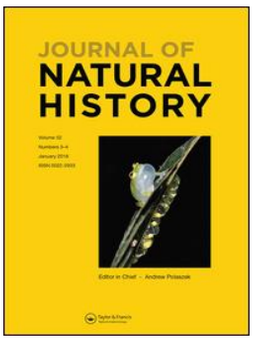

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Target-enriched DNA sequencing from historical type material enables a partial revision of the Madagascar giant stream frogs (genus *Mantidactylus*)

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ABSTRACT

The subgenus *Mantidactylus* is a group of frogs endemic to Madagascar, including the largest anuran species on the island. Although these frogs are common and widely distributed, their taxonomy remains unclear. Two species are currently recognised, *M. grandidieri* and *M. guttulatus*, with another available name, *Rana pigra*, considered to be a synonym of *M. grandidieri*. However, molecular studies have suggested the presence of several cryptic species within the group. Additionally, due to the lack of prominent morphological features, allocating the available names to evolutionary lineages has proven challenging. In the present study, we take a first step towards solving these problems by using fragments of the 16S mitochondrial gene and RAG1 nuclear gene from all over the range of the subgenus to describe its genetic diversity. We also use a newly designed target enrichment laboratory protocol to sequence three mitochondrial fragments from five name-holding museum specimens (as old as 120 years) in order to determine to which lineages the existing names should be applied. The study of the 16S mitochondrial gene revealed 7 geographically separated lineages, distinct enough to be considered candidate species. Out of the five museum specimens analysed, four successfully yielded DNA sequences and could be

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attributed to one of the aforementioned lineages. Therefore, the name *Mantidactylus grandidieri* should be applied to the populations from North-Eastern Madagascar, while *M. guttulatus* refers to populations from inland localities along the Eastern coast of the island. On the other hand, the holotype of *Rana pigra* did not yield enough genetic material to allow definitive identification. While our data were not sufficient to assess the status of the four lineages distributed along the Eastern coast, the populations from North-Western Madagascar were highly distinct on both the mitochondrial and nuclear markers. We thus describe them as a new species, *M. radaka* sp. nov.

http://www.zoobank.org/urn:lsid:zoobank.org:pub:1EDDAF0D-FE37-490A-B09E-E136A0C5CB35

Introduction

Type specimens are an important source of information for taxonomists as they allow name allocation to taxa (Banks et al. 1993). They provide an indication of morphological features of the species described, and are linked to valuable information, such as the date and location of collection. In taxonomic revisions of species complexes, attributing a type specimen – and thereby a scientific name – to the correct taxon can prove challenging, especially with cryptic species that show poor morphological divergence. This is particularly true in the case of species without clear diagnostic morphological features, or when the state of specimen preservation does not permit the evaluation of morphological or other diagnostic characteristics. Information that can be helpful in these cases, such as collection locality, is often missing or imprecise for old type specimens, making their identity even more difficult to disentangle (Cong et al. 2019).

In this context, DNA data can be extremely valuable, assuming that the focal species are genetically distinct, and that genetic data are available to compare with the type specimens. However, sequencing molecular markers from historical specimens can prove challenging, due to degradation of DNA over time or to the specimen preservation techniques used (Wandeler et al. 2007). Specific laboratory workflows have been developed to overcome this issue (e.g. Gansauge and Meyer 2013), which have helped to resolve many taxonomic problems (e.g. Hind et al. 2014; Shokrala et al. 2015; Prosser et al. 2016; Cong et al. 2019) as well as other biological questions (reviewed in Burrell et al. 2015). However, these approaches have so far rarely been successful when applied to specimens preserved in wet collections (i.e. most amphibians, reptiles and fishes). An important aspect is that the chemical composition of the preservatives used in these collections over time is often unknown. For instance, many specimens collected since around 1895 have been fixed with formaldehyde before being stored in ethanol for long-term preservation (Musiał et al. 2016). While this method enables long-term conservation of morphological features, formaldehyde alters the DNA by cross-linking other macromolecules (e.g. proteins) to it such that its extraction, amplification and sequencing become difficult (Tang 2006; Gilbert et al. 2007).

While some authors have successfully sequenced mtDNA fragments (Friedman and DeSalle 2008; Ruane and Austin 2017; McGuire et al. 2018), and even complete mitochondrial genomes (Hykin et al. 2015; Li et al. 2015, 2016; Evans et al. 2019) from specimens in wet collections, standard workflows are still rare. In this context, short-read sequencing

through Massively Parallel Sequencing (MPS) platforms is a very promising approach to apply 'museomics' to wet collections. Targeting of DNA fragments commonly used as barcodes for lineage characterisation via DNA hybridisation capture could be an efficient way to identify enigmatic museum specimens and solve taxonomic problems. As an attempt to provide a workflow combining MPS and targeted DNA barcode sequences to be applied to alcohol-preserved anuran amphibians, we here revise the taxonomy of the Madagascar giant stream frogs (genus *Mantidactylus*, subgenus *Mantidactylus*). Using an MPS-based approach, we aim to assign the existing type specimens, preserved in wet collections for up to 136 years, to genetic clades represented by more recently collected material in order to correctly apply nomenclature.

The genus *Mantidactylus* Boulenger, 1895 (family Mantellidae Laurent, 1946) is an apparently diverse, but largely understudied group of frogs, endemic to Madagascar. The current taxonomy accepts 32 species, divided into six subgenera. Although recent DNA barcoding surveys revealed the existence of almost 70 undescribed candidate species (Vieites et al. 2009; Randrianiaina et al. 2011; Rosa et al. 2012; Perl et al. 2014) characterised by high mitochondrial DNA divergences, only limited taxonomic work has focused on this genus in the past 20 years (Glaw and Vences 1999, 2004, 2006; Vences and Glaw 1999, 2004; Mercurio and Andreone 2007; Vences et al. 2018; Scherz et al. 2019).

Within this genus, the species of the subgenus *Mantidactylus* stand out because of their very large size, being Madagascar's largest native frogs. Two species are currently accepted: *Mantidactylus grandidieri* Mocquard, 1895 and *M. guttulatus* (Boulenger, 1881) (Glaw and Vences 2007; AmphibiaWeb 2019). One more taxon, *Rana pigra* Mocquard, 1900, was described, but the name was later synonymised with *M. guttulatus* by Boulenger (1918), and then with *M. grandidieri* by Blommers-Schlösser (1979). Molecular studies have revealed that several lineages within the genus may deserve species status (Vieites et al. 2009; Perl et al. 2014), but due to the lack of interspecific morphological differentiation combined with intraspecific variation, their status is unclarified, and it is uncertain to which populations the described names should be applied. We here use a comprehensive molecular dataset covering all the known range of the subgenus *Mantidactylus* to delimit candidate species within this subgenus, and include samples of type specimens of *M. grandidieri, M. guttulatus* and *R. pigra*, housed in the British Museum of Natural History (BMNH) and the Muséum National d'Histoire Naturelle (MNHN), in the genetic analysis. Doing so, we aim to (i) clarify species boundaries within the subgenus *Mantidactylus*, (ii) determine to which lineages the existing names should be applied and (iii) allocate new names to undescribed lineages when relevant. This study is also intended to serve as a pilot to assess the efficiency of genetic analysis of historical wet collection material for taxonomic revision.

Materials and methods

Sample collection

Frog individuals were collected during day or night along streams in both rainforest and secondary habitat, over multiple expeditions to Madagascar from 2000 to 2016. Specimens were anaesthetised and then euthanised by an overdose of MS222 or chlorobutanol, fixed in 95% ethanol, and thereafter transferred to 75% ethanol for long-term

storage. Before fixation, tissue samples of thigh muscle or tongue were taken and separately preserved in 99–100% ethanol.

Field numbers refer to the zoological collections of F. Andreone (FAZC, FA), A. Crottini (ACZC, ACP), P.-S. Gehring (PSG), M. D. Scherz (MSZC), C. R. Hutter (CRH), D. R. Vieites (DRV), M. Vences and F. Glaw (FGMV, ZCMV, FGZC) and the tissue collection of M. Vences (MVTIS). MAVOA refers to tissues collected by the team of Madagasikara Voakajy. Preserved voucher specimens studied are deposited in the Zoologische Staatssammlung München (ZSM), Germany, and Museo Regionale di Scienze Naturali di Torino, Italy (MRSN). Additional specimens (not analysed morphologically herein) were deposited at the Université d'Antananarivo, Madagascar, Mention Zoologie et Biodiversité Animale (UADBA) and the University of Kansas (KU) Biodiversity Institute. We took samples of historical type specimens preserved in the Muséum National d'Histoire Naturelle (MNHN), Paris, France, and the Natural History Museum (formerly British Museum of Natural History; BMNH), London, UK. For some sequences retrieved from GenBank, voucher specimens are tagged with field numbers RAN (R. A. Nussbaum), deposited in the Museum of Zoology, University of Michigan (UMMZ).

Target enrichment laboratory workflow for historical type specimens

DNA extractions from numerous specimens of historical wet collections, in many cases formalin-fixed, showed that these often contain substantial amounts of highly fragmented DNA, therefore not accessible using standard PCR-based amplification (e.g. Hykin et al. 2015; Li et al. 2016; Evans et al. 2019). We therefore set out to develop a bait-capture approach, specifically to obtain DNA information from historical collections of anurans from Madagascar. Because of the existing dense sampling of ethanol-preserved tissues of almost all described Malagasy anuran species, as well as numerous undescribed candidate species (Vieites et al. 2009; Perl et al. 2014), we did not aim to obtain sequences of a large number of markers from the historical specimens; instead, we followed a strategy to maximise the probability of capture of three mitochondrial gene fragments that have been sequenced as standard barcoding and phylogenetic markers from Malagasy frogs, i.e., 16S rRNA (16S), cytochrome b (cob) and cytochrome oxidase subunit 1 (cox1). For these genes, we compiled and aligned representative sequences for each species and candidate species as available from Vences et al. (2005a, 2005b), Vieites et al. (2009), Wollenberg et al. (2011), Perl et al. (2014) and Scherz et al. (2016). Because our main target was *Mantidactylus*, we included 58 cob (415 bp) and 72 cox1 (510 bp) sequences of this genus only, as well as 610 16S (ca. 415 bp) sequences of all species of Malagasy anurans available. An additional 57 ND2 sequences (389 bp) of chameleons were added to be able to capture sequences of this group for another project. The 797 sequences in total were processed by Arbour Biosciences to produce a bait set using the myBaits approach. For this, N bases were replaced with T. RepeatMasker was used to soft mask simple repeats with a 0.1% threshold. Finally, 18,928 baits of 70 nucleotides in length with 5 x tiling density were designed. These were BLASTed against seven complete or partial mitochondrial genomes of Malagasy frogs (from Kurabayashi et al. 2008), and their hybridisation melting temperature estimated. Non-specific baits were filtered out based on melting temperature criteria, and baits that were 99% identical were collapsed, leaving a final number of 5962 baits.

