

## Diversity, elevational variation, and phylogeographic origin of stump-toed frogs (Microhylidae: Cophylinae: *Stumpffia*) on the Marojejy massif, northern Madagascar

ANDOLALAO RAKOTOARISON<sup>1,3,\*</sup>, MARK D. SCHERZ<sup>1,2,\*</sup>, MOLLY C. BLETZ<sup>1</sup>, JARY H. RAZAFINDRAIBE<sup>3</sup>, FRANK GLAW<sup>2</sup> & MIGUEL VENCES<sup>1</sup>

<sup>1</sup>Zoological Institute, Braunschweig University of Technology, Mendelssohnstr. 4, 38106, Braunschweig, Germany

<sup>2</sup>Zoologische Staatssammlung München (ZSM-SNSB), Münchhausenstr. 21, 81247 München, Germany

<sup>3</sup>Mention Zoologie et Biodiversité Animale, Faculté des Sciences, Université d'Antananarivo, Antananarivo 101, Madagascar

\* These authors contributed equally to this study

Corresponding author: MIGUEL VENCES, e-mail: m.vences@tu-braunschweig.de

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**Abstract.** Stump-toed frogs (genus *Stumpffia* Boettger, 1881) are a diverse group of small-bodied frogs endemic to Madagascar. Seven species of this genus occur on Marojejy, a steep massif in northeastern Madagascar. Here we examine the elevational distribution, phylogenetic position, biogeographic origin, and genetic differentiation of this *Stumpffia* assemblage. We show that none of these species is another's closest relative, but rather they are all independent lineages that probably colonised the Marojejy Massif through repeated immigration events. All of the lineages on Marojejy are most closely related to species south and southwest of the massif, except one lineage, formerly known as *Stumpffia* sp. Cao7, but here assigned to *S. sorata* as a deep conspecific lineage (and referred to as *S. cf. sorata*), which occurs also in Sorata, 90 km north of Marojejy. The species on Marojejy are typically restricted to narrow elevational ranges, but at least two species, *S. cf. sorata* and *S. tridactyla*, occur over elevations spanning 1000 metres. We assessed the genetic variability of these populations, and found considerable haplotype separation in fragments of the mitochondrial 16S rRNA and nuclear Rag-1 genes, suggesting some disruption of gene flow associated with elevation. We discuss the biogeographic implications of our findings and, based on previously published data, the evolution of non-overlapping bioacoustic parameters among the diverse assemblage of *Stumpffia* species on the Marojejy massif.

Key words. Amphibia, Anura, *Stumpffia sorata*, *Stumpffia* sp. Cao7, *Stumpffia tridactyla*, candidate species, deep conspecific lineage.

### Introduction

The Marojejy massif in northern Madagascar is one of the hotspots of diversity of stump-toed frogs of the genus *Stumpffia* Boettger, 1881, a Madagascar-endemic group of microhylids that contains 40 species after the revision of RAKOTOARISON et al. (2017). The largest part of the massif is included in Madagascar's network of protected areas as Marojejy National Park (PATEL 2015). Six species of *Stumpffia* are known to occur in Marojejy National Park, all but one apparently being micro-endemic to the massif, and with distinct patterns of elevational distribution: *Stumpffia achillei* and *S. diutissima* occur only in the lowland forests from 300–750 m a.s.l., while *S. grandis* is apparently only found at around 1330 m a.s.l., and *S. roseifemoralis* also is most common at higher elevations; on the contrary, *S. tridactyla* and *S. sp. Cao7* [a can-

didate species briefly discussed by RAKOTOARISON et al. (2017) but not taxonomically described], have much larger elevational distributions, with the former occurring from 1330–2026 m a.s.l., and the latter from 310–1330 m a.s.l. (RAKOTOARISON et al. 2017). A seventh species of *Stumpffia* occurring at Marojejy, *S. sp. Ca11*, is reported for the first time herein (see below) and is known from a single individual only.

All of these leaf-litter frogs are typically miniaturised and range in adult body size from ca. 9 mm to almost 30 mm. They usually have small ranges, not only in elevation as summarized above for several species, but also in terms of geographic area since most species are known from just one or two localities (RAKOTOARISON et al. 2017) – patterns typically found in small-sized amphibians in Madagascar (BROWN et al. 2016). Their biogeography apparently involved multiple instances of moderately long-

distance (>100 km) dispersal mixed with localised diversification (RAKOTOARISON et al. 2017).

Three main questions arise from the diversity of *Stumpffia* species on this mountain massif: (1) Which species are the closest relatives of the massif-endemics, (2) does their phylogeny suggest in-situ speciation on the mountain itself, and (3) how much gene flow is there in species distributed over large elevational ranges on the massif? In the present study, we address these questions, based on new survey data gathered in 2016 and DNA sequences of additional individuals collected during this survey, to shed further light on the biogeography of frogs on the Marojejy massif in particular, and in northern Madagascar in general.

### Materials and methods

We conducted fieldwork on Marojejy in November–December 2016. Field camps and dates were as follows: Camp o (14.4463° S, 49.7852° E, 310 m a.s.l.), 14–15 and 25–27 November; Camp ‘Mantella’ (14.4377° S, 49.7756° E, 456 m a.s.l.) 16–17 and 21–24 November; Camp ‘Simpona’ (14.4366° S, 49.7434° E, 1325 m a.s.l.) 17–21 November; Camp ‘Marojejia’ (14.4350° S, 49.7606° E, 774 m a.s.l.) 30 November–4 December. Sites above Camp ‘Simpona’ including a site for which we coined the name ‘Buzzard Rock’ (14.4406° S, 49.7400° E, ca. 1560 m a.s.l.) and the *Pandanus* forest (14.44755° S, 49.7337° E, 2026 m a.s.l.) and areas between these sites were visited on 19 November 2016.

Specimens were collected at night or day by searching in the leaf litter guided by the calling of males and through opportunistic searches. Specimens were anaesthetised and subsequently euthanised in MS-222 solution, fixed in 90% ethanol and preserved in 70% ethanol. Vouchers were deposited in either the Zoologische Staatssammlung München (ZSM) or the amphibian collections of the Mention Zoologie et Biodiversité Animale of the University of Antananarivo (UADBA-A). FGZC and ZCMV refer to F. GLAW and M. VENCES field numbers, respectively.

