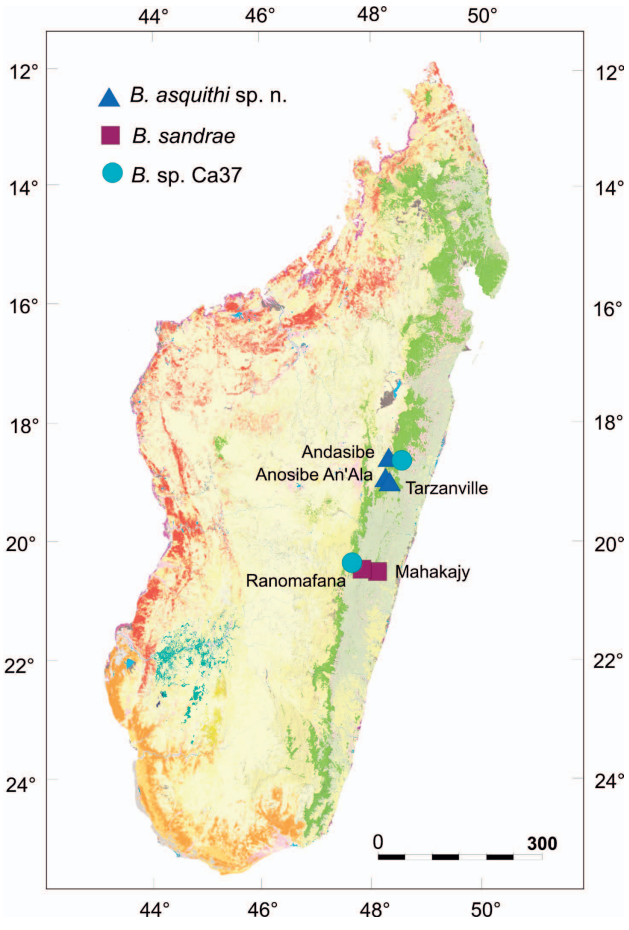

Figure 3. Map of Madagascar showing confirmed locality records of the focal lineages, *Boophis asquithi* sp. n., *B.* sp. Ca37, and *B. sandrae*.

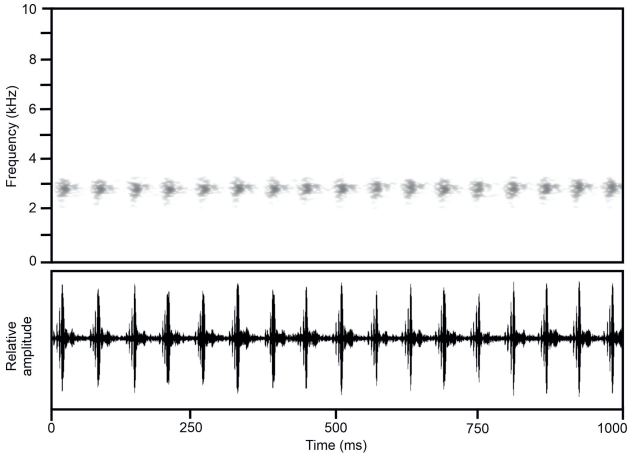

Figure 4. Spectrogram and oscillogram of advertisement calls provisionally assigned to *Boophis sandrae* recorded on 1 January 1996 at Ranomafana. The same advertisement calls have already been reported in Glaw et al. (2010) and the recording published in Vences et al. (2006). Since the calling voucher has not been sequenced, its attribution to *B. sandrae* is tentative.

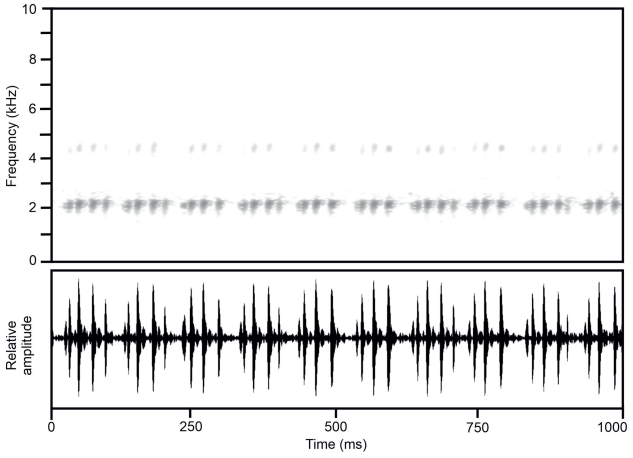

Figure 5. Spectrogram and oscillogram of advertisement calls provisionally assigned to *Boophis asquithi* sp. n., recorded on 15 January 1995 at Andasibe at 24.5°C air temperature. Since the specimen could not be seen calling and no voucher directly associated with this call has been collected, its attribution to *B. asquithi* remains tentative.