Tissue samples were taken in 2018 and 2019 from five museum specimens of the holdings of BMNH and MNHN, corresponding to all name-bearing specimens in the

subgenus *Mantidactylus*. All specimens were collected at the end of the nineteenth century and are currently stored in ethanol (ca. 70%), with an unknown history of fixation and preservation. However, at the BMNH it is suspected that the specimens were directly fixed in pure spirits of wine as this was the standard practice for preservation of 'lower vertebrates' during the nineteenth century, when the types of *M. guttulatus* were registered (Günther 1880). Specimens were taken out of their jars, and samples of muscle tissue from the thigh (and additionally, liver in some specimens) were taken, using a clean and DNA-free pair of scissors and forceps for each individual. Tissue samples were stored and transported in clean vials filled with ca. 1 ml 100% ethanol, which had been filled in a laboratory where no DNA work on *Mantidactylus* had previously been carried out.

DNA from historical samples was extracted, converted into a single-stranded library and sequenced following a protocol developed by NS, ML, and collaborators: the samples were taken to a clean lab and were washed prior to extraction with Qiagen® PE buffer. DNA was then extracted from muscle tissue following Dabney et al. (2013). Extracts were incorporated into a single-stranded DNA (ss-DNA) library, which has been shown to increase library complexity for both ancient (Gansauge and Meyer 2013) as well as Formalin-Fixed Paraffin-Embedded samples (Stiller et al. 2016). After adapter ligation using custom adapter sequences from Gansauge and Meyer (2013), libraries were amplified and indexed with custom Illumina indexing primers.

Ss-DNA libraries were captured for aforementioned target sequences using the Arbour Biosciences MyBaits kit as described in the following protocol: we used 14.5 µL of each indexed library in 24-h reactions at a hybridisation temperature of 65°C following the MyBaits target enrichment protocol, except for reducing the bait volume to 2.75 µL per reaction and substituting the missing 2.75 µL with nuclease-free water. After hybridisation, the DNA-RNA hybrid molecules were bound to streptavidin-coated magnetic beads and the reactions were washed and eluted according to the MyBaits protocol. Final elution was in 30 µL of 10 mM Tris-Cl, 0.05% TWEEN-20 solution (pH 8.0).

To increase target capture success, the procedure was performed twice, i.e. the amplified captured library was used as a template for repeating the process as in Li et al. (2013, 2015), Paijmans et al. (2016, 2017), Springer et al. (2015) and Templeton et al. (2013). Enriched libraries were pooled in equimolar amounts (10 nM) and sequenced on an Illumina NextSeq500/550 platform using a 75 cycle SE high-output kit aiming at 3 million reads per sample.

Identification of type specimens using target enrichment data

The raw Illumina reads from the five museum specimens were trimmed and quality checked using Trimmomatic 0.38 (Bolger et al. 2014) to remove Illumina adapters and low-quality bases. Leading and trailing ends with a quality below 10 were removed, as well as sliding windows of 2 bp with average quality below 25. Reads shorter than 36 bp were filtered out. All bases with a quality score below 30 were masked with 'N' using seqtk (https://github.com/lh3/seqtk).

For the three targeted mitochondrial genes (16S, cox1 and cob), a library of reference sequences was built, including references from the different candidate species identified in the *Mantidactylus* subgenus using the 16S tree, as well as one sequence from each of the following species: *Mantidactylus curtus, Boophis goudotii* and *Stumpffia grandis*. These

last three sequences were added to make sure that the recovered reads were specifically matching the sequences from the subgenus *Mantidactylus*, and not mitochondrial regions conserved across a larger phylogenetic scale in frogs. The processed Illumina reads were then aligned to the reference sequences with vsearch 2.3.4 (Rognes et al. 2016) using a similarity threshold of 98%. The full pipeline was wrapped into a bash script available at https://github.com/rancilhac/Museoscript. We then used the matching reads to assign the museum specimens to a species using two approaches (see complementary Figure S1 for a graphical summary of the pipeline): first, the reads producing unique matches with the reference sequences (i.e. aligning to only one sequence in the vsearch analysis) were extracted and counted, while those matching several sequences were ignored as they would not allow us to discriminate between lineages. Secondly, all the reads that matched one or more reference sequence were aligned to a reference sequence using CodonCode Aligner 6.0.2 (CodonCode Corp.), using a majority-based alignment approach. For this, we first considered that in some of our vsearch analyses, samples matched uniquely to *Mantidactylus* sp. Ca56, a lineage known from Betampona on Madagascar's east coast only; although this assignment was unlikely, in a conservative approach to avoid reference bias, we used a sequence of *Mantidactylus* sp. Ca56 as reference for alignment. As the obtained consensus sequences did not cluster with this species in preliminary phylogenetic analyses, we then used reference sequences of the North-Eastern lineage and Inland Eastern lineage, respectively, for building the final consensus sequence, obtaining virtually identical consensus sequences as in the initial approach. After aligning these consensus sequences with the 16S sequences obtained from fresh samples using MEGA, we inferred a phylogenetic tree and assigned the museum specimens to the lineages with which they clustered (c.f. details below).

A visual representation of per base coverage of the 16S fragment of archival type specimens was computed in bedtools v.2.26.0 (Quinlan and Hall 2010). Mean, minimum and maximum coverage values per nucleotide site were computed in Geneious R6 (https://www.geneious.com; Kearse et al. 2012).

Molecular phylogenetics

We extracted total genomic DNA by standard salt extraction from all available fresh samples of the subgenus *Mantidactylus* and amplified fragments of DNA using polymerase chain reactions (PCRs). DNA sequences of the 3′ fragment of the mitochondrial 16S rRNA gene (16S) were amplified and sequenced using previously established protocols described elsewhere with primers 16SAr-L and 16SBr-H (e.g., Vences et al. 2003). Furthermore, fragments of the nuclear recombination-activating gene 1 (RAG-1) were obtained with primers Rag1-Manti-F1 (CGTGACAGAGTSAAAGGAGT) and Rag1-Manti-R1 (TCAATGATCTCTGGAACGTG) from Vences et al. (2018) with the following PCR protocol: 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 finally 600 s at 72°C, combined with a nested RAG-1 PCR to obtain additional sections of this gene, with primers and protocols as in Rakotoarison et al. (2015). Further, one additional sample (KU 340853 [CRH729]) had 16S data extracted from the raw Illumina reads from a much larger sequence capture dataset (Hutter et al. 2019).

Sequences were resolved on automated capillary DNA sequencers and error-checked with CodonCode Aligner (Codon Code Corp.). All newly obtained DNA

sequences were submitted to GenBank (accession numbers MT187991–MT188029, MT188687–MT188690, MT193311–MT193398). For the 3′ 16S fragment, the newly determined sequences were combined with all homologous sequences from GenBank for the subgenus *Mantidactylus*. These sequences were combined with the consensus sequences obtained from the museum specimens (c.f. details above) and aligned. The final 16S alignment (514 bp) was used to infer a maximum likelihood tree using IQTREE (Nguyen et al. 2014) under a K2P+I substitution model. Branch support was assessed using 1000 ultrafast bootstrap replicates (Hoang et al. 2017). The tree was rooted using a sequence of *Mantidactylus (Brygoomantis) ulcerosus* (Boettger 1880) as the outgroup. Mega X (Kumar et al. 2018) was used to calculate the average p-distances between the lineages delimitated using the phylogenetic tree. The museum specimens were not considered for this last analysis.

We inferred alleles (haplotypes) of the nuclear RAG-1 gene using the PHASE algorithm (Stephens et al. 2001) in DnaSP (version 5.10.3; Librado and Rozas 2009). We then constructed a ML tree from the phased and unpartitioned RAG-1 sequences using the Jukes-Cantor substitution model in MEGA X, and entered this tree with the phased alignment in Haploviewer, written by G. B. Ewing (http://www.cibiv.at/~greg/haploviewer), to build a network following the methodological approach of Salzburger et al. (2011).

Morphometric measurements of preserved specimens

Morphological measurements of type specimens (Table 1) were taken in 2018 and 2019 using a digital calliper to 0.1 mm by MV at MNHN and BMNH. The following measurements were taken: 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), length and width of the femoral gland (FGL, FGW), inner metatarsal tubercle length and height (IMTL, IMTH), diameter of the third finger disc (F3D) and diameter of the fourth toe disc (T4D). These features were also measured in 17 specimens attributed to either *M. grandidieri* or *M. guttulatus* at ZSM in 2019 by LR and TB. Webbing formulae follow Blommers-Schlösser (1979); femoral gland terminology follows Glaw et al. (2000).

Results

Mitochondrial and nuclear sequences reveal several distinct lineages of Madagascar giant stream frogs

The phylogenetic tree reconstructed from 514 bp of the mitochondrial gene for 16S rRNA (Figure 1) shows seven highly supported divergent lineages, all corresponding to geographically distinct groups of populations. Uncorrected 16S pairwise distances between these lineages (Figure 2) range between 1.9% and 4.9%; hence, in many cases, they differ by less than 3%, the threshold used by Vieites et al. (2009) for this gene fragment to define

Table 1. Morphometric measurements of specimens of the two nominal species *Mantidactylus guttulatus* and *M. grandidieri*, and the newly described species *M. radaka* sp. nov. (all in mm). Asterisks indicate name-bearing type specimens: Holotype of *M. radaka* sp. nov., syntypes of *M. grandidieri* and *M. guttulatus*, as well as the holotype of *Rana pigra* (here considered as junior synonym of *M. guttulatus*). Acronyms: M = male, F = female, SA = Sub-adult.