Tissue samples were taken by cutting pieces of leg muscle from the euthanised animals and preserved separately in 99% ethanol. Males and females were distinguished based on field observations (calling behaviour) or presence of a vocal sac in males, or eggs in females.

### DNA extraction and sequencing

Genomic DNA was extracted from muscle tissue samples preserved in 99% ethanol using a standard salt extraction protocol (BRUFORD et al. 1992). Separate analyses were carried out for fragments of two genes, the mitochondrial 16S rRNA (16S) gene and the nuclear recombination-activating gene 1 (Rag-1). Gene fragments were amplified via polymerase chain reaction with primers and protocols as in RAKOTOARISON et al. (2015, 2017): 16S was amplified with primers 16SL3 (AGCAAAGAHYWWACCTCGTAC-

CTTTTGCAT) and 16SAH (ATGTTTTTGATAAACAGCG), with 90 s at 94°C followed by 33 cycles of 45 s at 94°C, 45 s at 52°C, 90 s at 72°C, and a final extension step of 300 s at 72°C. Rag-1 was amplified with primers Rag1\_Coph\_F1 (CGTGATCGGGTAAAAGGTGT) and Rag1\_Coph\_R1 (TCGATGATCTCTGGAACGTG), with 120 s at 94°C followed by 35 cycles of 20 s at 94°C, 50 s at 53°C, 180 s at 72°C, and a final extension step of 600 s at 72°C. PCR products were cleaned with 0.15 units of Shrimp Alkaline Phosphatase (SAP) and 1 unit of Exonuclease I (New England Biolabs, Frankfurt am Main, Germany) incubated for 15 min at 37°C followed by 15 min at 80°C. Purified PCR products were sequenced on an automated DNA sequencer (Applied Biosystems ABI 3130XL). Sequencing reaction (10 µl) contained 0.2 or 0.3 µl of PCR product, 0.5 µl of BigDye 3.1 (Applied Biosystems, Darmstadt, Germany) and 0.3 µmol of primer. Sequences were checked and edited, and heterozygous positions in Rag-1 inferred in the software CodonCode Aligner 3.7.1 (Codon Code Corporation, Dedham, MA, USA). Newly determined sequences were submitted to GenBank (accession numbers MK452367–MK452379 and MK457424–MK457436).

### Sequence alignment and analysis

Sequences were aligned with those from previous studies in MEGA7 (KUMAR et al. 2016). For 16S we used the dataset from RAKOTOARISON et al. (2017). We included all sequences available for those species occurring in Marojejy, whereas for all other species we included only one sequence each. We then calculated a maximum-likelihood tree under a GTR+G model as previously determined by model testing (RAKOTOARISON et al. 2017) in MEGA7, with 2000 full heuristic bootstrap replicates to assess node support.

For Rag-1 we first trimmed all sequences to equal length and removed sequences containing ambiguities that could not be interpreted as heterozygotes. We then separated sequences into haplotypes using the Phase algorithm (STEPHENS et al. 2001) as implemented in DNAsp 5 (LIBRADO & ROZAS 2009). Phased sequences were subsequently used 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.

In addition, we also analysed sequences of a different stretch of the 16S gene which were obtained by an Illumina amplicon approach as described in VENCES et al. (2016). These short sequences of 201 bp were available for a larger number of specimens of *S. tridactyla* and *S. sp. Ca7* from different elevations in Marojejy and were thus used to analyse whether populations at different elevations on this massif show mitochondrial differentiation despite geographical proximity. To represent the encountered variation, consisting of only few mutations, we reconstructed haplotype networks as described above for Rag-1.

## Results

### Diversity and relationships of Marojejy-endemic *Stumpffia* lineages

A phylogenetic analysis of a DNA sequence alignment of 598 bp of a fragment of the 16S gene (Fig. 1) confirmed the presence of seven species-level lineages of *Stumpffia* on the Marojejy massif (Fig. 2). These include the six lineages already reported by RAKOTOARISON et al. (2017), plus a specimen of *Stumpffia* sp. Ca11, newly reported here. This specimen was collected by us in 2016 at 1330 m a.s.l. on Marojejy. This candidate species was previously known only from Ambolokopatrika to the southwest of Marojejy. While this single and short gene fragment is not suitable to reconstruct deep relationships within *Stumpffia* (see the better resolved multi-gene tree in RAKOTOARISON et al. 2017), it illustrates that the *Stumpffia* from Marojejy are not each other's closest relatives, as each of them (except *S. grandis*, which has no obvious sister lineage) is grouped with high support with other species from different sites.

Three species of *Stumpffia* on Marojejy are most closely related to species or lineages from Ambodivoangy, a low elevation locality ca. 90 km south of the mountain. In the following we give their respective genetic divergences as uncorrected pairwise distance in the 16S fragment typically used for DNA barcoding Madagascar's frogs (VIEITES et al. 2009) from RAKOTOARISON et al. (2017): *S. tridactyla* is deeply divergent (10.7% uncorrected p-distance) from its sister species *S. contumelia* from Ambodivoangy, *S. diutissima* is fairly strongly divergent (8.5%) from its sister, *S. pardus*; and *S. roseifemoralis* from Marojejy is also strongly divergent (9.6%) from its sister lineage, an unconfirmed candidate species from Ambodivoangy called *S. sp. Ca57* by RAKOTOARISON et al. (2017). *Stumpffia achillei* is sister to *S. analanjirofo* from Nosy Mangabe and the surrounding mainland, ca. 110 km south of Marojejy, and these two lineages are separated by just 3.0% uncorrected p-distance. The sister lineage of *Stumpffia* sp. Ca11 from Marojejy and Ambolokopatrika is *S. kibomena* from Andasibe, more than 500 km SSW of Marojejy, and these lineages are separated by 6.3% uncorrected p-distance. This species may therefore warrant description, but we will deal with its taxonomy and that of other red-bellied *Stumpffia* elsewhere, once that sufficient specimens are available for analysis.

*Stumpffia* sp. Ca07 is the only lineage whose closest relative is found north of the massif, being sister to specimens of *S. sorata* from the Sorata massif, 90 km NNW of Marojejy, separated by 3.3% uncorrected p-distance. These lineages belong to a clade (Clade A of RAKOTOARISON et al. 2017) with its centre of endemism in northern Madagascar, in contrast to the other species, which all belong to clades with their centres of endemism in eastern or northeastern Madagascar (RAKOTOARISON et al. 2017).