the initial pulse always containing less call energy than subsequent pulses. Numerical call parameters of 48 analyzed calls from two call series recorded (both > 20 s in duration, but both not completely recorded) are as follows: call duration (= note duration) 62–80 ms (70.8 ± 7.3 ms); inter-call intervals (= inter-note intervals) 16–38 ms (28.6 ± 6.5 ms); call repetition rate within regular call series ranges from approximately 590–615 calls/min; 4–5 pulses/note (4.5 ± 0.5); pulse duration 5–11 ms (8.2 ± 1.7 ms); pulse repetition rate within notes 44.4–90.9 pulses/s (59.6 ± 18.5 pulses/s); dominant frequency 2229–2377 Hz (2278 ± 40 Hz); prevalent bandwidth 2000–2400 Hz, with two weak additional peaks in call energy at around 4400 and 6600 Hz.

Advertisement calls of *B. elenae* from Maharira (Ranomafana National Park) mainly differed from the recordings described above by a distinctly longer note duration and lower call repetition rate (= note repetition rate) within call series, as illustrated in Figure 6. For a detailed call description, see Glaw et al. (2010).

Morphology and coloration

All specimens assigned to *B. sandrae, B.* sp. Ca36 and *B.* sp. Ca37 are very similar to each other (Figs 7–10, Table 1). We could not detect any obvious and constant differences in coloration from the available pictures in life. However, some differences seem to exist in body size: male body size appears to be smallest in *B. sandrae* (SVL 35.5–38.4 mm), intermediate in *B.* sp. Ca36 (37.8–40.5 mm), and largest in *B.* sp. Ca37 (43.6–48.2 mm). The same is true for females, where *B. sandrae* has a SVL of 50.3–52.7 mm, *B.* sp. Ca36 of 56.5 mm, and *B.* sp. Ca37 of 62.6–63.0 mm. The available photos (Figs 7–10) also indicate that in *B.* sp. Ca36, the iris periphery is usually cream with a light shade of blue, while in the two photos that can be reliably assigned to *B. sandrae* and *B.* sp. Ca 37, respectively, the posterior iris periphery is light blue (Fig 7, Fig 10).

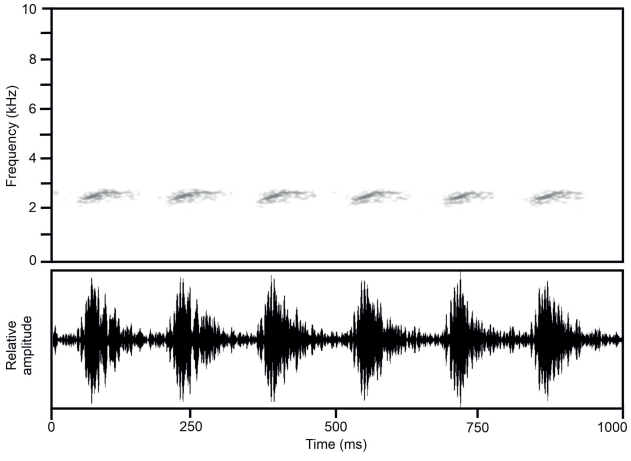

Figure 6. Spectrogram and oscillogram of advertisement calls of *Boophis elenae* from Maharira, Ranomafana, shown here for comparative purposes. The same call has already been reported in Glaw et al. (2010) and the recording published in Vences et al. (2006). Although no voucher specimen can be unambiguously assigned to the recording, calling specimens were observed in the field and the call therefore can be reliably assigned to *B. elenae* (M. Vences pers. obs. in 2004).

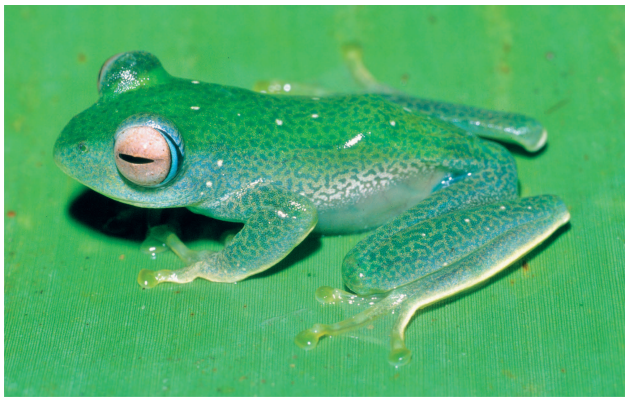

Figure 7. Male holotype of *Boophis sandrae*, ZMA 20133 (ZCMV 352), from Andranoroa river, Ranomafana, in life.