Museum number	Field number	Sex	SVL	HW	HL	TD	ED	END	NSD	NND	FORL	HAL	HIL	FOTL	FOL	FGL	FGW	TIBL	IMTL	IMTH	F3D	T4D
M. radaka sp. nov.																						
* ZSM 644/2001	FGMV 2001.132	M	93.0	42.4	38.8	3.9	11.4	7.5	5.5	8.5	49.3	24.9	140.1	63.4	45.5	18.1	9.1	46.8	7.3	3.0	2.6	2.8
ZSM 180/2010	ZCMV 12567	M	88.3	39.1	35.1	3.3	9.1	6.2	4.9	8.7	45.5	23.4	141.6	61.1	45.2	11.7	4.9	44.6	5.5	2.8	2.0	2.4
ZSM 581/2014	DRV 06072	F	84.9	34.6	32.0	2.7	8.9	6.8	5.1	6.5	45.5	21.8	132.5	57.1	42.1	–	–	40.1	6.1	2.5	2.3	3.2
ZSM 1800/2010	ZCMV 12345	F	98.1	39.2	37.4	3.8	10.8	6.2	6.6	7.6	47.8	23.9	146.6	64.7	46.7	–	–	45.6	7.0	2.6	2.5	3.2
ZSM 1802/2010	ZCMV 12596	F	102.8	46.8	40.6	3.5	9.2	7.4	6.7	9.1	51.3	25.3	150.1	66.3	49.2	–	–	47.0	6.4	3.1	2.2	3.2
ZSM 97/2016	MSZC 80	F	102.3	43.5	41.9	4.4	11.1	7.1	6.0	9.1	52.7	24.6	159.7	70.2	49.3	–	–	48.9	6.2	3.1	2.9	3.1
M. grandidieri																						
* MNHN 1895.255		M	88.1	38.9	36.1	4.9	10.9	6.4	7.2	7.7	55.4	24.3	131.4	62.0	43.2	7.5	6.0	–	–	–	–	–
ZSM 5076/2005	ZCMV 2158	M	80.9	33.6	32.6	4.5	10.3	6.9	4.0	7.2	52.1	21.4	124.0	54.3	40.4	7.3	5.6	37.6	5.7	2.5	1.8	2.4
* MNHN 1883.580		F	90.4	37.1	37.6	5.0	10.6	6.5	7.3	8.5	54.0	26.0	151.0	67.1	43.7	4.1	3.7	–	–	–	–	–
ZSM 5077/2005	ZCMV 2159	F	90.7	37.2	33.2	5.0	9.1	8.9	3.8	8.0	60.6	24.6	143.8	64.6	44.8	6.9	4.2	46.4	5.3	2.1	2.0	2.5
ZSM 276/2005	FGZC 2682	F	112.5	47.9	45.2	6.7	14.5	9.7	4.9	10.6	55.9	29.0	152.3	72.1	52.4	6.3	4.8	50.2	7.7	3.4	3.0	4.0
ZSM 262/2005	FGZC 2114	F	116.3	45.0	41.1	6.9	10.8	8.6	5.3	9.9	59.0	28.6	157.0	73.8	54.9	4.0	2.5	50.5	7.5	2.7	1.9	3.1
ZSM 263/2005	FGZC 2119	F	109.8	42.5	42.1	6.9	11.9	8.5	7.4	9.8	61.9	31.1	153.9	70.1	50.9	4.4	3.8	49.0	6.3	2.7	2.1	3.1
ZSM 264/2005	FGZC 2120	F	117.5	46.2	43.5	6.8	11.8	7.2	5.3	9.6	61.6	31.6	158.0	74.8	56.2	4.4	3.0	49.9	8.7	3.4	2.4	3.0
M. guttulatus																						
ZSM 714/2003	FGMV 2002.362	M	89.8	36.5	35.1	5.3	8.6	7.6	4.5	8.2	–	22.1	115.0	58.2	39.3	5.3	3.9	43.0	4.9	2.4	1.7	2.6
ZSM 266/2005	FGZC 2660	M	97.5	42.9	42.7	8.0	13.1	7.5	5.2	8.1	58.1	24.1	136.6	63.4	44.5	7.7	5.3	45.6	6.3	3.5	1.8	2.5
ZSM 1013/2003	FGMV 2002.438	F	109.0	43.0	39.6	6.2	11.0	7.2	5.9	9.5	51.5	27.2	146.8	68.7	48.0	5.5	4.2	47.7	6.4	3.2	2.2	2.9
ZSM 300/2000		SA F	87.0	34.5	33.5	4.5	10.9	6.2	5.1	7.6	47.4	23.8	129.9	56.5	39.1	5.8	3.4	43.9	4.5	2.0	1.2	2.2
ZSM 474/2006	ZCMV 2342	F	115.1	43.5	40.1	6.2	14.7	8.4	6.5	8.5	78.4	25.7	153.9	66.0	47.0	5.9	4.8	49.5	6.3	3.4	2.4	2.9
* BMNH 1947.2.25.48		F	122.3	50.3	50.4	4.3	13.0	7.7	10.3	9.0	64.3	34.7	171.7	79.6	54.4	4.2	3.7	51.1	11.8	2.9	1.6	2.4
* BMNH 1947.2.25.49		F	123.7	50.3	51.6	5.1	14.4	8.0	7.7	10.9	63.1	31.3	166.0	73.0	52.5	4.7	3.0	50.6	6.3	3.4	1.8	2.1
* BMNH 1947.2.25.50		F	125.6	51.1	49.0	4.6	13.5	8.0	7.9	10.5	62.8	33.2	179.1	77.3	54.1	5.0	4.1	52.8	7.9	3.1	2.5	2.7
* BMNH 1947.2.25.51		F	116.5	44.2	46.4	4.6	13.7	8.0	8.0	10.3	59.8	30.3	151.7	69.4	48.2	5.0	5.0	47.7	7.6	3.2	2.1	2.2
* BMNH 1947.2.25.52		SA	95.8	37.6	36.8	4.7	10.4	5.6	6.6	8.0	50.6	23.6	130.6	57.7	40.7	3.4	4.7	41.0	5.9	2.2	1.3	1.5
MNHN 1899.410 (*R. pigra*)		M	118.8	46.8	49.7	6.0	15.0	7.8	8.2	10.5	71.2	33.2	162.5	72.0	51.7	6.9	6.0	–	–	–	–	–

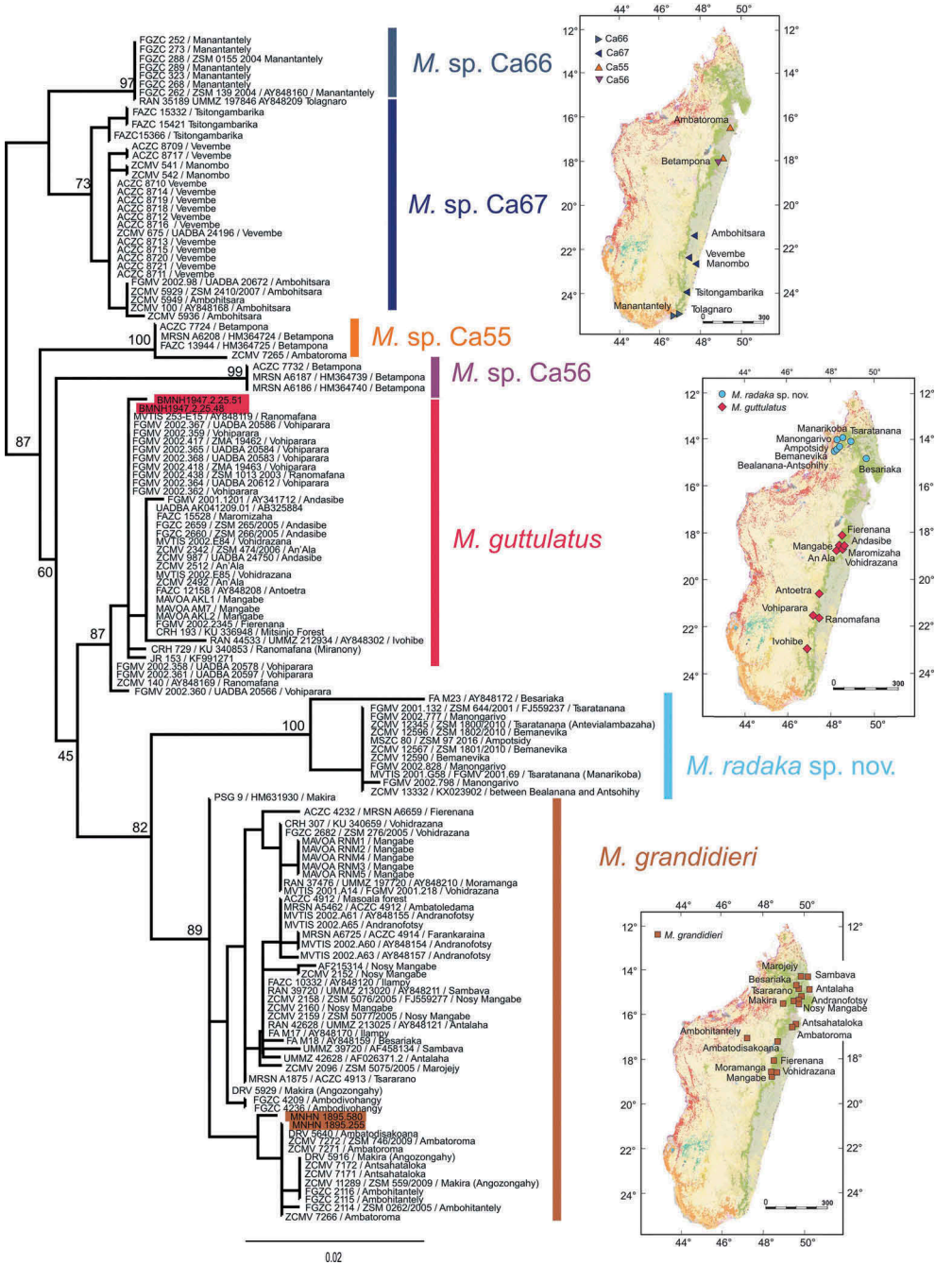

Figure 1. Maximum likelihood phylogenetic tree obtained from 514 bp of the mitochondrial 16S rRNA gene. The values at the nodes are the bootstrap supports (not given for intra-lineage nodes for improved clarity). The type specimens of *M. guttulatus* and *M. grandidieri* from the London and Paris museum collections are highlighted in red and brown, respectively.

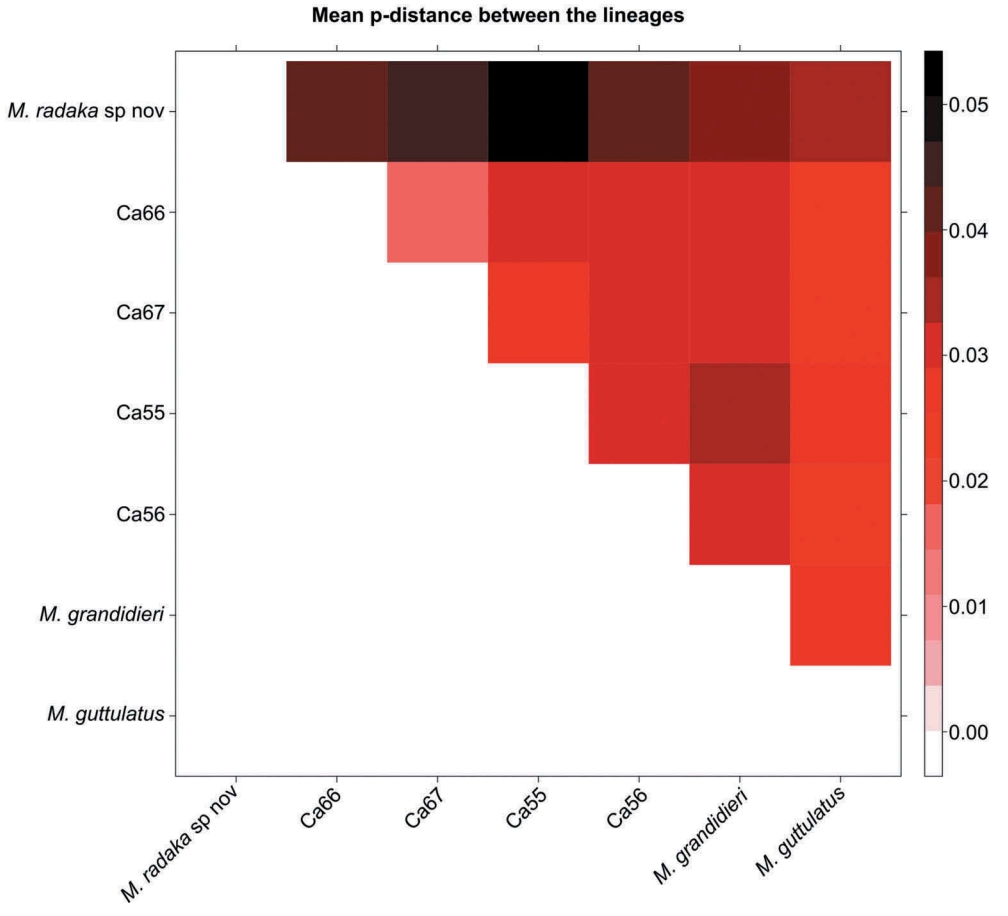

Figure 2. Diagonal matrix visualising the mean uncorrected genetic distances (p-distances) in the mitochondrial 16S rRNA gene between the different lineages in the subgenus *Mantidactylus*, calculated from 514 bp of the 16S mitochondrial gene.

candidate species. Yet, the syntopic occurrence of some of these lineages, with apparently limited admixture in mtDNA and one nuclear gene, RAG-1 (cf. Figures 2 and 3, discussed in more detail below), suggests that at least some of them may represent valid species. To allow an easier discussion, we here opt for a consistent definition of all lineages differing by 1.9% or more as candidate species; yet, we emphasise that this does not imply all of them merit future recognition as distinct species, and once hybrid zones are studied in more detail, several of them may be relegated to deep conspecific lineages.