### Taxonomic status of *Stumpffia* sp. Ca07

The taxonomic status of *Stumpffia* sp. Ca07 warrants comment in light of our new sequence data. In our mitochondrial tree (Fig. 1), it was recovered as sister to *S. sorata* with high support. The uncorrected p-distance to this lineage of 3.3% in the typically-used barcoding fragment of the 16S gene (VIEITES et al. 2009) and of 4.2–4.5% in the other fragment of the same gene analysed here exceeds the threshold of 3% established by VIEITES et al. (2009) to identify a lineage as a candidate species. A comparison of Rag-1 sequences (335 bp) revealed a high variation of alleles (haplotypes) within *S. sp. Ca07* in Marojejy, with up to 7 mutations among alleles (Fig. 3). Samples from low elevation on Marojejy had an exclusive allele differing from the one found at higher elevation. Haplotypes of *S. sorata* from its type locality (Sorata Massif) differed by only a single mutation from the nearest allele of *S. sp. Ca07* (from Marojejy Massif, Camp Simpona at 1330 m a.s.l.), but no allele sharing was observed.

Genetic evidence that *S. sp. Ca07* is a separate species from *S. sorata* is therefore equivocal; the two lineages are on the one hand clearly distinct in mitochondrial DNA, but only at a relatively low level. Given their separation of 90 km, but connection through more or less continuous forest at 1330 m a.s.l., and the absence of surveys from the intervening forests, it seems likely that intermediate populations exist that would connect these two extremes of their distribution, and that might break up the 16S distance and have allele sharing in nuclear genes.

We compared the morphology of specimens of *S. sp. Ca07* to *S. sorata*, and found them to be extremely similar (measurements in Table 1, compared with data from RAKOTOARISON et al. (2017); see also Revised circumscription of *Stumpffia* cf. *sorata* in Supplementary Appendix). The only difference between these lineages that appears to be more or less consistent is that specimens from Marojejy tend to be slightly smaller than those from Sorata (SVL: 11.3–15.1 mm vs. 15.6–16.0 mm) and have marginally longer feet (FOTL/SVL: 0.67–0.76 vs. 0.63–0.65). As bioacoustic data are not available from *S. sorata*, we cannot compare the calls of *S. sp. Ca07* (described in RAKOTOARISON et al. 2017) to those of that species.

In light of this evidence, to avoid taxonomic inflation, we here consider *S. sp. Ca07* in a preliminary way as a deep conspecific lineage of *S. sorata*, and refer to it herein as *S. cf. sorata*, until its status can be clarified by further data on the genetics, morphology, and bioacoustics of intervening populations between Marojejy and Sorata.

### Elevational distribution and gene flow in *Stumpffia* on Marojejy

There is considerable variability among species in elevational range sizes (Fig. 3): *S. achillei* was found from 480–

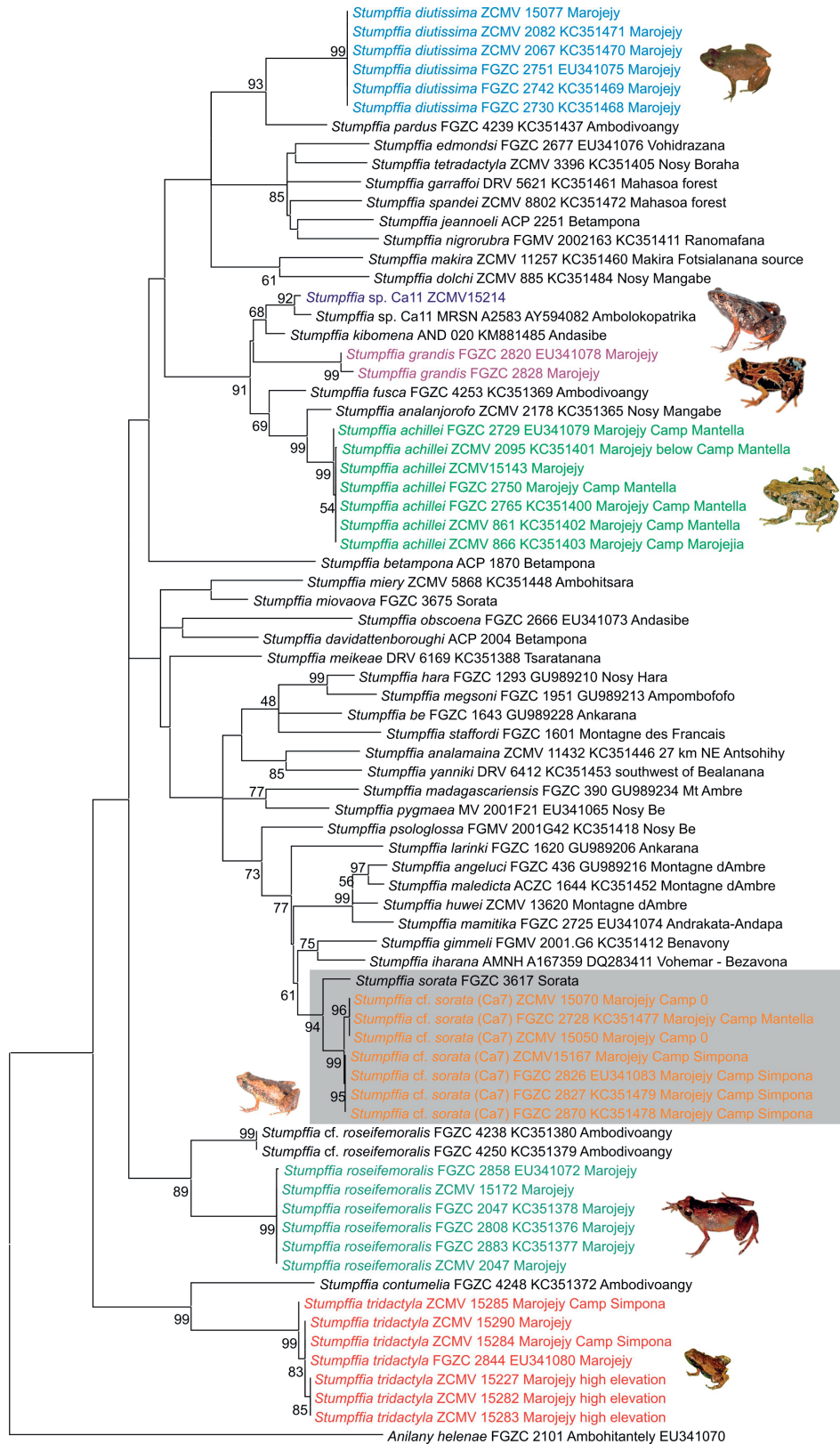


Figure 1. Maximum Likelihood tree inferred from 598 bp of the mitochondrial 16S gene, with all available samples of *Stumpffia* occurring in Marojejy (coloured), plus one sequence each of all other nominal species of the genus. Numbers at nodes are support values in percent from a bootstrap analysis (2000 replicates; only values >50% are shown). Specimens assigned to *S. sorata* are shown in a grey box.

