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Two New Microhylid Frogs of the Genus *Rhombophryne* with Superciliary Spines from the Tsaratanana Massif in Northern Madagascar

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Abstract: The *Rhombophryne serratopalpebrosa* (Guibé 1975) species complex consists of numerous cryptic narrow-mouthed frogs (Microhylidae) mostly restricted to northern Madagascar. We here provide an updated phylogeny of the genus *Rhombophryne*, and formally define the *R. serratopalpebrosa* group, characterized by the possession of small, fleshy superciliary spines. We describe two new species belonging to this group from the Tsaratanana Massif in northern Madagascar: *Rhombophryne ornata* sp. nov. and *R. tany* sp. nov. Both of the new species have fewer superciliary spines above each eye than any other member of the group (2 vs. 3–4). *Rhombophryne ornata* sp. nov. is unusual in having fairly elaborate dorsal markings, and is unique in having reddish thighs. In contrast, *R. tany* sp. nov. is a truly cryptic species characterized by a combination of characters unique within the *R. serratopalpebrosa* group. Micro-CT scans revealed differences between the skeletons of these species and other members of the group: the pubis is unossified in both species, and *R. ornata* sp. nov. lacks ossification in the epiphyses of its limb bones and in many of its small bones. We give a full osteological description of these frog species to facilitate ongoing research concerning this group. Genetic data suggest that these two syntopic species might be sister to each other, but they show a substantial genetic divergence of 3.9% and 8.4% uncorrected pairwise distance in the mitochondrial 16S rRNA and cytochrome oxidase subunit I genes, respectively. As these species have a limited extent of occurrence, and are known only from a single location in a forest that is declining in quality, we propose they be listed as Vulnerable B1ab(iii) on the International Union for Conservation of Nature Red List.

Key words: 3D modeling; Anura; Microhylidae; Osteology; *Rhombophryne serratopalpebrosa* group; Taxonomy; X-ray micro-computed tomography

Cophylinae is a diverse radiation of microhylid frogs endemic to Madagascar (Andreone et al. 2005a), accounting for ~81% of the described microhylid diversity of the island (AmphibiaWeb 2015). Its greatest diversity and endemism are concentrated in the rainforests of northern Madagascar (Glaw and Vences 2007; Wollenberg et al. 2008), with only few species known from the drier regions in the west of the island (Glaw et al. 2007; Andreone and Randrianirina 2008). Cophylinae currently comprises seven genera (AmphibiaWeb 2015; Scherz et al. 2015; currently valid nominal species numbers in parentheses): *Anodonthyla* (11 spp.), *Cophyla* (6 spp.), *Madecassophryne* (1 sp.), *Platypelis* (13 spp.), *Plethodontohyla* (10 spp.), *Rhombophryne* (12 spp.), and *Stumpffia* (16 spp.). Peloso et al. (2015) have proposed the synonymization of *Stumpffia* with *Rhombophryne*, but we here refrain from adopting this rearrangement pending further investigation, and all references to *Rhombophryne* herein refer to that genus sensu Glaw and Vences (2007).

Within the genus *Rhombophryne*, 13 candidate species were identified by DNA barcoding studies (Vieites et al. 2009; Perl et al. 2014). Three of these 13 candidates have already been described: *R. mangabensis* Glaw, Köhler and Vences 2010, *R. matavy* D'Cruze, Köhler, Vences and Glaw 2010, and *R. vaventy* Scherz, Ruthensteiner, Vences and Glaw 2014. A fourth recently described species, *R. longicrus* Scherz, Rakotoarison, Hawlitschek, Vences and Glaw 2015, was not included in candidate estimates. Additionally, we have recently redescribed *R. serratopalpebrosa* (Guibé 1975) to facilitate further work on a complex of morphologically similar species with superciliary spines that has been difficult to resolve (Scherz et al. 2014). Because of the paucity of external morphological characters, and the lack of genetic data on museum specimens collected before tissue sampling was commonplace, emphasis is placed on osteology in this complex, acquired using nondestructive X-ray micro–computed tomography (micro-CT).

The present paper is part of an ongoing integrative revision of the genus *Rhombophryne*. We present an updated molecular phylogeny of *Rhombophryne* based on new and existing DNA sequence data, and formally define the *Rhombophryne serratopalpebrosa* group. We then describe two new species from the Tsaratanana Massif, an area of high endemism with many threatened and fragmented forests (Raxworthy and Nussbaum 1996; Andreone et al. 2005b, 2009; Rakotoarison et al. 2012).

Materials and Methods

Specimens were killed with chlorobutanol solution, fixed in 90% ethanol, preserved in 70% ethanol, and deposited in the Zoologische Staatssammlung München (ZSM) in Germany, and the Universite d'Antananarivo Départment de Biologie Animale (UADBA) in Madagascar. Field numbers refer to those of either Miguel Vences (ZCMV) or David R. Vieites (DRV).