Taxonomy

Considering the substantial genetic divergence (around 3%) from the closest nominal species, *B. sandrae,* and the genealogical concordance in a mitochondrial and a nuclear-encoded gene, along with some (although faint) differences in body size, we are convinced that the *B. sandrae* complex contains more than one species. We here describe one of the identified lineages (*B.* sp. Ca36) as new species, *B. asquithi* sp. n., while we leave the status of *B.* sp. Ca37 pending, due to insufficient data.

***Boophis asquithi* sp. n.**

LSID: urn:lsid:zoobank.org:act:ECD34E32-0F53-4FCA-B0F8-7A80189B540A

Holotype. ZSM 278/2010 (field number FGZC 4424), adult male, collected in a forest near Tarzanville, Anosibe An'Ala region (geographical coordinates -19.32435°, 48.21988°, 881 m above sea level), eastern Madagascar, on 12 April 2010 by F. Glaw, J. Köhler, P.-S. Gehring, K. Mebert, E. Rajeriarison and F. M. Ratsoavina (Figs 8a, b, 11).

Paratypes. ZSM 279/2010 (FGZC 4425), adult female found in amplexus with holotype, same collection data as holotype; ZSM 277/2010 (FGZC 4397), UADBA (FGZC 4396) and UADBA (FGZC 4398), three adult males, collected at Anosibe An'Ala (-19.43492°, 48.20074°, 636 m above sea level), eastern Madagascar, on 11 April 2010 by F. Glaw, J. Köhler, P.-S. Gehring, K. Mebert, E. Rajeriarison and F. M. Ratsoavina; ZSM 130/2016 (FGZC 4951), adult male, col-

Table 1. Measurements of specimens of the *Boophis sandrae* complex with reliable molecular identification (all in mm), plus two relevant ZFMK specimens without molecular data. For abbreviations of measurements, see Materials and methods. Other abbreviations: HT, holotype; PT, paratype; PT (Bs) paratype of *B. sandrae*; M, male; F, female; na, not available.

Locality	Catalogue number	Field number	Status	Sex	SVL	HW	HL	TD	ED	END	NSD	NND	HAL	FORL	HIL	FOTL	FOL	TL
Boophis sandrae																		
Ranomafana, Andranoroa river	ZMA 20133	ZCMV 352	HT	M	35.8	12.1	12.9	2.0	5.5	2.4	3.3	4.3	10.2	20.8	58.2	25.7	15.7	18.9
Ranomafana village	ZMA 20134	ZCMV 585	PT	M	37.3	13.6	13.9	2.2	5.5	2.7	3.6	4.5	11.8	23.4	65.0	29.2	17.4	21.0
Ranomafana, Kidonavo bridge	ZSM 237/2006	ZCMV 3187	PT	M	35.5	11.7	12.7	2.0	5.1	2.4	3.4	4.2	10.4	21.8	58.2	26.4	16.2	18.5
Ranomafana, Ambatolahy forest	ZMA 20136	ZCMV 631	PT	M	36.7	13.0	13.7	2.0	5.2	2.7	3.1	4.7	11.2	22.1	65.2	29.5	17.7	19.9
probably Ranomafana	ZMA 20137	ZCMV 634	PT	M	38.4	14.2	14.1	2.1	5.6	3.0	3.2	4.6	11.3	23.2	65.5	28.7	17.0	20.6
Ranomafana, Andranoroa river	ZMA 20132	ZCMV 327	PT	F	52.7	17.5	17.9	3.0	6.3	3.5	4.8	6.1	15.4	32.1	87.3	38.5	24.0	27.0
Mahakajy near Ranomafana	ZSM 238/2006	ZCMV 3371	PT	F	50.3	17.2	18.2	2.6	6.0	3.7	4.1	5.3	14.6	31.0	85.0	37.0	22.3	27.2
***Boophis* sp. Ca 37**																		
Ranomafana, Ambatovory	ZSM 235/2006	ZCMV 3003	PT (*Bs*)	M	43.6	14.8	15.8	2.3	5.9	3.0	3.9	4.9	13.7	26.1	81.1	36.4	22.0	26.0
Ranomafana village	ZMA 20135	ZCMV 586	PT (*Bs*)	M	48.2	16.4	17.3	2.5	6.5	3.1	4.6	5.9	15.4	29.2	88.8	39.1	23.8	28.0
Ranomafana, Ambatolahy river	ZSM 236/2006	ZCMV 3058	PT (*Bs*)	F	63.0	20.8	21.5	3.4	7.2	4.4	5.6	7.0	19.2	39.2	108.4	49.5	30.7	33.7
Ranomafana, road near ValBio	ZSM 464/2009	ZCMV 8539		F	62.6	21.9	21.9	3.2	7.5	3.7	5.3	7.3	19.6	37.9	110.1	47.3	29.6	33.3
***Boophis asquithi* sp. n.**																		
Tarzanville	ZSM 278/2010	FGZC 4424	HT	M	40.5	13.2	13.6	2.1	5.4	2.8	3.7	5.2	12.3	24.4	67.1	28.8	16.8	21.3
Anosibe An'Ala	ZSM 277/2010	FGZC 4397	PT	M	37.8	13.4	13.9	2.2	5.2	3.0	3.6	4.8	11.7	22.6	66.5	28.5	17.4	20.8
Andasibe	ZSM 130/2016	FGZC 4951	PT	M	38.1	12.2	13.5	2.1	5.3	2.4	4.6	3.0	11.8	23.4	65.2	29.0	17.4	21.6
Tarzanville	ZSM 279/2010	FGZC 4425	PT	F	56.6	19.6	19.2	2.5	6.4	4.1	5.1	7.4	16.9	34.4	97.3	41.8	26.5	30.4
***Boophis* cf. *asquithi* (or Ca37)**																		
Andasibe	ZFMK 60027	NA	–	M	42.6	15.4	16.1	2.3	6.1	3.0	3.8	5.0	13.9	26.2	76.1	33.4	20.5	24.0
Andasibe	ZFMK 50646	NA	–	F	52.6	18.2	17.8	3.0	6.1	4.1	na	5.9	16.5	35.3	92.7	41.8	25.1	29.3