750 m (restricted to bamboo forests), and *S. diutissima* from 310–750 m. *Stumpffia roseifemoralis* was found mostly at 1330 m around Camp Simpona, but one record (in need of confirmation; see Discussion) exists from at 481 m, and is therefore possibly distributed over this wide elevational range. *Stumpffia grandis* and *S. sp. Ca11* were found only at 1330 m, *S. sorata* was found from 310–1330 m, and *S. tridactyla* from 1330 m to the peak at nearly 2300 m.

We analysed genetic differentiation related to elevation in two of these species, *S. tridactyla* and *S. sorata*, the elevational ranges of which both span over 1000 m a.s.l. In *S. sorata*, specimens from low elevations shared a unique haplotype that differed by at least two steps from high-elevation specimens in Rag-1 sequences (Fig. 4). Additionally, four specimens from 1326 m a.s.l. differed by two mutations from three specimens collected between 310 and 481 m a.s.l. in a short segment of the mitochondrial 16S rRNA gene (Fig. 5). In *S. tridactyla*, three specimens found at 2026 m a.s.l. differed by 1–2 mutations from four specimens collected between 1326–1573 m a.s.l. in the same 16S fragment (Fig. 5).

## Discussion

### Phylogeographic origin of *Stumpffia* species on Marojejy

A previous phylogenetic study included various *Stumpffia* species from Marojejy (WOLLENBERG et al. 2008) and placed two of these Marojejy lineages into a clade (*S. grandis* and *S. sp. 4*, the latter corresponding to *S. achillei*), suggesting that these might represent an event of in-situ diversification on the massif. The wider analysis of RAKOTOARISON et al. (2017) confirmed by additional data herein (Fig. 1) uncovered and described an unprecedented number of additional species of this genus, and revealed that the sister lineages of the Marojejy *Stumpffia* species all are allopatrically distributed, not occurring on this massif.

Many of these occur to the south of Marojejy, i.e., in the Makira-Masoala lowland forest (localities Ambodivoangy and Nosy Mangabe) or the adjacent Ambolokopatrika forest, while *S. sorata* is found in Sorata to the north. All of these sister lineages occur in the north-eastern or north-