To describe the distribution of the various lineages (species and candidate species), we here follow the geographical zonation scheme for Madagascar of Boumans et al. (2007). In the South-East, extending slightly northwards in the Southern Central East, we found populations belonging to a mitochondrial clade, composed of two lineages that so far have not received candidate species numbers in the scheme established for Malagasy frogs by Vieites et al. (2009). Following that numbering system, the last named candidate species in *Mantidactylus* is *Mantidactylus* sp. Ca65 in Cocca et al. (2018), and we therefore use the next consecutive numbers to refer to the South-East lineages here newly

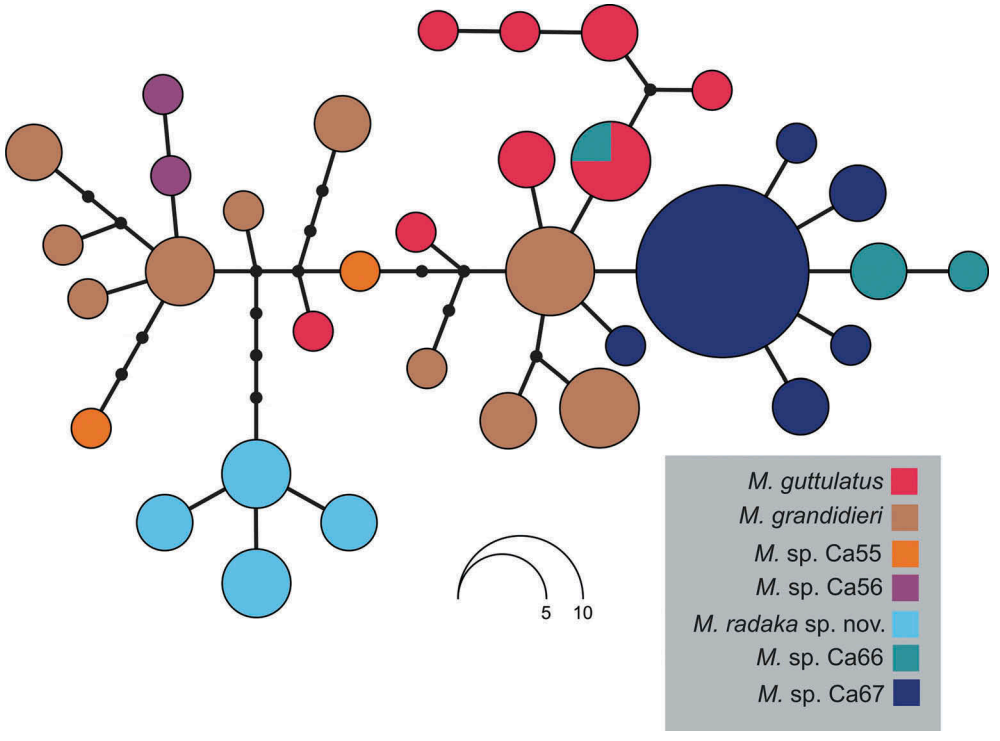

Figure 3. Haplotype network of the subgenus *Mantidactylus* based on 1227 bp of the nuclear RAG-1 gene from 39 samples. Small black dots represent additional mutational steps.

identified in the subgenus *Mantidactylus*. One of these lineages is restricted to the extreme South East (locality Manantantely and near Tolagnaro) and is here called *Mantidactylus* sp. Ca66; the second lineage appears to have a wider distribution, from Tsitongambarika in the South East northwards to Ambohitsara in the Southern Central East, and is here called *Mantidactylus* sp. Ca67. These are the two least divergent lineages in 16S (pairwise uncorrected distance of 1.9%) and it is possible that future research will reveal them to be conspecific. All known localities of these two candidate species are in the lowlands (<600 m above sea level), relatively close to the east coast.

Two additional geographically restricted lowland lineages are found in the Northern Central East, *Mantidactylus* sp. Ca55 and *Mantidactylus* sp. Ca56. Both of these were found in sympatry in Betampona, and our analysis also provides evidence for occurrence of the former lineage further north, at a site named Ambatoroma. These two sympatric candidate species differ by 3.4% pairwise 16S distance.

The highlands of the Southern Central East and the Northern Central East, from Ivohibe to Fierenana, are occupied by one rather widespread lineage that, based on analysis of type material (see below), corresponds to the nominal species, *M. guttulatus*. In a small area of the Northern Central East around Moramanga (localities Fierenana, Mangabe, Vohidrazana), *M. guttulatus* co-occurs with another lineage that spreads northwards into the Northern Central East and westwards into the highlands in Ambohitantely but also occurs at various coastal lowland sites, coming into sympatry with *Mantidactylus* sp. Ca55 in Ambatoroma. This lineage has previously been dubbed *Mantidactylus* sp. Ca57 (e.g.,

Vieites et al. 2009), but based on analysis of type material (see below), it corresponds to the nominal species, *M. grandidieri*. Pairwise distances between the locally sympatric species *M. grandidieri* and *M. guttulatus*, and *M. grandidieri* and *Mantidactylus* sp. Ca55, are 3.0% and 3.4%, respectively.

In the locality Besariaka, *M. grandidieri* occurs sympatrically with a further species that is restricted to mid-altitude and highland localities of the North East and Sambirano regions. This species, previously referred to as *M. guttulatus* (e.g., Vences et al. 2004; Glaw and Vences 2007; Schulze et al. 2016) is the genetically most divergent lineage in the subgenus *Mantidactylus* (Figure 2), differing by 3.2–4.9% from all other lineages, and by 3.8% from the locally sympatric *M. grandidieri*.

The haplotype network inferred from 1227 bp of the nuclear RAG-1 gene (Figure 3) revealed a large number of haplotypes, which, however, were in most cases only connected by single mutational steps. Except for one allele shared between *M. guttulatus* and *Mantidactylus* sp. Ca66 we did not find allele sharing between main lineages, but the alleles of most lineages did not form well-defined clusters: most individuals of the South East lineages, *Mantidactylus* sp. Ca66 and *Mantidactylus* sp. Ca67, formed a cluster of seven alleles, separated from other lineages by at least one step; and all sequences of the North East/Sambirano lineage formed a cluster of three alleles, separated by a minimum of five steps from all other alleles.

As a conclusion, the subgenus *Mantidactylus* possibly contains as many as seven distinct species, corresponding to the mitochondrial lineages. However, the lack of well-preserved voucher specimens for many of these, and the small number of samples available for analysis especially from sympatric areas, do not allow for a conclusive and comprehensive delimitation of all of these. We suspect that such an analysis would also reveal that some of these lineages hybridise more widely and may constitute deep conspecific lineages. However, our data do provide good integrative evidence for distinctness of the North East/Sambirano lineage which has (i) the highest and most consistent divergence in the mitochondrial (16S) gene (Figure 2), (ii) occurs sympatrically with *M. grandidieri* in Besariaka, (iii) has a distinct divergence in the nuclear RAG-1 gene without allele sharing with any other lineage, and (iv) has consistent morphological differences in femoral gland size and in some other morphological characters, as discussed below. We therefore conclude that this lineage corresponds to a distinct species that we describe herein as *Mantidactylus radaka* sp. nov.

Target enrichment DNA sequencing allocates historical types to lineages

The existence of more than one species of giant Stream Frog in the subgenus *Mantidactylus* has been assumed for many years (e.g., Blommers-Schlösser and Blanc 1991; Glaw and Vences 1994, 2007; Vieites et al. 2009) but allocating existing scientific names (*nomina*) to the various populations has proven extremely difficult due to an apparent combination of high morphological variation within and lack of morphological differentiation among several of the lineages. In the previous section, for ease of reading, we have already assigned these names to lineages, anticipating our results reported in the following, based on DNA sequences obtained from historical type material.

As summarised in Blommers-Schlösser and Blanc (1991) and Frost (2019), three species-level nomina exist for the subgenus *Mantidactylus*: (i) *Rana guttulata* Boulenger, 1881,

originally based on five syntypes: BMNH 1947.2.25.48–52 (formerly 1880.7.15.10–12 and 1881.1.3.29.15–16) from 'Betsileo' (later changed to 'S. E. Betsileo' by Boulenger 1882), of which BMNH 1947.2.25.51 was designated lectotype by Blommers-Schlösser and Blanc (1991); (ii) *Mantidactylus grandidieri* Mocquard, 1895, based on two syntypes MNHN 1883.580 and 1895.255 from 'Madagascar … côte Est'; and (iii) *Rana pigra*, 1900, based on holotype MNHN 1899.410, from 'forêt d'Ikongo'.

Our sampling included all name-bearing type specimens of these nomina plus one additional paralectotype of *Rana guttulata*. Using target enrichment, we successfully recovered DNA from these five museum specimens, the total number of Illumina reads ranging from 677,442 to 1,658,433. After processing, the number of reads ranged from 159,890 to 928,481, representing 24–56% of the raw reads. The length of the processed reads ranged from 36 to 76 bp (mean 49.4 bp). Of the five samples, four had several thousand reads mapping to our reference sequences with a similarity threshold of 98%, while the type specimen of *Rana pigra* only yielded a few reads. This last sample was therefore removed from most downstream analyses. The details for each sample are given in Table 2.

The patterns of unique matches to the reference sequences varied depending on the sample considered (Figure 4). For all samples, the number of reads matching the cox1 gene in the target species was notably lower than for the other two genes, and matching reads for cob were overall less frequent than those for 16S. For the BMNH samples, the reads consistently matched best to the highland lineage from the Southern and Northern Central East in all three studied mitochondrial genes (16S, cob, cox1). For the MNHN samples, the results are less clear. In both samples, more than 3000 reads matched the *M. radaka* sp. nov. reference sequence vs, respectively, 1300 and 600 matching the *M. grandidieri* sequence. Very few reads matched to the cox1 reference sequences, and only to *M. grandidieri*. Finally, some reads matched to the cob sequences of both Ca56 and *M. guttulatus*.

Table 2. Summary of the number of reads recovered in the different museum samples, and the 16S consensus sequences obtained from them. Coverage (per nucleotide of the consensus sequence) is given as mean ± SD, followed by range in parentheses. The sixth column gives the complete length of the assembled fragment for each sample, followed by the number of nucleotides that could not be reliably reconstructed (region covered either by no reads or by low quality reads).