lected along the road to Andasibe near Hotel Feon'ny Ala (ca -18.942221°, 48.417243°, ca 940 m a.s.l.), eastern Madagascar, on 24 December 2015 by F. Glaw, D. Prötzel and L. Randriamanana. The two paratypes from the UADBA collection were not available for morphological examination but were unambiguously identified genetically (Fig. 1).

Description of the holotype. Adult male in good state of preservation, SVL 40.5 mm (Fig. 11). Body slender; head slightly longer than wide, slightly wider than body; snout rounded in dorsal view, obtuse in lateral view, nostrils directed laterally, nearer to eye than to tip of snout; canthus rostralis straight in dorsal view, loreal region slightly concave; tympanum distinct, rounded, TD 39% of eye diameter; supratympanic fold moderately distinct; vomerine odontophores distinct, well separated in two round patches, positioned posteromedian to choanae; choanae medium-sized, rounded. Posterior part of the tongue removed as tissue sample, tongue originally bifid, free posteriorly. Arms slender, subarticular tubercles single, round; metacarpal tubercles not recognizable; fingers moderately webbed and with (poorly recognisable) lateral dermal fringes; webbing formula 1(–), 2i(1), 2e(1), 3i(2), 3e(1), 4(1); relative length of fingers 1<2<4<3 (finger 2 distinctly shorter than finger 4); finger discs distinctly enlarged; distinct, well developed nuptial pad on inner side of first finger, unpigmented. Hindlimbs slender; tibiotarsal articulation reaching nostril when hindlimb is adpressed along body; lateral metatarsalia separated by webbing; inner metatarsal tubercle small, distinct, elongated; no outer metatarsal tubercle; webbing between toes well-developed, lateral dermal fringes present; webbing formula 1(0), 2i(0), 2e(0), 3i(1), 3e(0), 4i(1), 4e(1), 5(0); relative length of toes 1<2<5=3<4; toe discs enlarged. Skin on dorsal surfaces smooth, very finely granular on throat, slightly more coarsely granular on chest and belly; an apparently glandular (whitish) area ventrally of the cloacal opening; no distinctly enlarged tubercles in the cloacal region.