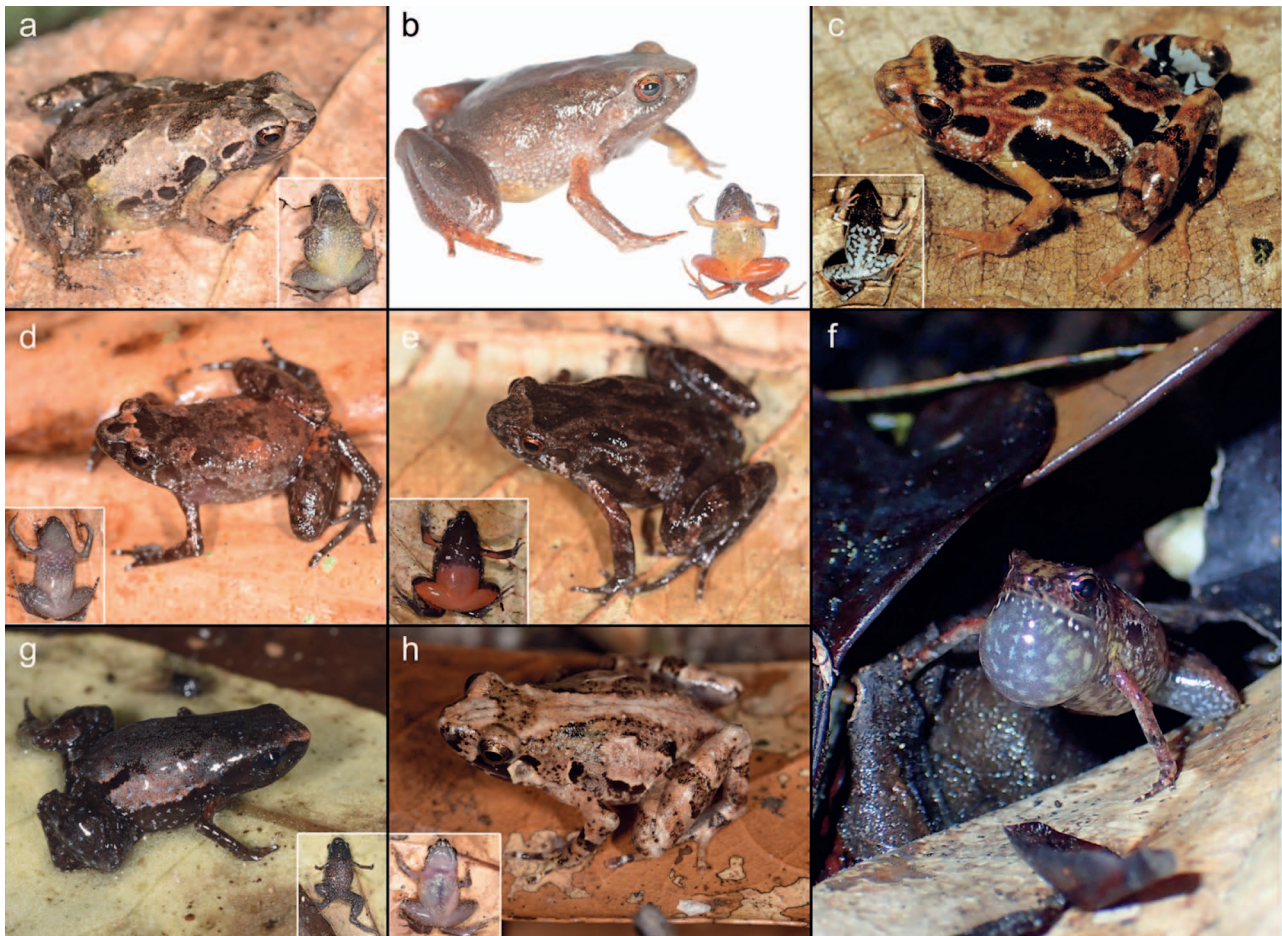


Figure 2. *Stumpffia* species occurring in Marojejy National Park: (a) *Stumpffia* sp. Ca7 (here considered as *S. cf. sorata*: ZSM 555/2016 (ZCMV 15182); (b) *Stumpffia roseifemoralis*, ZSM 529/2016 (ZCMV 15172); (c) *Stumpffia grandis* (not assigned to voucher specimen); (d) *Stumpffia diutissima*, ZSM 547/2016 (ZCMV 15117); (e) *Stumpffia* sp. Ca11, ZSM 551/2016 (ZCMV 15214); (f) *Stumpffia* cf. *sorata*, ZSM 544/2016 (ZCMV 15181); (g) *Stumpffia tridactyla* (specimen collected, but specimen number uncertain); (h) *Stumpffia achillei*, ZSM 536/2016 (ZCMV 15149).

Table 1. Morphometric measurements of *Stumpffia* cf. *sorata* from Marojejy (all in mm). ND: Not Determined

Collection number (Field nr)	Sex	SVL	HW	HL	TD	ED	END	NSD	NND	FORL	HAL	HIL	FOTL	FOL	TIBL
ZSM 379/2005 (FGZC 2826)	ND	14.6	5.1	4.9	1.1	1.6	1.3	0.9	2.1	7.9	2.7	21.1	10.0	6.0	7.4
ZSM 537/2016 (ZCMV 15047)	ND	11.4	4.2	4.6	1.2	2.0	1.1	0.9	1.4	7.6	2.4	16.8	8.7	5.0	6.4
ZSM 538/2016 (ZCMV 15048)	ND	11.3	4.1	4.6	1.1	1.9	1.0	0.9	1.2	7.7	2.3	16.9	8.7	5.2	6.3
ZSM 539/2016 (ZCMV 15049)	ND	14.0	4.7	4.8	1.3	2.0	1.1	1.1	1.2	8.5	2.7	20.5	8.9	6.0	6.4
ZSM 540/2016 (ZCMV 15050)	ND	12.6	4.8	4.4	1.2	2.0	1.1	0.9	1.6	6.8	2.8	19.3	8.9	5.2	6.3
ZSM 541/2016 (ZCMV 15069)	ND	11.6	4.2	4.7	1.2	2.0	1.1	0.8	1.1	7.5	2.9	16.7	8.8	5.2	6.4
ZSM 542/2016 (ZCMV 15070)	ND	12.0	4.5	4.7	1.2	2.0	1.1	1.0	1.4	7.7	2.7	20.8	8.7	5.2	6.4
ZSM 543/2016 (ZCMV 15168)	ND	14.8	6.0	4.9	1.2	2.2	1.4	0.9	1.9	6.7	2.9	20.7	9.8	6.7	6.6
ZSM 544/2016 (ZCMV 15181)	Male	14.6	5.3	5.0	1.2	2.2	1.4	0.9	1.5	8.6	3.2	22.4	11.6	6.8	7.7
ZSM 545/2016 (ZCMV 15182)	Male	15.1	5.0	5.2	1	2.5	1.2	1.1	1.6	6.1	2.9	22.3	10.3	5.8	6.8
ZSM 550/2016 (ZCMV 15212)	ND	15.1	5.1	5.2	1.1	2.4	1.2	1.1	1.5	6.0	2.8	22.1	10.1	5.5	6.5
ZSM 554/2016 (ZCMV 15281)	Juvenile	8.5	3.2	3.4	0.3	1.5	0.9	0.8	1.1	4.3	1.9	12.7	5.7	2.9	4.0
ZSM 555/2016 (ZCMV 15187)	ND	14.4	5.1	4.6	0.9	2.5	1.5	0.9	1.4	7.5	3.0	ND	ND	ND	ND
ZSM 556/2016 (ZCMV 15167)	Juvenile	9.2	3.4	3.5	0.3	1.7	0.8	0.8	1.1	4.5	2.0	13.9	6.1	3.6	4.4

ern geographic regions of Madagascar (BROWN et al. 2016) at distances <200 km, suggesting a mechanism of regional diversification (WOLLENBERG et al. 2008) in allopatry, possibly in part via divergence in montane refugia (RAXWORTHY & NUSSBAUM 1995).

Of particular interest is the occurrence of *S. sorata* both in Marojejy (here reported as *S. cf. sorata*) and the Sorata Massif revealed here. We have recently published several species descriptions arising from a herpetological survey

of the Sorata massif, undertaken in 2012 (SCHERZ et al. 2015, 2017, 2018a, b, RAKOTOARISON et al. 2017, PRÖTZEL et al. 2018). In the majority of these cases, a close affinity has been suggested between the taxa of Sorata and Marojejy; several species have their sisters lineages on Marojejy (e.g. *Gephyromantis* (*Duboimantis*) *grosjeani* sister to *G. (D.) tandroka*; *Rhombophryne longicrus* sister to *R. minuta*), while others are apparently conspecific across the massifs (e.g. *Calumma uetzi*, *Gephyromantis* (*Vatomantis*) *lomo-*