Catalogue number	Number of raw reads	Number of processed reads	16S fragment – reads matching reference	16S fragment – length/missing	16S fragment – coverage
M. guttulatus					
BMNH 1947.2.25.51 (lectotype)	1,658,433	928,481 (56%)	42,228	457 bp/2 bp	4537 ± 4923 (1–18,535)
BMNH 1947.2.25.48 (paralectotype)	1,066,500	516,158 (48%)	80,448	431 bp/0 bp	8572 ± 9672 (5–38,086)
MNHN 1899.410 (holotype of *Rana pigra*)	677,442	159,890 (24%)	NA	NA	NA
M. grandidieri					
MNHN 1883.580 (syntype)	1,437,053	554,256 (39%)	66,902	451 bp/8 bp	6549 ± 9372 (1–35,403)
MNHN 1895.255 (syntype)	1,121,605	493,613 (44%)	76,569	414 bp/19 bp	8297 ± 11,825 (58–45,765)

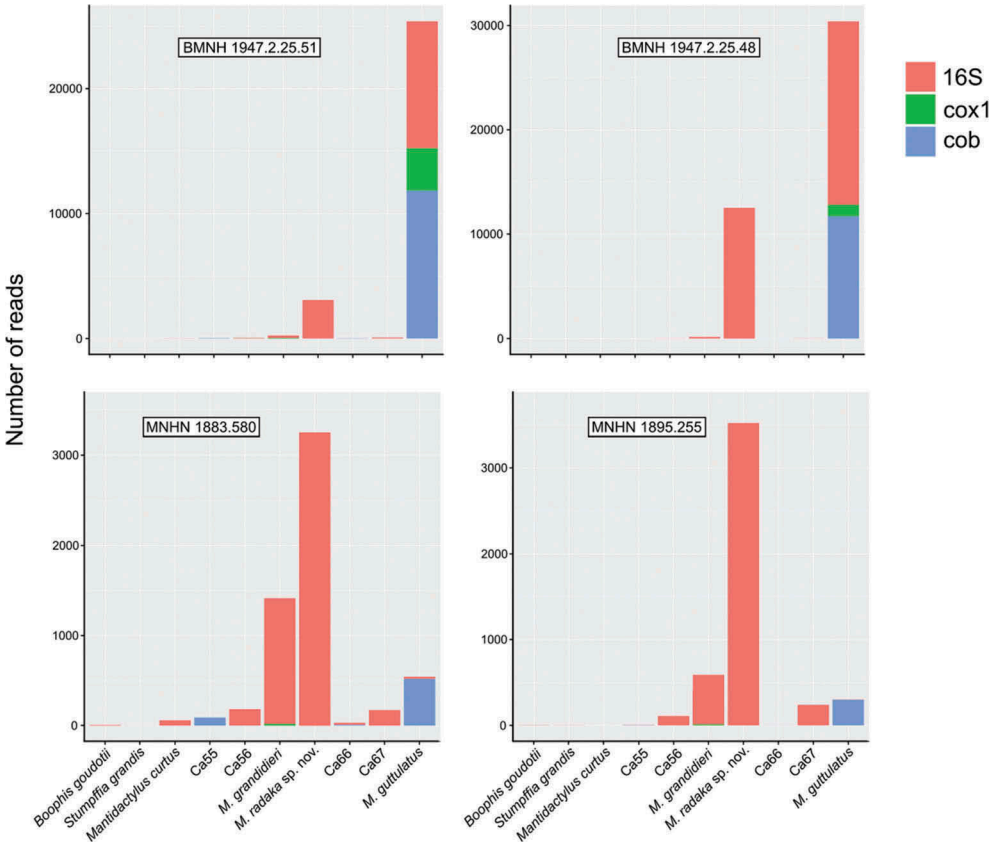

Figure 4. Stacked barplots showing the number of reads uniquely matching different reference sequences for the three targeted mitochondrial genes with a similarity threshold of 98%. The *Rana pigra* type was not included because the number of reads was too low.

From these reads, we assembled consensus sequences, which for 16S had an average coverage of 4537 to 8572 (the details for each sample are given in Table 2 and Figure 5). The consensus sequences obtained by aligning reads to different reference sequences were almost identical, except for stretches where the reference sequences differed by indels, and in exploratory phylogenetic analyses clustered in the same clades in the tree. We therefore used our preferred consensus sequences (obtained by alignment to those reference sequences closest to their preliminary phylogenetic clustering) for final analysis. These sequences were aligned to the main 16S alignment and could be successfully placed in the phylogenetic tree (Figure 1). Additional alignments of the consensus sequences for cob, and, where available, cox1 sequences of the historical types, with single representatives of the various *Mantidactylus* lineages, yielded results fully concordant with those of the 16S gene (Supplementary Figures S2 and S3).

We found that the lectotype and paralectotype of *Rana guttulata* from the London museum clustered with the lineage occurring predominantly in central eastern Madagascar to which we therefore apply the name *M. guttulatus* herein (Figure 1), as anticipated in the previous section. The two analysed syntype specimens of *M. grandidieri* from the Paris museum instead clustered with the lineage from the Northern Central East

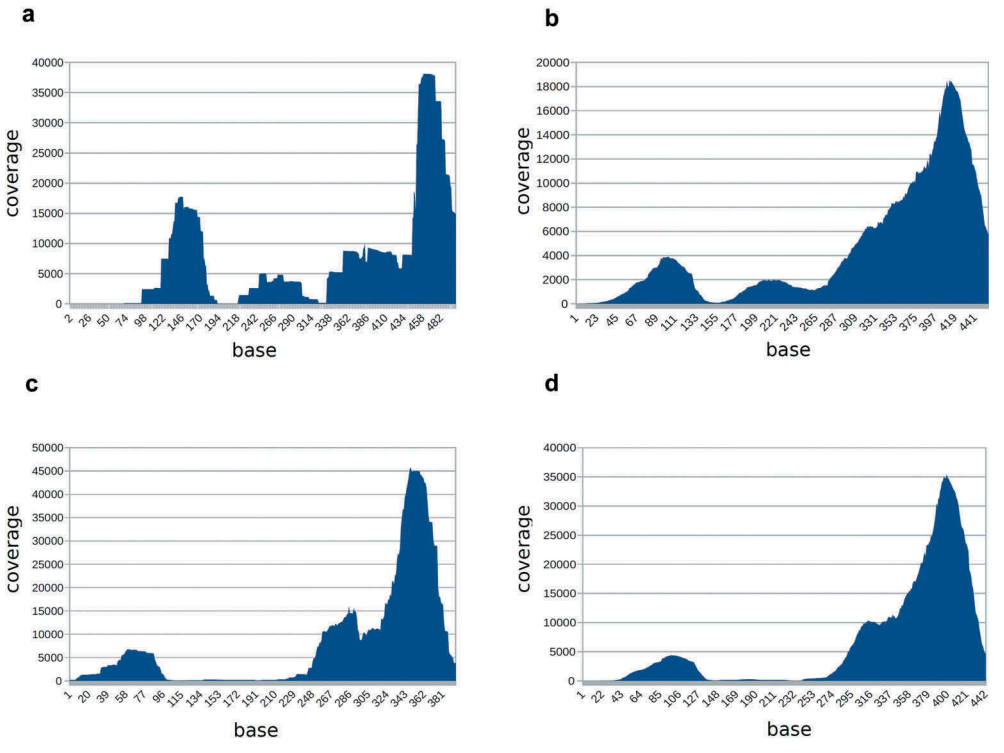

Figure 5. Per-base coverage plots for the 16S fragment in four *Mantidactylus* type specimens from the MNHN and BMNH collections. (a) BMNH 1947.2.25.48 (paralectotype of *Rana guttulata*); (b) BMNH 1947.2.25.51 (paralectotype of *Rana guttulata*); (c) MNHN 1895.255 (syntype of *M. grandidieri*); (d) MNHN 1883.520 (syntype of *M. grandidieri*).

and North East that previously had been named *Mantidactylus* sp. Ca57, which is herein renamed accordingly as *M. grandidieri*.

The holotype of the third nomen *Rana pigra* (MNHN 1899.410) yielded no reads matching *Mantidactylus* 16S or cox1 sequences. However, a total of 16 reads of this sample matched the terminal stretch of the target cob sequence (Supplementary Figure S3). In their majority, they agreed with a sequence of the lineage assigned to *M. guttulatus*. This assignment would also agree with the type locality (Ikongo forest), which is located roughly between Ranomafana and Ivohibe, within the distribution area of this lineage. However, given the very low number of reads, this result clearly remains in need of confirmation.

Lineages of Madagascar giant stream frogs show weak morphological differentiation

The morphometric comparison between lineages in the subgenus *Mantidactylus* is currently hampered by a lack of genetically identified adult voucher specimens, and no satisfying quantitative analysis was therefore possible. Morphometric data for all measured individuals in Table 1, and photographs in Figures 6–9 revealed only little morphological differentiation among lineages. Previous studies proposed to distinguish *M. grandidieri* and *M. guttulatus* by a more granular dorsal skin texture, wider terminal

Figure 6. Photographs of living specimens of *Mantidactylus radaka* sp. nov. (a, b) Male holotype ZSM 644/2001 (field number FGMV 2001.132) from Manarikoba forest, Tsaratanana Massif. (c–f) Female paratype ZSM 1800/2010 (ZCMV 12345) from Camp 1 (Antevialambazaha), Tsaratanana Massif. (g, h) Female paratype ZSM 97/2016 (MSZC 0080) from Ampotsidy. (i, j) Male paratype MSZC 0120 (uncatalogued in UADBA) from Ampotsidy. (k) Unidentified specimen from Camp 0 (Ankijagna Lagnana), Tsaratanana Massif. (l) Paratype ZSM 582/2014 (DRV 6073) from Camp 0 (Ankijagna Lagnana). (m, n) Unidentified female specimen from Manongarivo (Camp 0), probably preserved in UADBA collection.

discs of fingers and toes, and wider head of the latter (e.g., Blommers-Schlösser and Blanc 1991), but based on our measurements, examination of available vouchers, and photographs, these differences do not seem to be consistent. In fact, to judge from photographs (Figures 6 and 7) and the type specimens, and contrary to previous assumptions, most specimens of *M. guttulatus* appear to have a rather smooth dorsal skin, whereas some individuals of *M. grandidieri* are rather wide-headed and moderately granular on the back. Based on comparison of one female (ZSM 1013/2003) and one male (ZSM 266/2005) whose sex we confirmed by dissection, the male's tympanum appeared to be larger (ratio TD/SVL 0.082 vs 0.057), and its femoral glands were roughly of equal size to those of the female, but more distinct and prominent.