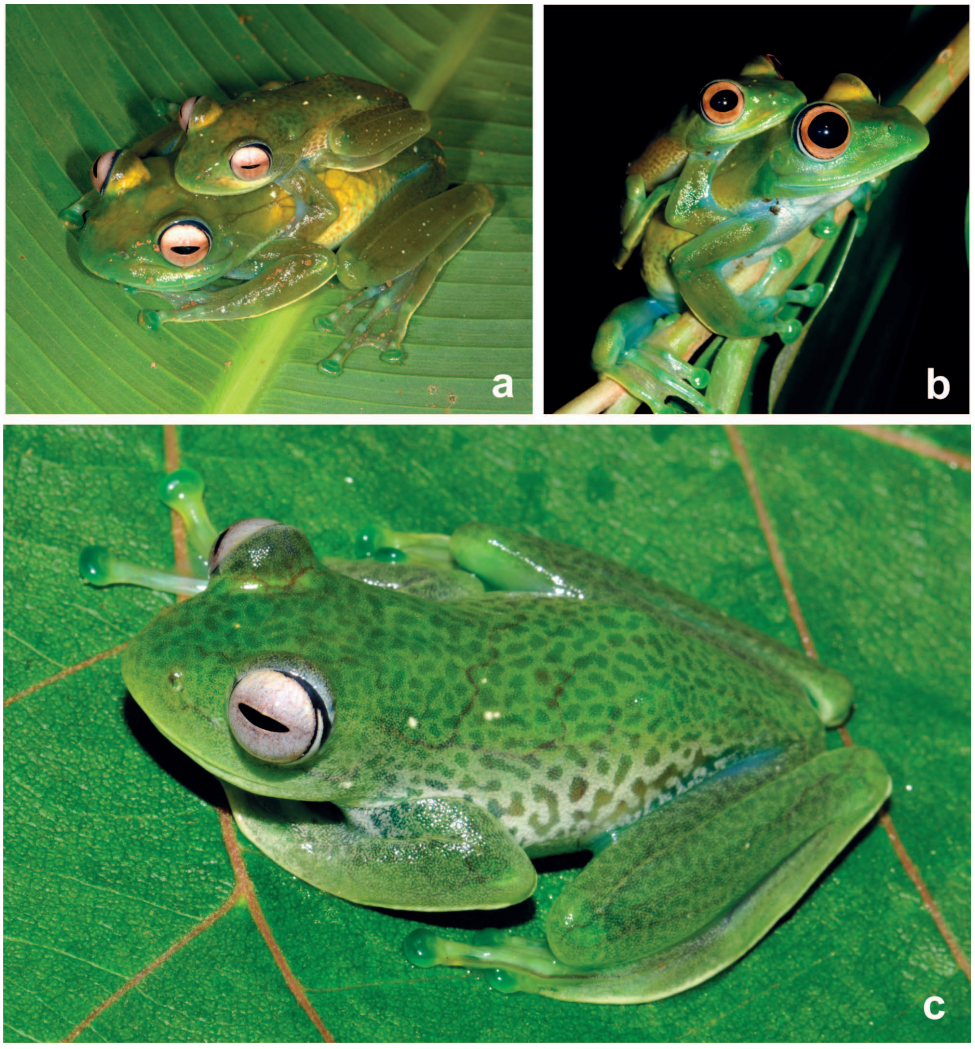

Figure 8. *Boophis asquithi* sp. n. in life: (a,b) Male holotype, ZSM 278/2010 (FGZC 4424), in amplexus with female paratype ZSM 279/2010 (FGZC 4425), from near Tarzanville; (c) Male paratype (ZSM 130/2016) from Andasibe.

After more than ten years in preservative, ground colour of flanks, dorsal and ventral surfaces uniformly creamy yellow. Skin above eyes dark grey. Nostril internally bordered with fine dark pigment.

In life (Fig. 8), ground colour of upper surface of head and dorsum light green with darker green spotting. A pale grey zone at midflanks with dark green-grey reticulations, starting behind the insertion of forelimbs; below this zone unpigmented. A bluish shade is present on external edge of upper eyelid, around the insertion of forelimbs and the borders of the concealed parts of the hindlimbs. During the day, iris colour around the horizontal pupil is copper, external parts of the iris (especially dorsally and ventrally) silvery. At night the iris forms a homogeneous red ring around the open and almost round pupil. Posterior iris periphery black, followed by cream with a faint bluish shade. Eye periphery dark blue posteriorly. White lateral fringes along the lateral edge of lower arm, tarsus, and heel. Webbing yellowish green. Small, distinct white spots scattered on the anterior part of the head and on the back, lower hindlimbs with numerous white dots. Dorsal surfaces of fingers and toes green, terminal discs blueish-green. Ventral life coloration of holotype unknown.

Variation. Morphometric data of three paratypes are provided in Table 1. The two males are similar to the holotype in size, general morphology and coloration. The female paratype ZSM 279/2010, which was found in axillary amplexus with the holotype, is distinctly larger than the males. Photographs of additional individuals, some of them probably deposited in UADBA and most likely belonging to *B. asquithi*, are shown in Figure 9.

Etymology. The specific name is dedicated to Mr. John David Asquith, in recognition of his support of biodiversity research and nature conservation through the BIOPAT initiative.