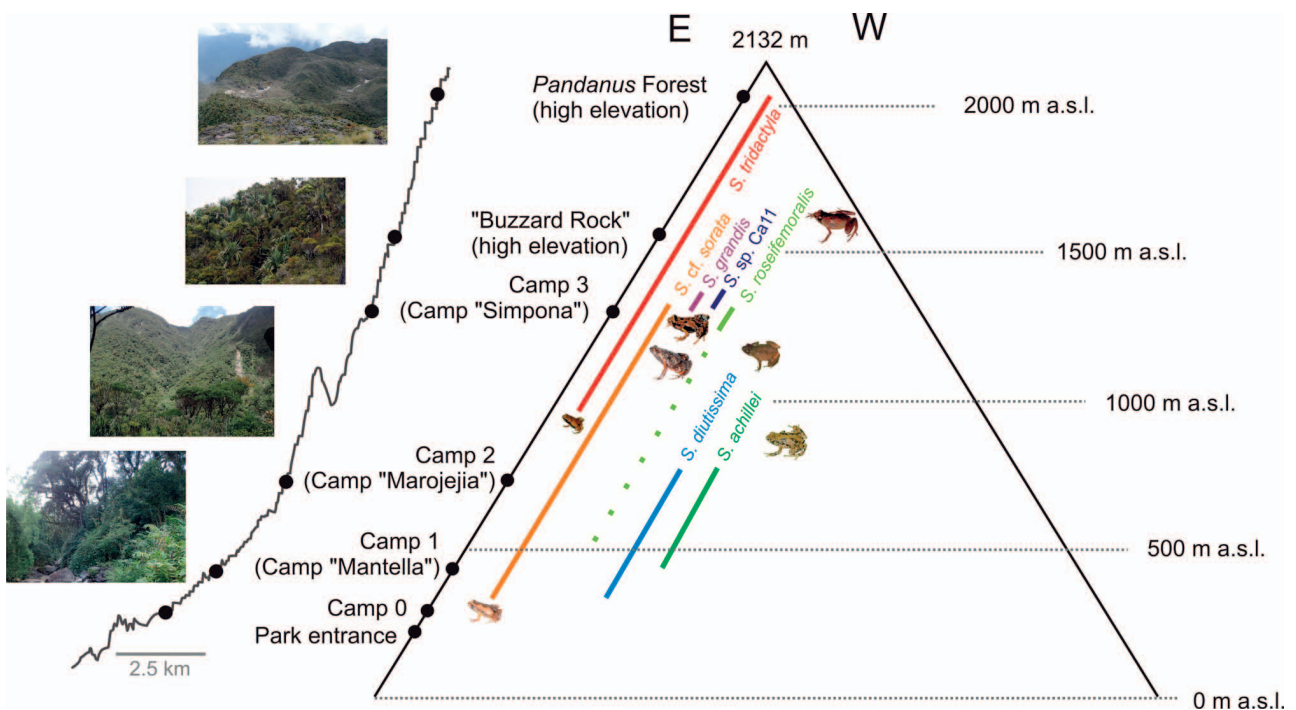


Figure 3. Schematic graph showing known elevational distribution of species of *Stumpffia* on the Marojejy Massif, along with photos of the habitat at various elevations and the elevational profile of the track up the Marojejy summit, with the collection sites mentioned in the text and Supplementary materials.

*rina*, *G. (Duboimantis) schilfi*, and possibly also *Rhombophryne vaventy*). The variability in genetic differentiation between lineages found on Marojejy and Sorata, from conspecific populations with low degrees of genetic differentiation, to distinct species with high degrees of genetic and phenotypic differentiation, suggests a long and possibly on-going history of recurrent vicariance and biotic reconnection of these rainforests. A detailed dating of these events is not possible due to the lack of thorough time-calibrated phylogenies for the majority of the taxa concerned, but will be a promising endeavour for future studies.

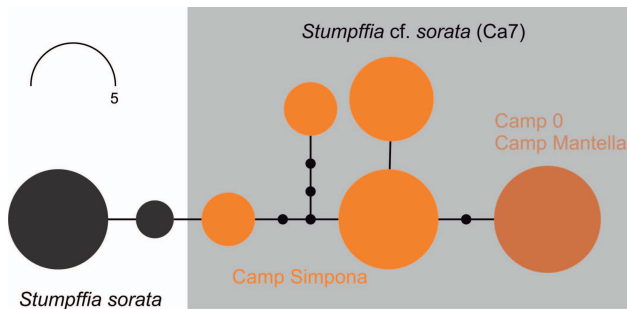


Figure 4. Haplotype network based on Rag-1 sequences (335 bp) of 12 specimens of *S. cf. sorata* from Marojejy, and 4 specimens of *S. sorata* from the Sorata Massif, showing separation of their haplotypes by a single step, and high variation within *S. cf. sorata*. Note that low-elevation specimens of *S. cf. sorata* (Camp 0/Mantella) share an exclusive haplotype.

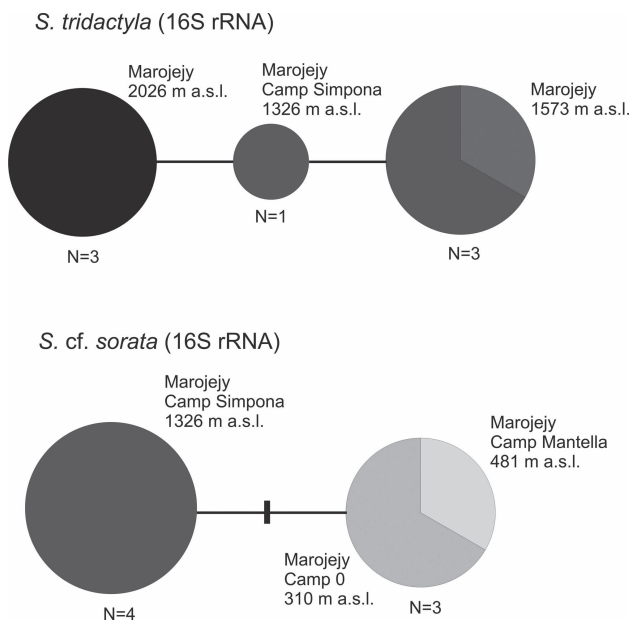


Figure 5. Haplotype networks based on 221 bp of the mitochondrial 16S gene. The networks show a low but consistent differentiation between samples of *S. tridactyla* and *S. cf. sorata* from different elevations on the Marojejy massif, respectively. Note that this is a different 16S fragment than used in the phylogenetic tree in Figure 1.

*Stumpffia sorata* is among the species apparently shared between the Sorata massif and the Marojejy massif. Particularly noteworthy in this case is that *S. sorata* is not restricted just to high elevations like other species shared between these two sites, but instead occurs across almost 1 km elevational range in Marojejy. Whether or not the species formerly occurred over such a broad range in Sorata is unknown, but forest below 1300 m is practically eradicated on that massif, so low-elevation populations are probably extinct there. They may, however, persist on the neighbouring Andravory massif. Although *S. sorata* occurs from 300–1330 m a.s.l. on Marojejy, the lineage genetically closest to that in Sorata is found at 1330 m a.s.l. At elevations around 1120 m a.s.l. there is continuous (and forested) connection between these massifs, which constitutes roughly the minimum possible single-elevation distance between the localities. At higher elevations there is no continuity, while at lower elevations, there is continuity, but the distance travelled is greater, encompassing the perimeter of additional, lower massifs. Anthropogenic deforestation has also recently broken continuity in some areas, especially at lower elevations. Thus, gene flow between populations on these two massifs, as in *S. sorata*, is probably currently greatest or at least easiest at 1100–1330 m a.s.l. This pattern could be explicitly tested in the future based on population genomic data sets and resistance mapping and least-cost path analysis (CHAN et al. 2011). Major climate transitions may have pushed species toward and away from the ‘optimal’ elevations for gene flow, and the timing of divergence between sister-lineages from Sorata and Marojejy should be compared to identify whether the degree and timing of differentiation are correlated.