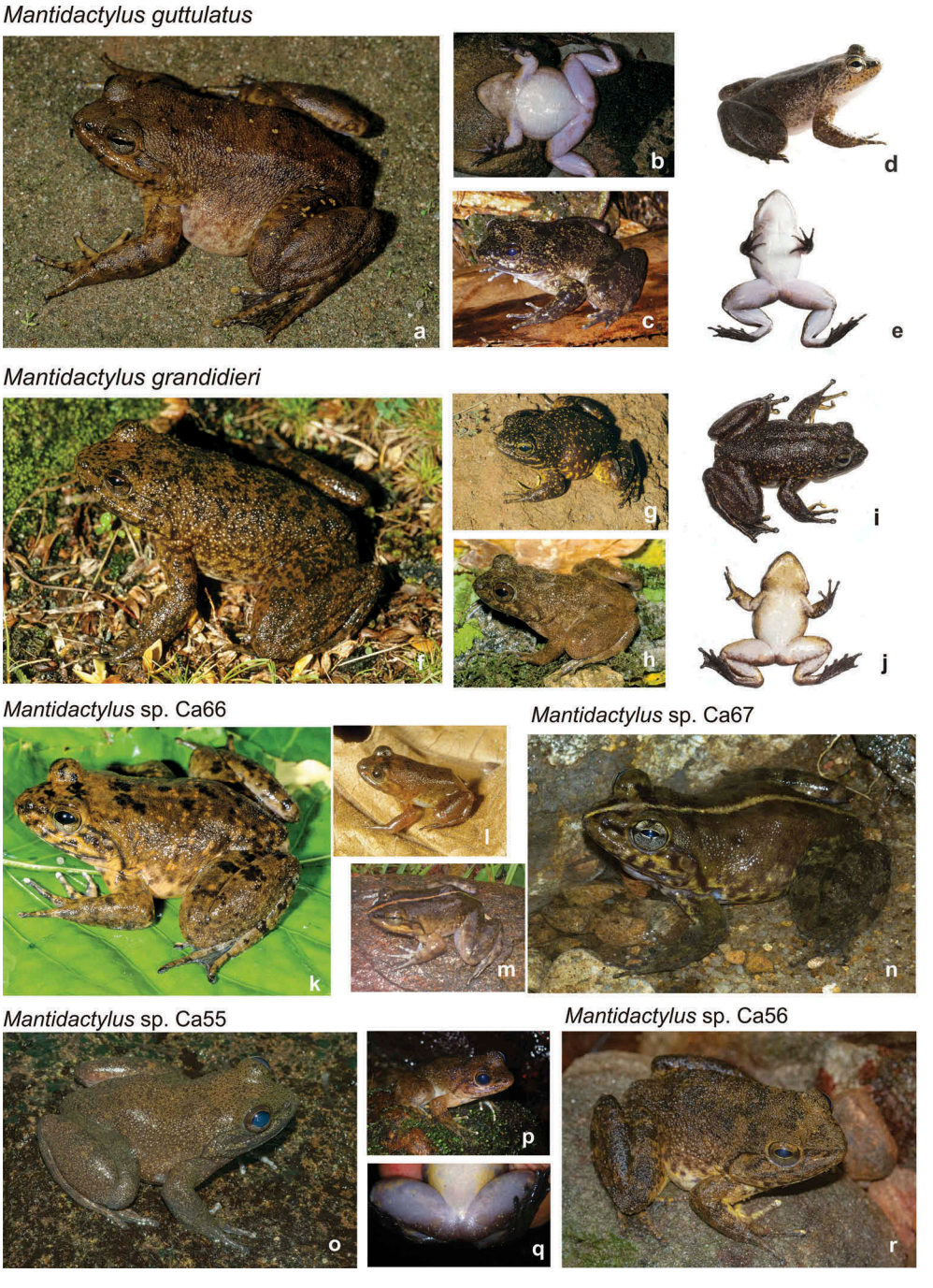

Figure 7. Photographs of living specimens of *Mantidactylus (Mantidactylus) guttulatus, M. (M.) grandidieri*, and of three candidate species. (a, b) *M. (M.) guttulatus*, female ZSM 1013/2003 (FGMV 2002.438) from Ranomafana. (c) Unidentified specimen from Ranomafana, assigned tentatively to *M. (M.) guttulatus* (no genetic evidence). (d, e) *M. (M.) guttulatus*, specimen KU 340853 (CRH729) from Ranomafana. (f) *M. (M.) grandidieri*, specimen ZSM 5077/2005 (ZCMV 2159) from Nosy Mangabe. (g) *M. (M.) grandidieri*, specimen ZSM 276/2005 (FGZC 2682) from Vohidrazana. (h) *M. (M.) grandidieri*, unidentified specimen (probably subadult) from Andranofotsy. (i, j) *M. (M.) grandidieri*, specimen KU

Figure 8. Lateral views of the heads of preserved adult males of *Mantidactylus (Mantidactylus) radaka* sp. nov. in comparison with *M. (M.) guttulatus* and *M. (M.) grandidieri*. Note the more distinct and larger tympanum (indicated by yellow arrows) in the latter two species. Not to scale.

The available photographs (Figure 7) indicate that the candidate species *Mantidactylus* sp. Ca66 and Ca67 from the South East appear to have a rather narrow head and an

340659 (CRH307) from Vohidrazana. (k) *M. (M.)* sp. Ca66, unidentified specimen from Manantantely (belonging to the series included in molecular analysis, voucher probably uncatalogued in UADBA collection). (l) *M. (M.)* sp. Ca66, juvenile from Nahampoana, tentatively assigned to this lineage (no genetic evidence). (m) *M. (M.)* sp. Ca67, unidentified specimen from Vevembe forest. (n) *M. (M.)* sp. Ca67, FAZC 15,332 from Tsitongambarika. (o) *M. (M.)* sp. Ca55, specimen FAZC 13,944 from Betampona. (p, q) *M. (M.)* sp. Ca55, unidentified specimen from Betampona. (r) *M. (M.)* sp. Ca56, specimen FAZC 13,579 from Betampona. Pictures: M, from J. E. Randrianirina; O-R, from G. M. Rosa.

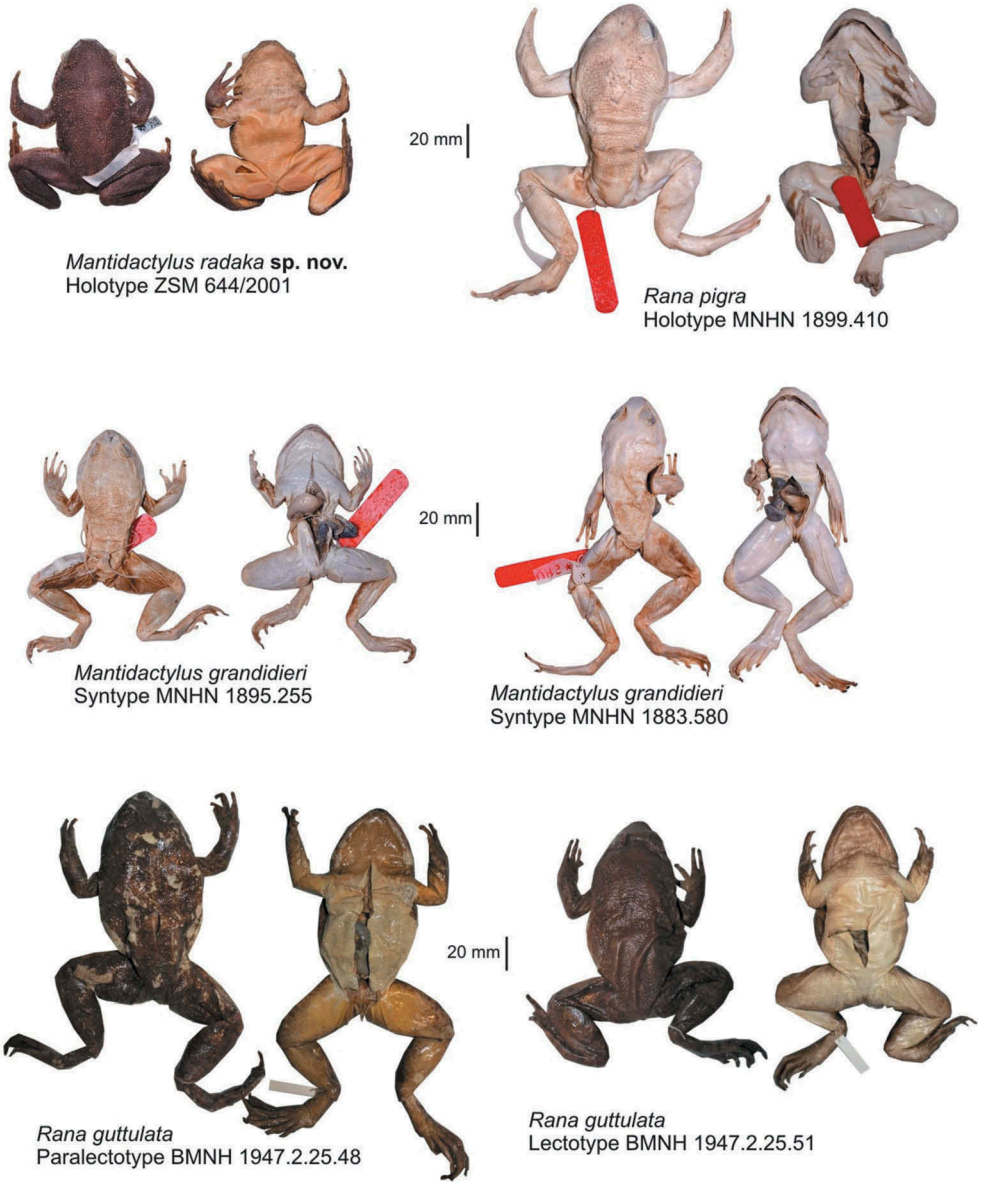

Figure 9. Preserved type specimens of the four nomina in the *Mantidactylus* subgenus *Mantidactylus* and one of the paralectotypes of *Rana guttulata*.

especially smooth dorsal skin, which may constitute a diagnostic character for future taxonomic revision of these lineages.

The most obvious morphological difference encountered was the shape and size of the femoral glands in the lineage from the Sambirano and North East regions, which is described as a new species in the following: in this lineage, the males have large and ovoid femoral glands, while they are smaller and rounded in males of *M. guttulatus* and *M. grandidieri*. On the other hand, the femoral glands in the females of the new species are indistinct (only tiny rudiments recognisable upon dissection), while always recognisable

externally in the two other species. Furthermore, the tympanum of the new species appears to be smaller than in most specimens of *M. grandidieri* and *M. guttulatus*, and often hidden. As an exception, the measured tympanum diameter of the lectotype and paralectotypes of *M. guttulatus* was also rather low, which however may be a preservation artefact, or due to measuring differences in different years of examination (2018 vs 2019).

Systematics

Mantidactylus (Mantidactylus) guttulatus (Boulenger, 1881)
Rana guttulata Boulenger, 1881

Lectotype. BMNH 1947.2.25.51, designated lectotype by Blommers-Schlösser and Blanc (1991), from the region of Betsileo (S.E. Betsileo), collected by Bartlett.

Paralectotypes. Four specimens, BMNH 1947.2.25.48–50, BMNH 1947.2.25.52, with same collection locality and data as lectotype.

Junior synonym. *Rana pigra* Mocquard, 1900. Holotype: MNHN 1899.410, from 'forêt d'Ikongo'.

Referred material. For field numbers of additional specimens referred to *M. guttulat us* genetically, see Figure 1. For morphological measurements of types and five additional specimens in the ZSM collection, see Table 1.

Remarks. *Mantidactylus (M.) guttulatus* is a large nocturnal stream-dwelling frog, distributed at elevations from 810 m a.s.l. (Vohidrazana) to ca. 1500 m a.s.l. (Antoetra). It is typically found in slow-moving parts of small streams in rainforest, and almost, nothing is known about its natural history. Based on genetic data herein, confirmed localities are (from north to south) Fierenana, Andasibe, Maromizaha, Mangabe region, An'Ala and Vohidrazana in the Northern Central East, and Antoetra, Vohiparara, Ranomafana and Ivohibe in the Southern Central East of Madagascar (map in Figure 1). If *Rana pigra* is correctly assigned as a junior synonym to *M. guttulatus*, then a further locality would be Ikongo Forest.