Natural history. Calls probably emitted by *B. asquithi* were sometimes heard from higher positions in trees around Andasibe in the rainy season, but unfortunately, call recordings are not available of any voucher specimen. The

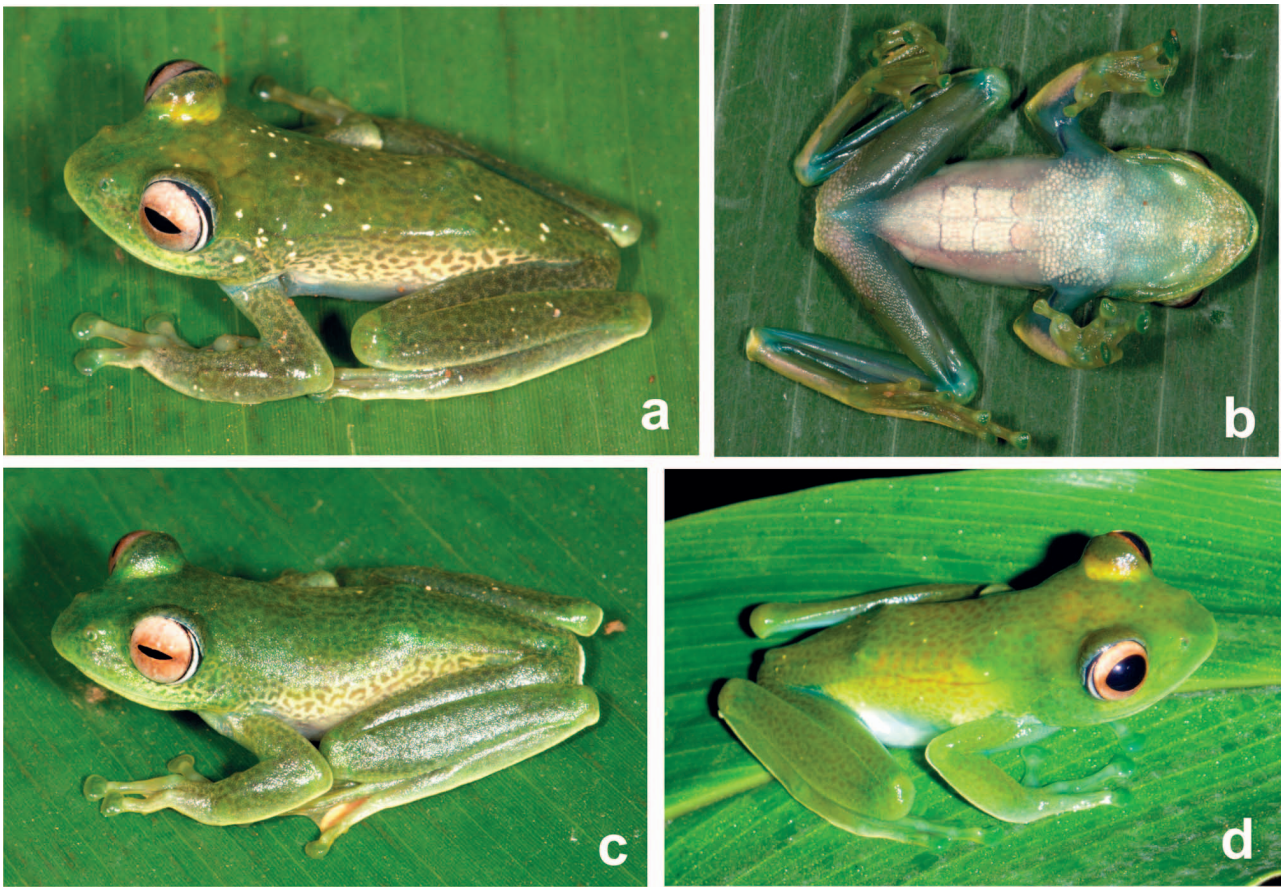

Figure 9. Additional specimens from near Tarzanville (region of Anosibe An'Ala) in life, probably all representing adult males of *Boophis asquithi* sp. n.: (a, b) dorsolateral and ventral views of a specimen photographed on 12 April 2010; (c) specimen photographed on 12 April 2010; (d) specimen, photographed on 11 April 2010. Because these photos cannot be reliably assigned to voucher specimen numbers, their species assignment cannot be fully ascertained; however, all vouchers collected from these sites during these field campaigns were genetically verified to belong to *B. asquithi* sp. n.

genetically unconfirmed male ZFMK 60027 was calling on 15 January 1995 (at night) from vegetation ca 3 m above the ground. The vocal sac was relatively large, but did not inflate during the vocalization, suggesting that the vocalizations were possibly produced both during expiration and inspiration. The couple from near Tarzanville (Fig. 8) was found in axillary amplexus along a small river in the evening of 12 April 2010. The female had numerous yellowish eggs in its body cavity. A second couple in axillary amplexus probably assignable to this species was found inactive (sleeping) during the day along the road. ZSM 130/2016 was sitting at night on a leaf in the vegetation along the road, less than 1 m above the ground and did not vocalize.