*Stumpffia sorata* is also the only *Stumpffia* species present on the Marojejy massif that belongs to a clade that has its biogeographic centre of diversity and probable origin in northern Madagascar (RAKOTOARISON et al. 2017). Marojejy represents a southeastern expansion of this clade, and we consider it likely that *S. sorata* originated in Sorata and arrived in Marojejy via dispersal along the connecting mountain chain.

#### Bioacoustic and morphological divergence in syntopic *Stumpffia* on Marojejy

Biogeographically, the available data suggest that the community of *Stumpffia* species on Marojejy is due to community assembly through independent colonisations rather than in-situ diversification. However, the species making up this community show a clear differentiation in morphology and bioacoustics, suggesting community assembly may not have been random. Marojejy National Park is home of six nominal species (*S. tridactyla*, *S. grandis*, *S. roseifemoralis*, *S. diutissima*, *S. achillei* and *S. sorata*) and one candidate species (*S. sp. Ca11*). Morphologically, the Marojejy *Stumpffia* species are remarkable by the presence of the miniaturised species *S. tridactyla* (8.6–10.6 mm) and the large sized species *S. grandis* (19.3–23.7 mm). The four

other nominal species *S. roseifemoralis* (16.2–18.4 mm), *S. diutissima* (13.4–20.0 mm), *S. achillei* (14.6–19.7 mm) and *S. sorata* (11.3–15.1 mm) have more or less overlapping body sizes. A similar pattern is seen in Montagne d'Ambre National Park in north Madagascar, where there is a single highly miniaturised species (*S. madagascariensis*), several species of intermediate size that are elevationally segregated (*S. huwei*, *S. maledicta*, and *S. achillei*), and two larger species recently discovered (unpubl. data).

Differentiation of advertisement calls of the *Stumpffia* species from Marojejy is remarkable. The species that occur in strict syntopy differ strongly in call duration: *S. diutissima* (53–56 ms) and *S. achillei* (36–52 ms) have short calls, and *S. tridactyla* (101–198 ms) has an intermediate call duration. *S. sorata* (290–299 ms) and *S. roseifemoralis* (276–280 ms) have a relatively long call duration (RAKOTOARISON et al. 2017). Inter-call interval, although strongly influenced by temperature and motivation of the calling male (KÖHLER et al. 2017), also shows extreme inter-species differences, with *S. achillei* having the shortest of all values in the genus (507–582 ms), *S. roseifemoralis* (2891–3304 ms) and *S. sorata* (2764–3250 ms) having very long intervals, and *S. tridactyla* (969–1121 ms) and *S. diutissima* (1775–2200 ms) intermediate intervals (RAKOTOARISON et al. 2017). Temporal distribution of calling may also differ among species, but at present data are still too inadequate to give a clear pattern.

The distinct differentiation among the calls of all species in temporal variables means that the calls of these frogs can be relatively easily distinguished by the human ear, permitting, with some experience, the assessment of the presence of the different species without requiring the often time-consuming capture of the frogs themselves. Syntopic anuran species very rarely have similar and almost never identical advertisement calls (KÖHLER et al. 2017). In Marojejy, this pattern is likely caused by local processes of character displacement into non-overlapping ranges of temporal and spectral variables. However, a number of other hypotheses could be advanced on this topic, e.g. that only species with such distinct calls have been able to successfully integrate into the local community, or that bioacoustic distinction between frogs instead results from their phylogenetic disparity. Testing among these and other alternatives will only be possible once bioacoustic data for the sister lineages of the Marojejy species become available; so far, these are almost completely lacking.

#### Endemism and the effect of elevation on gene flow in *Stumpffia*

The current species of *Stumpffia* on Marojejy do not appear to have sympatrically split from each other on the massif. However, the two species reliably found occurring over wide elevational bands, *S. tridactyla* and *S. sorata*, do show a genetic divergence related to elevation. Stump-toed frogs tend to have small ranges in terms of physical area, as well as narrow elevational distributions (RAKOTOARI-

SON et al. 2017). In Marojejy, most *Stumpffia* species are restricted to the massif itself and most also occur over only a narrow elevational range, according to current knowledge. Exceptions are *S. cf. sorata* and *S. tridactyla*, and possibly also *S. roseifemoralis*, which occur over ca. 1000 m elevation difference. However, for *S. roseifemoralis*, the occurrence at low elevations is currently only based on a single record (ZSM 373/2005 = FGZC 2808; RAKOTOARISON et al. 2017) and requires confirmation. The two species with verified exceptionally large elevational ranges, *S. cf. sorata* and *S. tridactyla*, possess divergent, exclusive haplotypes in a fragment of the mitochondrial 16S rRNA gene (Fig. 5) and nuclear Rag-1 gene (Fig. 4) at different elevations. This suggests that gene flow up and down the slope is impeded to some degree. A more comprehensive sampling along multiple elevational transects would be necessary to understand if, indeed, elevational (and thus adaptive) divergence is at play here. Alternative explanations that require testing include non-adaptive mechanisms such as isolation-by-distance, or different micro-refuges in which refugial populations became genetically divergent in episodes of unfavourable climate, and subsequently expanded their ranges across the massif.

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#### References

- BROWN, J. L., N. SILLERO, F. GLAW, P. BORA, D. R. VIEITES & M. VENCES (2016): Spatial biodiversity patterns of Madagascar's amphibians and reptiles. – *PLoS One*, **11**: e0144076.
- BRUFORD, M. W., O. HANOTTE, J. F. Y. BROOKEFIELD & T. BURKE (1992): Single-locus and multilocus DNA fingerprint. – pp. 225–270 in: HOELZEL, A.R. (ed.) *Molecular Genetic Analysis of Populations: A Practical Approach*. – IRL Press, Oxford.
- CHAN, L. M., J. L. BROWN & A. D. YODER (2011): Integrating statistical genetic and geospatial methods brings new power to phylogeography. – *Molecular Phylogenetics and Evolution*, **59**: 523–537.
- KÖHLER, J., M. JANSEN, A. RODRÍGUEZ, P. J. R. KOK, L. F. TOLEDO, M. EMMRICH, F. GLAW, C. F. B. HADDAD, M.-O. RÖDEL & M. VENCES (2017): The use of bioacoustics in anuran taxonomy: theory, terminology, methods and recommendations for best practice. – *Zootaxa*, **4251**: 1–124.
- KUMAR, S., G. STECHER & K. TAMURA (2016): MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. – *Molecular Biology and Evolution*, **33**: 1870–1874.