The definition of this species has had a very convoluted history, and many populations and specimens have intermittently been named *M. guttulatus*. A complete revision of all these uses in the literature is beyond the scope of this paper. Glaw and Vences (2007) defined populations from Tsaratanana as *M. guttulatus*, and calls and tadpoles of the lineage occurring at Tsaratanana were also described under this name (Vences et al. 2004; Schulze et al. 2016). However, this population corresponds to the new species *M. radaka* sp. nov. described below. The tadpole described by Altig and McDiarmid (2006) as *M. guttulatus* actually belongs to *M. majori*, a representative of the subgenus *Hylobatrachus* (Randrianiaina et al. 2011).

Mantidactylus (Mantidactylus) grandidieri Mocquard, 1895

Syntypes. Two specimens, MNHN 1883.580 and MNHN 1895.255 collected by Humblot and Grandidier, from 'Madagascar … côte Est'.

Referred material. For field numbers of additional specimens referred to *M. grandidieri* genetically, see Figure 1. For morphological measurements of types and additional six specimens in the ZSM collection, see Table 1.

Remarks. *Mantidactylus (M.) grandidieri* is a large nocturnal stream-dwelling frog, distributed at elevations from near sea level (Nosy Mangabe) to ca. 1500 m a.s.l. (Ambohitantely), but appears to be more common at low elevations <900 m a.s.l. It is found in slow-moving parts of streams in rainforest, often among rocks and boulders, and almost nothing is known about its natural history. Based on genetic data herein, confirmed localities are (from north to south) Marojejy, Sambava, Besariaka, Tsararano, Antalaha, Masoala, Ilampy, Ambodivoangy, Andranofotsy, Nosy Mangabe, Angozongahy (west slope of Makira Reserve), Antsahataloka, Ambatoroma, Ambohitantely, Ambatodisakoana, Fierenana, Vohidrazana, Moramanga and the Mangabe region.

As with *M. guttulatus*, the definition of this species has changed multiple times in the past. A complete revision of all these uses in the literature is beyond the scope of this paper. Glaw and Vences (2007) partially following Blommers-Schlösser and Blanc (1991) used the name *M. grandidieri* primarily to refer to highland populations in the Northern Central East and Southern Central East, which, according to the present revision, are to be referred to as *M. guttulatus*. Instead, populations of *M. grandidieri* were named *Mantidactylus* sp. aff. *grandidieri* 'North' by Glaw and Vences (2007), *Mantidactylus* sp. 57 by Vieites et al. (2009) and *Mantidactylus* sp. Ca57 by Perl et al. (2014).

Mantidactylus (Mantidactylus) radaka new species

Holotype. ZSM 644/2001 (field number FGMV 2001.132), adult male, collected on the Tsaratanana massif, Manarikoba forest, Antsahamanara, 'Camp I' (14.04500°S, 48.78528°E, ca. 1000 m above sea level), Diana Region, former Antsiranana province, northern Madagascar on 4–9 February 2001 by F. Andreone, F. Mattioli, J. Randrianirina & M. Vences.

Paratypes. ZSM 1800/2010 (field number ZCMV 12,345, female), collected on 10 June 2010 on the Tsaratanana massif, Camp 1 (14.17413°S, 48.94521°E, 1589 m above sea level), Sofia Region, former Mahajanga province, northwestern Madagascar, by M. Vences, D. Vieites, R. D. Randrianiaina, F. Ratsoavina, S. Rasamison, A. Rakotoarison, F. Randrianasolo, E. Rajeriarison & T. Rajoafiarison. ZSM 1801/2010 (field number ZCMV 12,567, male), collected on 29 June 2010 at Bemanevika river (14.48251°S, 48.62723°E, 1109 m above sea level), Sofia Region, former Mahajanga province, Madagascar, by M. Vences, D. Vieites, R. D. Randrianiaina, F. Ratsoavina, S. Rasamison, A. Rakotoarison, F. Randrianasolo, E. Rajeriarison & T. Rajoafiarison. ZSM 1802/2010 (field number ZCMV 12,596, female), collected on 28 June 2010 at Bemanevika, Camp 2

(14.35991°S, 48.59022°E, 1538 m above sea level), Sofia Region, former Mahajanga province, Madagascar, by M. Vences, D. Vieites, R. D. Randrianiaina, F. Ratsoavina, S. Rasamison, A. Rakotoarison, F. Randrianasolo, E. Rajeriarison & T. Rajoafiarison. ZSM 581/2014 (field number DRV 6072, female), ZSM 582/2014 (field number DRV 6073, male), ZSM 583/2014 (DRV 6074, male) and ZSM 584/2014 (field number DRV 6097, male), collected on 9 June 2010 at Tsaratanana massif, camp 0 (Ankijagna Lagnana, 14.23989°S, 48.97208°E, 1162 m above sea level), Sofia Region, former Mahajanga province, Madagascar, by D. Vieites, M. Vences, R. D. Randrianiaina, S. Rasamison, A. Rakotoarison, F. Randrianasolo, E. Rajeriarison & T. Rajoafiarison. ZSM 95/2016 (field number MSZC 0189), collected on 13 January 2016 at Ampandrana forest (26.6 km SW of Bealanana on the RN31, 14.71788°S, 48.57548°E, 1038 m above sea level), Sofia Region, former Mahajanga province, Madagascar, by M. D. Scherz & M. Rakotondratsima. ZSM 96/2016 (field number MSZC 0205), collected on 14 January 2016 at Andranonafindra forest (30 km SW of Bealanana on the RN31, 14.73654°S, 48.54846°E, 1204 m above sea level), Sofia Region, former Mahajanga province, Madagascar, by M. D. Scherz & M. Rakotondratsima. ZSM 97/2016 (field number MSZC 0080, female), collected on 22 December 2015 on Ampotsidy mountains, 15.7 km NNW of Bealanana (8.7 km NNW of Beandrarezona, 14.41456°S, 48.71148°E, 1400 m above sea level), Sofia Region, former Mahajanga province, Madagascar, by M. D. Scherz, J. Borrell, L. Ball, T. Starnes, E. Razafimandimby, D. H. Nomenjanahary & J. Rabearivony.

Diagnosis

The new species is assigned to the genus *Mantidactylus* based on the presence of an intercalary element between terminal and subterminal phalanges of fingers and toes (verified by external observation only), of a central depression in femoral glands and of a (very) rudimentary femoral gland in the female. Within *Mantidactylus*, it is assigned to the nominal subgenus *Mantidactylus* by combination of (1) very large body size (male SVL 88–93 mm, female SVL 85–103 mm), (2) absence of dorsolateral colour border, (3) absence of a distinct frenal stripe, (4) absence of large yellowish patches or stripes in the inguinal region or between colouration of flanks and belly, (5) fully webbed feet and (6) riparian habits, living very close to or in streams. The assignment of the species to this group is also supported by its molecular phylogenetic relationships.

From the two described species in the subgenus *Mantidactylus*, the new species is distinguished as follows:

From *M. guttulatus* by a longer relative hindlimb length (HIL/SVL 1.46–1.60 versus 1.28–1.43); larger femoral glands in males (FGL/SVL 0.13–0.20 versus 0.06–0.08), which are ovoid (versus round in *M. guttulatus*), and indistinct femoral glands in females (versus always recognisable in *M. guttulatus*); and possibly by an often-hidden tympanum and smaller relative tympanum size (TD/SVL 0.032–0.047 versus 0.052–0.082) [however not applying to the *M. guttulatus* type specimens (0.035–0.049), possibly due to preservation artefacts].

From *M. grandidieri* by an often-hidden tympanum and smaller relative tympanum size (TD/SVL 0.032–0.047 versus 0.055–0.063); larger femoral glands in males (FGL/SVL 0.13–0.20 versus 0.09); which are ovoid (versus round in *M. grandidieri*); and indistinct femoral glands in females (versus always recognisable in *M. grandidieri*).

Description of the holotype. Adult male in excellent state of preservation. For measurements, see Table 1. Body rather stout. Head wider than long, slightly wider than body.

Snout blunt. Nostrils directed dorsolaterally, slightly protuberant, nearer to tip of snout than to eye. Canthus rostralis poorly distinct, loreal region concave. Upper part of the tympanum hidden under the tympanic fold. Tympanum small, horizonal diameter of tympanum 34% of horizontal eye diameter. Supratympanic fold distinct, straight above the tympanum, curving gently towards the axilla. Tongue ovoid, distinctly bifid posteriorly. Vomerine teeth form two rounded aggregations, positioned posterolateral to choanae. Choanae ovoid. Subarticular tubercles single. Outer metacarpal tubercle not recognisable, inner metacarpal tubercle present and distinct. Fingers without webbing. Relative length of fingers: I<II<IV<III. Finger discs enlarged. Nuptial pads absent. Foot slightly shorter than tibia (97%). Lateral metatarsalia separated. Inner metatarsal tubercle present. Outer metatarsal tubercle not recognisable. Webbing formula: 1(0), 2i(0.5), 2e(0), 3i(1), 3e(0), 4i(1), 4e(0.5), 5(0). Relative length of toes: I<II<III=V<IV. Skin on the upper surface strongly rugose with numerous small warts, these becoming larger on the flanks. Ventral side smooth. Femoral glands very large and distinct in external view.

Colour in preservative (after 18 years in preservative) dorsally brown, with tiny yellowish dots on the warts. Venter beige, with indistinct mottling on the throat. The inner three fingers are lighter than the fourth one. Colouration in life (Figure 6) similar to that in preservative, with larger yellowish dots particularly on the flanks. Uniformly greenish-brown iris. Light tips on the inner three fingers.

Etymology. The species name is a noun in apposition to the genus name, and is the Malagasy word for large frogs in general (as opposed to 'sahona', which usually refers to small frogs), and those of the subgenus *Mantidactylus* in particular.

Variation. No obvious difference in tympanum diameter between males and females (TD/SVL female 0.032–0.043, male 0.037–0.047). The paratypes and additional photographed specimens in general agree with the holotype in morphology and colouration. In preserved females, femoral glands are not recognisable externally. In life, their femoral glands are visible as tiny black patches (cf. photos; Figure 6F, H). Some females (ZSM 1800/2010 in particular) have very dark throat colouration. In a young male (ZSM 582/2014), femoral pores are already distinct (both externally and internally) and ovoid.

Natural History. A large nocturnal frog typically found in relatively calm stretches of streams, including small and shallow streams with sandy bottom in rainforest (e.g., Tsaratanana), and more rarely also in shallow stretches of wider streams with rocky bottom in more open areas at the edge of rainforest (e.g., near Bemanevika). In Besariaka, individuals of this species also showed an apparently distinct natural history and behaviour, preferring running water (usually moving in the central parts of streams), while *M. grandidieri* appeared associated more closely with stream banks. *Mantidactylus radaka* sp. nov. is the only species of the subgenus for which some natural history information is available: calls and tadpoles were described, from Tsaratanana and from a site between Bealanana and Antsohihy (close to the collection locality of ZSM 96/2016), respectively, by Vences et al. (2004) and Schulze et al. (2016), under the name *M. guttulatus*.