Vocalization. Advertisement calls probably belonging to this species (but perhaps instead assignable to *B.* sp. Ca37) have been described in the section Bioacoustics; see above.

Larval stages. Raharivololoniaina et al. (2006) reported on tadpoles collected at Andasibe whose DNA sequences identify them as *B. asquithi* (included in Fig. 1: LR228, LR230, LR234, LR249). However, the detailed tadpole description and drawing provided by these authors refers to the specimen LR214, which genetically belongs to *B.* sp. Ca37; the larval stages of *B. asquithi* therefore remain undescribed.

Figure 10. Female specimen of *Boophis* sp. Ca37, ZSM 236/2006 (ZCMV 3058), from Ambatolahy River, Ranomafana. Note that this specimen has been designated as paratype of *B. sandrae* (and figured as such in Glaw et al. 2010) but based on our new molecular data, belongs to *B.* sp. Ca37.

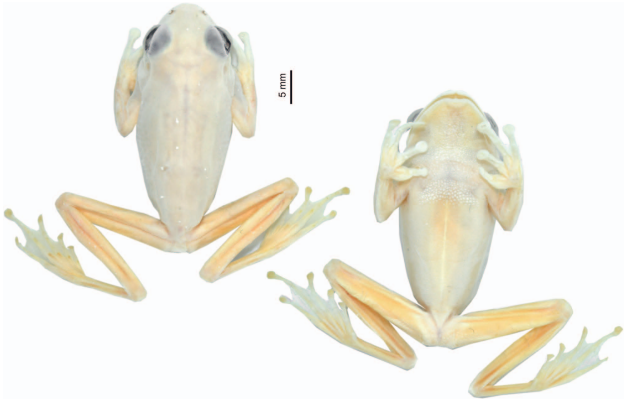

Figure 11. Preserved holotype of *Boophis asquithi* sp. n. (ZSM 278/2010) in dorsal (left) and ventral views (right).

Discussion

All known species in the *Boophis luteus* group emit loud advertisement calls in the rainy season and sometimes also during the dry season. Calling males often perched high in the trees and their vocal sacs only moderately inflate during call emission. Paradoxically, calls of these frogs are therefore usually ubiquitous in Madagascar's mid-elevation rainforests, yet observations of males in the moment of emitting their calls are comparatively rare. After observation and identification of a calling male high in a tree, an added difficulty is to catch and collect this individual without losing track of it and confusing it with other frogs that may be perched on the same tree or branch. These challenges resulted in a combination of usually many available call recordings but few unambiguously assigned call vouchers in this group of frogs.

Still, it is surprising to what extent these challenges affect the taxonomic conundrum surrounding the *B. luteus* group in eastern Madagascar. When Andreone (1993) initially described *Boophis elenae*, he distinguished the new species from *B. luteus* by the absence of a bright red coloured ring on the iris (typical for *B. luteus*), and by divergent advertisement calls. The original description includes informative photographs showing the coloration of the holotype from Vohiparara in life, including its distinct iris coloration with a silvery-whitish ground colour and well-defined thin red horizontal lines/rings around the pupil (Andreone 1993). Given this highly species-specific pattern in the name-bearing type specimen, the identity of *B. elenae* is beyond doubt. Also, the calls of *B. elenae* have been reliably assigned. The call description in Andreone (1993) does not refer to the holotype and is based on a rather noisy recording from Ambatolahy, but the re-description of the species' advertisement call in Glaw et al. (2010) is based on a recording from Maharira forest within Ranomafana National Park, where multiple individuals could be heard and seen calling (M. Vences, pers. obs. in 2004), although the available recording cannot be assigned unambiguously to one of the collected and sequenced specimens (several of which are included in Fig. 1).

On the other hand, not a single call recording can be unambiguously assigned to either *B. asquithi, B. sandrae,* or *B.* sp. Ca37. This suggests that these specimens often call from less exposed positions high in trees, and may be rather shy, quickly interrupting call emission when disturbed. At Andasibe we have spent many hours in vain trying to locate and collect specimens from very high positions in trees while emitting the call that we here assign tentatively to *B. asquithi*. At Ranomafana, we often heard a particular

call that Andreone (1993) had assigned to *B. albilabris,* a species at that time considered to be a close relative of *B. luteus*. Although we were able to collect specimens probably emitting these calls (later on described as *B. sandrae*; Glaw et al. 2010) we did not succeed in observing a (sequenced) voucher specimen calling, and therefore cannot exclude these calls may instead belong to *B.* sp. Ca37.

Despite the uncertainty surrounding the bioacoustic differentiation of the focal frog lineages, their genetic divergence in two unlinked loci under syntopic conditions provides clear evidence that more than one biological species are involved – and the evidence for morphological differences in body size and probable bioacoustic differentiation is in agreement with this hypothesis.