- LIBRADO, P. & J. ROZAS (2009): DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. – *Bioinformatics*, **25**: 1451–1452.
- PATEL, E. R. (2015): Extensive new boundary demarcation of Marojejy National Park. – Duke Lemur Center SAVA Conservation Madagascar – Conservation news from the Sambava-Andapa-Vohemar-Antalaha region of NE Madagascar, **4**: 1–3.
- PRÖTZEL, D., M. VENCES, O. HAWLITSCHKEK, M. D. SCHERZ, F. M. RATSOAVINA & F. GLAW (2018): Endangered beauties: micro-CT cranial osteology, molecular genetics and external morphology reveal three new species of chameleons in the *Calumma boettgeri* complex (Squamata: Chamaeleonidae). – *Zoological Journal of the Linnean Society*, **184**: 471–498.
- RAKOTOARISON, A., A. CROTTINI, J. MÜLLER, M.-O. RÖDEL, F. GLAW & M. VENCES (2015): Revision and phylogeny of narrow-mouthed treefrogs (*Cophyla*) from northern Madagascar: integration of molecular, osteological, and bioacoustic data reveals three new species. – *Zootaxa*, **3937**: 61–89.
- RAKOTOARISON, A., M. D. SCHERZ, F. GLAW, J. KÖHLER, F. ANDREONE, M. FRANZEN, J. GLOS, O. HAWLITSCHKEK, T. JONO, A. MORI, S. H. NDRIANTSOA, N. RAMINOSOA RASOAMAMPIONONA, J. C. RIEMANN, M.-O. RÖDEL, G. M. ROSA, D. R. VIEITES, A. CROTTINI & M. VENCES (2017): Describing the smaller majority: Integrative taxonomy reveals twenty-six new species of tiny microhylid frogs (genus *Stumpffia*) from Madagascar. – *Vertebrate Zoology*, **67**: 271–398.
- RAXWORTHY, C. J. & R. A. NUSSBAUM (1995): Systematics, speciation and biogeography of the dwarf chameleons (*Brookesia*; Reptilia, Squamata, Chamaeleontidae) of northern Madagascar. – *Journal of Zoology*, **235**: 525–558.
- SALZBURGER, W., G. B. EWING & A. VON HAESLER (2011): The performance of phylogenetic algorithms in estimating haplotype genealogies with migration. – *Molecular Ecology*, **20**: 1952–1963.
- SCHERZ, M. D., A. RAKOTOARISON, O. HAWLITSCHKEK, M. VENCES & F. GLAW (2015): Leaping towards a saltatorial lifestyle? An unusually long-legged new species of *Rhombophryne* (Anura, Microhylidae) from the Sorata massif in northern Madagascar. – *Zoosystematics and Evolution*, **91**: 105–114.
- SCHERZ, M. D., O. HAWLITSCHKEK, F. ANDREONE, A. RAKOTOARISON, M. VENCES & F. GLAW (2017): A review of the taxonomy and osteology of the *Rhombophryne serratopalpebrosa* species group (Anura: Microhylidae) from Madagascar, with comments on the value of volume rendering of micro-CT data to taxonomists. – *Zootaxa*, **4273**: 301–340.
- SCHERZ, M. D., A. RAKOTOARISON, F. M. RATSOAVINA, O. HAWLITSCHKEK, M. VENCES & F. GLAW (2018a): Two new Madagascar frog species of the *Gephyromantis* (*Duboisimantis*) *tandroka* complex from northern Madagascar. – *Alytes*, **36**: 130–158.
- SCHERZ, M. D., O. HAWLITSCHKEK, J. H. RAZAFINDRAIBE, S. MEGSON, F. M. RATSOAVINA, A. RAKOTOARISON, M. C. BLETZ, F. GLAW & M. VENCES (2018b): A distinctive new frog species (Anura, Mantellidae) supports the biogeographic linkage of the montane rainforest massifs of northern Madagascar. – *Zoosystematics and Evolution*, **94**: 247–261.
- STEPHENS, M., N. J. SMITH & P. DONNELLY (2001): A new statistical method for haplotype reconstruction from population data. – *American Journal of Human Genetics*, **68**: 978–989.
- VENCES, M., M. L. LYRA, B. R. G. PERL, M. C. BLETZ, D. STANKOVIC, R. GEFFERS, C. F. B. HADDAD, S. STEINFARTZ, C. MARTINS LOPES, M. JAREK & S. BHUJU (2016): Freshwater vertebrate metabarcoding on Illumina platforms using double-indexed primers of the mitochondrial 16S rRNA gene. – *Conservation Genetics Resources*, **8**: 1–5.
- VIEITES, D. R., K. C. WOLLENBERG, F. ANDREONE, J. KÖHLER, F. GLAW & M. VENCES (2009): Vast underestimation of Madagascar's biodiversity evidenced by an integrative amphibian inventory. – *Proceedings of the National Academy of Sciences of the USA*, **106**: 8267–8272.
- WOLLENBERG, K. C., D. R. VIEITES, A. VAN DER MEIJDEN, F. GLAW, D. C. CANNATELLA & M. VENCES (2008): Patterns of endemism and species richness in Malagasy cophyline frogs support a key role of mountainous areas for speciation. – *Evolution*, **62**: 1890–1907.

### Supplementary data

- Revised circumscription of *Stumpffia* cf. *sorata*, with a list of newly assigned material and an expanded diagnosis.
- Supplementary Figure S1. *Stumpffia* cf. *sorata* (ZSM 544/2016) adult male specimen from Marojejy.
- Supplementary Figure S2. *Stumpffia* cf. *sorata* (ZSM 544/2016) adult male specimen from Marojejy, in life.
- Supplementary Figure S3. Colour variation of *Stumpffia* cf. *sorata* on Marojejy.