Distribution. Localities span an elevational range from 690 m a.s.l. (Manongarivo Special Reserve, Camp 0) up to at least 1590 m a.s.l. (Tsaratanana Massif, Antevialambazaha/Camp 1).

Based on genetic data herein, confirmed localities are (from north to south) the Tsaratanana Massif (localities Manarikoba forest and Antevialambazaha/Camp 1), Manongarivo, Ampotsidy, Bemanevika, several sites between Bealanana and Antsohihy (e.g. Ampandrana and Andranonafindra), and Besariaka.

Conservation. During fieldwork in 2016, this species was found to be rather abundant, even where the forest was heavily degraded, as long as some riparian habitat remained. However, it is also actively hunted as a source of food by local people in the Bealanana District (Figure 10). When asked, two locals stated that they no longer hunted these frogs in their home village (Ampandrana, ca. 14.704°S, 48.589°E), because they are no longer found in sufficient abundance in its vicinity. We managed to collect specimens from the river that flows towards Ampandrana only by going upstream (14.71788°S, 48.57548°E), and these frogs were considerably more abundant 5 km further SSW along the Route National 31 (at Andranonafindra), where there are no further villages for another 15 km. Thus, it seems evident that anthropogenic pressures are having impacts on local populations. However, the extent of those impacts is currently unclear, and given the apparently very large clutches that these frogs have (see the egg-laden ovaries evident in Figure 10C)

Figure 10. *Mantidactylus (Mantidactylus) radaka* sp. nov. being prepared for human consumption. (a) Frogs and crabs are collected from broad streams. Then (b) the frogs are gutted and skinned, and the head, hands and feet removed. The frog is then rinsed in the stream, leaving (c) cleaned animals for cooking in a stew. Note the ovaries full with hundreds of eggs.

and their stream-dwelling tadpoles, it is possible that up-stream populations may seed down-stream ones, and the species may thus be rather resilient to exploitation and able to recover quickly if pressures cease.

At present, the distribution of this species appears to be rather large: a minimum convex polygon of known localities has an area of 12,534 km^2. Over that area, there is no doubt that the habitat of the species is declining in extent and quality, and, although it is found in numerous locations, its habitat is also severely fragmented. Therefore, this species qualifies as Vulnerable under the International Union for Conservation of Nature (IUCN) Red List of Threatened Species, criterion B1ab(iii) (IUCN 2012).

Candidate species *Mantidactylus* sp. Ca55, Ca56, Ca66, Ca67

Besides the three species now recognised, our analysis has identified four additional lineages in the subgenus *Mantidactylus* (Figure 1), for which we have herein used the candidate species names *Mantidactylus* sp. Ca55, Ca56, Ca66 and Ca67. At present, data are clearly insufficient to take taxonomic decisions regarding these lineages.

Mantidactylus sp. Ca55 and Ca56 occur sympatrically in Betampona, but no specimens of either candidate species were available for morphological examination in this study. Both of these candidate species are crepuscular-nocturnal. In Betampona, *Mantidactylus* sp. Ca56 seems to be both more abundant and more widespread, but precise data on its fine-scale distribution within this reserve are thus far lacking. *Mantidactylus* sp. Ca56 inhabits all large and fast-flowing streams across the reserve, while *Mantidactylus* sp. Ca55 seems to prefer lateral branches of the fast-flowing streams, where water is shallower and runs much more slowly. Photos of genetically identified individuals of the two candidate species (Figure 7) do not immediately reveal obvious morphological differences between them. They do not appear to be sister species based on the mitochondrial data (Figure 1), and in the nuclear RAG-1 gene, the two examined samples do not share alleles. If confirmed with a larger number of samples, such a concordance between mitochondrial and nuclear gene signals in sympatry would clearly be indicative of a status as separate species (Padial et al. 2010).

In contrast, *Mantidactylus* sp. Ca66 and Ca67 appear to be allopatric sister lineages distributed in the South East. Their mitochondrial divergence is low, and they may be conspecific. However, their distinctness from other species and candidate species of the subgenus *Mantidactylus* is supported by exclusive RAG-1 alleles of numerous individuals sequenced, and by their apparently very smooth skin. Thus, they are likely to represent at least one new and as yet undescribed species. In the forest at Manantantely, *Mantidactylus* sp. Ca66 was nocturnal and living in rocky streams in rather pristine low-elevation rainforest.

Discussion

Massively-Parallel-Sequencing-based method successfully allowed the molecular barcoding of old museum specimens preserved in wet collections

Using a Massively Parallel Sequencing (MPS) approach, we attempted amplification and sequencing of mitochondrial DNA from five historical frog specimens, all of them preserved for over 120 years in wet collections, with an unknown history of preservatives.

After filtering, we ended up with hundreds of thousands of short reads for each specimen. In four of these samples, several thousand of these reads could be mapped to reference sequences of frogs from the subgenus *Mantidactylus*. However, for the fifth sample (MNHN 1899.410, the holotype of *Rana pigra*), only a few reads could be identified as *Mantidactylus* DNA, and thus we did not consider these data for further analyses. Why this particular specimen did not work remains unclear. One possible explanation could be that the specimen of *R. pigra* was the only one to be fixed with formaldehyde, since this fixative was not yet in general use when the four other samples were collected. However, formaldehyde fixation alone does not necessarily inhibit DNA isolation and subsequent capture with adequate baits – instead this preservation method mainly leads to severe fragmentation of DNA (Gilbert et al. 2007). Given the uncertainty regarding the fixation and preservation history of the *Rana pigra* sample, further analyses will be required to understand better why DNA sequencing failed.

We then used these reads to assign the four remaining specimens to a specific lineage using two approaches. Firstly, we quantified the number of reads that had unique matches to each sequence of a reference database representing the different lineages of the *Mantidactylus* subgenera, as well as three other frog species as negative controls. The underlying hypothesis is that the reference sequence with the most unique matches should correspond to the lineage to which the specimen belongs (this method was also used by Yuan et al. 2019). While this method worked well for the two *M. guttulatus* types, clearly indicating their belonging to the northeastern lineage, the results for the *M. grandidieri* types are less decisive. Indeed, the counts do not allow us to clearly distinguish between the two northern lineages, and the total number of reads matching to the reference sequences was around 10 times lower than in the *M. guttulatus* samples. Secondly, for each of the four samples, we generated consensus sequences of the 16S, cox1 and cob genes. These sequences were added to our phylogenetic tree, and the specimens attributed to the lineage in which they clustered. Doing so, we could confidently identify the type specimens, in concordance with the type localities when available. The two type specimens of *M. guttulatus* clustered within the inland eastern lineage, while the type specimens of *M. grandidieri* were included in the northeastern group. This result was consistently supported by the three targeted mitochondrial genes.

Although these two approaches yielded overall concordant results, interpreting them was not equally straightforward in both cases. Indeed, the 'phylogeny-based' approach proved rather easy to interpret, as long as the consensus sequence obtained from the reads was complete enough to ensure phylogenetic resolution. On the other hand, the approach based on unique matches proved to be sensitive to the reference sequences used. Using two very close references will reduce the number of unique matches observed, since most reads will match both of them. The presence of ambiguous or missing positions in the reference also proved to be a problem, by preventing the reads to align to the reference when using a conservative similarity threshold. Finally, the number of reads alone is not particularly valuable information, since a large number of reads may cover only a small part of the sequence. Considering these observations, the 'phylogeny-based' approach seems to be the safest and most straightforward approach to assign a specimen to a lineage. When using this approach however, aligning the reads to a set of reference sequences including more distant species as negative controls is a good way to ensure the absence of artefacts at the sequencing step, such as cross-contaminations.

Indeed, the baits we used are highly conserved, particularly for the 16S gene, and the risk of capturing DNA from other organisms (e.g. *Homo sapiens*, or other historical specimens that might have been conserved in the same jar) is substantial. In addition, such analysis can also provide interesting insights on the origin of the reads. Thus, these two approaches act complementarily, and in the present case allowed us to confidently identify our samples.

In line with previous studies (Hykin et al. 2015; Li et al. 2016; Ruane and Austin 2017; McGuire et al. 2018; Evans et al. 2019), we further demonstrate that MPS methods can be successfully applied to historical specimens preserved in wet collections. As exemplified in the present study, this approach can be very useful to solve complex taxonomic conundrums, particularly when morphologically cryptic lineages are involved. However, it could be applied to other biological questions, such as resolving the phylogenetic placement of rare or extinct taxa, or studying the dynamics of parasites through specimens of the host species collected at different times, among others (reviewed in Burrell et al. 2015).

The subgenus Mantidactylus *shows deep phylogenetic structure*

As in other groups of Malagasy frogs, the study of genetic variation within the subgenus *Mantidactylus* confirmed that the current taxonomy underestimates species richness. Using mitochondrial sequences, we were able to delimit seven well-supported candidate species, all of which are geographically separated, with little to no sympatry. In the present study, we take a first step to revise the taxonomy of this group by determining to which lineages the currently described names should be attributed, and describing a third lineage under a new name. As discussed above, the study of molecular data from museum samples successfully allowed us to attribute the type specimens of *M. (M.) guttulatus* and *M. (M.) grandidieri*. Therefore, the former name should be used for the lineage distributed in inland localities of Eastern Madagascar, from Ivohibe in the South to Fierenana in the North. On the other hand, the name *M. (M.) grandidieri* should be applied to the populations from the northeast of Madagascar, between Mangabe in the South and Marojejy in the North. Unfortunately, the type specimen of *Rana pigra*, although here treated as junior synonym of *M. (M.) guttulatus*, could not be identified with full certainty, and we cannot exclude that it may belong to the *Mantidactylus* sp. Ca66/Ca67 clade at this time.

While the status of the four lineages distributed along the eastern coast is difficult to assess due to our sparse sampling and few loci considered, the northwestern lineage stands out by its high divergence from all the other lineages. The monophyly of this group is fully supported on the 16S phylogenetic tree, and the p-distance matrix shows that it is the most distinct lineage of the group. In addition, it does not share any RAG-1 haplotypes with the other lineages. For all these reasons, as well as its geographic separation from the other lineages, we have described it as a new species: *Mantidactylus (M.) radaka*. While the molecular analysis of museum samples alone could not rule out that the name *Rana pigra* refers to that lineage, we are confident that this is very unlikely. Indeed, the type locality for *R. pigra* ('forêt d'Ikongo') is within the range of *M. (M.) guttulatus* whereas the range of *M. (M.) radaka* to our knowledge had not been visited by zoologists at the time of the description of *R. pigra* (absence of historical amphibian records from this area: see Blommers-Schlösser and Blanc 1991; Glaw and Vences 1994). However, further studies of the *R. pigra* type specimen will be needed to definitively confirm its synonymy with *M.*

(M.) guttulatus. Despite their molecular distinctiveness, the lineages of *Mantidactylus* show little morphological divergence. However, the new species tends to have a smaller tympanum, as well as very large and ovoid femoral glands.

The combination of MPS-based molecular study of type specimens with traditional phylogenetic and morphologic analyses proved to be an efficient and effective approach to resolve the taxonomy of the *Mantidactylus* subgenus. Even if our pipeline remains to be improved in many aspects, it could provide a standardised way to assess taxonomic questions within complexes of cryptic species. Further development of our approach could be the integration of coalescent-based species delimitation and discovery models applied on multi-locus data.

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