The present study provides an informative example on how taxonomy can be confused by underestimating the amount of cryptic diversity of organisms. In his initial study, Andreone (1993) did not consider the possibility that some of the frogs lacking the distinct iris coloration of *B. luteus* might belong to yet another species, in addition to the newly described *B. elenae*. This led him to define a paratype (MRSN A71.2) that probably belongs to a different species (*B. sandrae* or *B.* sp. Ca37). A similar conservative approach led also Glaw et al. (2010) to underestimate the species diversity in the *B. sandrae* complex; these authors included in the newly described species *B. sandrae* at least three paratypes without genetic data (ZMA 20135, ZSM 235/2006, ZSM 236/2006), which our present study revealed as belonging to *B.* sp. Ca37, and in the current study, we may be overly cautious again, not yet scientifically naming *B.* sp. Ca37 as new species despite evidence from mitochondrial and nuclear genes for it being an evolutionarily independent lineage. However, given that in integrative taxonomy, excessive splitting is more difficult to correct than excessive lumping (Miralles & Vences 2013), we opted for a conservative taxonomy where additional species may be described with new evidence in the future.

One obvious lesson learned, however, from the current example as well as from other, similar case studies in the Malagasy anuran fauna (e.g., Cocca et al. 2020), is an urgent recommendation to always designate, in anuran species, as holotype an individual that has been identified as unambiguously as possible. Typically, this will be a specimen with genetic data (preferably from multiple unlinked loci), or at least with clearly assignable advertisement call recording. The same applies, to a lesser degree, also to paratypes; but while an erroneous attribution of paratype specimens to species may cause some confusion, they eventually are without nomenclatural consequence given that paratypes have no immediate nomenclatural relevance in the International Code of Zoological Nomenclature. However, also paratypes can become relevant when the holotype is lost, as the Code recommends to choose neotypes from paratypes if these are still available in collections (recommendation 75A: ICZN 1999).

The status of *B.* sp. Ca37 remains to be clarified in future studies. While the data herein suggest it is likely another species-level lineage of large-sized, green-coloured *Boophis*, we propose the following information should be completed before drawing final taxonomic conclusions: (a) most importantly additional fieldwork and collections in the Andasibe region should be carried out to verify the putative syntopic occurrence of *B. asquithi* and *B.* sp. Ca37. If these two lineages indeed occur at the same site without genetic admixture, it would provide conclusive evidence for their status as separate species (Miralles et al. 2021); (b) targeted call recordings at Ranomafana and Andasibe are necessary. On one hand, by catching, measuring and genetically sampling multiple calling individuals of frogs of this complex, it will be possible to finally assign calls reliably to species; furthermore, such data will allow one to verify body size differences. On the other hand, such bioacoustic research also provides a test for the hypothesis of a third, yet unrecorded call attributable to this complex of species, and thus confirmation of bioacoustic differentiation among all three lineages; (c) lastly, detailed assessments of tadpole morphology may reveal further characters to differentiate the three lineages, as was previously found in other *Boophis* species (e.g., Randrianiaina et al. 2009). In fact, tadpoles of *B.* sp. Ca37 (wrongly reported as *B. sandrae*) were found to morphologically differ from those of *B. elenae* (Rasolonjatovo Hiobiarilanto et al. 2010). Interestingly, despite intensive surveys in the Ranomafana region, tadpoles of *B. sandrae* were not collected, although those of *B.* sp. Ca37 were encountered at several sites (Strauss et al. 2013). Future surveys should therefore specifically target the larval stages of *B. sandrae*.

The amount of cryptic diversity in the *B. sandrae* complex may continue surprising us. In another complex of green-coloured treefrogs, the *B. albipunctatus* complex, three species (*B. albipunctatus, B. sibilans, B. luciae*) occur in syntopy around Andasibe, of which *B. sibilans* was initially described as a subspecies of *B. albipunctatus* (Glaw & Thiesmeier 1993). Although the amount of genetic divergence among lineages in the *B. sandrae* complex is rather low, other studies have provided clear evidence for the existence of genetically closely related and morphologically cryptic species of Malagasy treefrogs in syntopy (Vences et al. 2012). However, lineages with such low genetic divergences may well occupy different positions in the "grey zone of speciation" (e.g., Dufresnes et al. 2020) and could also represent widely admixing, intraspecific lineages (Chan et al. 2021). Therefore, in such cases, additional scrutiny is required before naming them as new species. Basic field exploration and natural history assessments, including the targeted collection of voucher specimens, thus remain of paramount importance in Madagascar, even in well-known rainforests sites such as Analamazaotra-Mantadia National Park close to Andasibe, and Ranomafana National Park.

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