Measurements of external morphology were taken with calipers (±0.01 mm, rounded to the nearest 0.1 mm) following Scherz et al. (2014): Snout–vent length (SVL); maximum head width (HW); head length, from the maxillary commissure to the snout tip (HL); horizontal eye diameter (ED); eye–nostril distance (END); nostril–snout tip distance (NSD); internarial distance (NND); horizontal tympanum diameter (TDH); vertical tympanum diameter (TDV); hand length, from the metacarpal–radioulnar articulation to the tip of the longest finger (HAL); lower arm length, from the metacarpal–radioulnar articulation to the radioulna–humeral

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articulation (LAL); upper arm length, from the radioulna–humeral articulation to the trunk (UAL); forelimb length, given by the sum of HAL, LAL, and UAL (FORL); foot length, from the tarsal–metatarsal articulation to the tip of the longest toe (FOL); tarsal length, from the tarsal–metatarsal articulation to the tarsal–tibiofibular articulation (TARL); foot length including tarsus, from the tibiotarsal articulation to the tip of the longest toe (FOTL); tibiofibula length (TIBL); thigh length, from the cloaca to the femur–tibiofibular articulation (THIL); hindlimb length, given by the sum of FOL, TARL, TIBL, and THIL (HIL); maximum length of inner metacarpal tubercle (IMCL); and maximum length of the inner metatarsal tubercle (IMTL). Basic post-hoc measurements were taken from photographs of UADBA specimens laid on graphing paper, using the image analysis functionality of ImageJ v1.48 (Schneider et al. 2012). See Scherz et al. (2015) for a figure depicting how this scheme is applied.

For micro-CT scanning, specimens were placed in closed plastic containers filled with ethanol-saturated air (small amount of ethanol at the bottom of the vessel) and affixed to an oblique polystyrene panel. Scanning was performed with a Phoenix Nanotom m cone beam CT scanner (GE Measurement and Control, Wunstorf, Germany) at a voltage of 130 kV and a current of 120 mA for 25 min (ZSM1816/2010, 2000 projections) or a voltage of 140 kV and a current of 80 mA for 24 min (ZSM1815/2010, ZSM 1814/2010, and DRV 06156; 1440, 1440, and 2440 projections, respectively). Surface meshes of the skeleton were generated using the threshold tool in the segmentation editor of the software Amira v5.4.5 (FEI Visualization Sciences Group, Burlington, MA, USA; Stalling et al. 2005). For varying intensities in the volume data, the threshold was locally adjusted.

Skeletal figures were prepared from high surface mesh resolution models in Amira v5.4.5 using manual isolation and the snapshot tool. Portable document format (PDF) three-dimensional (3D) models were prepared following the procedures outlined by Ruthensteiner and Heß (2008). These models were examined in Adobe® Reader® XI (Adobe Systems Incorporated, San Jose, CA, USA), and comparative measures of osteological characters were taken using the built-in measurement tool. Osteological terminology follows Trueb (1973, 1993). Terminology for the septomaxilla is synthesised from Pugener and Maglia (2007) and Trueb (1968). Micro-CT scans do not render cartilages; therefore, these structures are omitted from our description.

Total genomic DNA was extracted from ethanol-preserved tissue samples using proteinase K (final concentration 1 mg/mL), and isolated with a standard salt extraction protocol (Bruford et al. 1992). A fragment of the mitochondrial large ribosomal subunit or 16S rRNA (16S) was amplified via polymerase chain reaction (PCR) using the primers 16Sar-L and 16Sbr-H (Palumbi et al. 1991). Mitochondrial cytochrome oxidase subunit I gene (COX1) sequences were taken from Perl et al. (2014).

Sequences were resolved on an ABI 3130xl automated DNA sequencer (Applied Biosystems) and deposited in GenBank (accession numbers KP895582–KP895585). We calculated a phylogenetic tree of the concatenated COX1 and 16S sequences by Bayesian inference with MrBayes v3.2 (Ronquist et al. 2012) after determining a GTR + I + G substitution model as best fitting the data using jModeltest (Darriba et al. 2012). We considered all sections of the 16S gene with more than two consecutive gaps in one or more sequences as ambiguous and excluded these sections from the alignment (total alignment length after exclusion: 1221 nucleotides); however, explorative analyses including these stretches resulted in an identical topology and similar support values. Results of two independent runs of 20 million generations, each comprising four Markov chains (three heated and one cold), were sampled every 10,000 generations. We assessed chain mixing and stationarity by examining the standard deviation of split frequencies and plotting the $-\ln L$ per generation using Tracer v1.5 software (Rambaut and Drummond 2007). Results were combined to obtain a 50%-majority rule consensus tree and the respective posterior probabilities of nodes, after discarding 25% of the generations as burn-in (all compatible nodes with probabilities <0.5 kept).

RESULTS

The candidate species *R.* Ca11 and *R.* Ca12 of Perl et al. (2014) differ by an uncorrected pairwise DNA distance (p distance) of 3.9% in the 16S locus, whereas intraspecific variation within *R.* Ca11 was substantially lower, with p distances of 0.2–0.4% (Table 1, *R. ornata* and *R. tany*). Therefore, these two candidate species are genetically divergent. Examination of specimens of each species revealed numerous morphological differences (including skeletal morphology) between them and all other currently valid nominal species of the genus *Rhombophryne*. The combined evidence of genetic, morphological (Table 2), and osteological differences corroborates the status of these two candidates as valid species in need of formal description. Therefore, we describe them as new species, below.

The molecular phylogeny of the genus *Rhombophryne*, based on newly generated and existing 16S rRNA and COX1 sequences, revealed that the species possessing superciliary spines—*R. coronata*, *R.* sp. "Ambolokopatrika," *R. vaventy*, and the herein described *R. ornata* and *R. tany*—form a monophyletic group with high support (Fig. 1). *Rhombophryne serratopalpebrosa* also possesses superciliary spines, but the holotype and only known specimen is too old for DNA extraction using standard methods (see Scherz et al. 2014) and, although we are confident that it belongs to this clade, its position is not known. Because this group is monophyletic and united by a derived morphological character state (i.e., superciliary spines), we provide here a formal definition.

The *Rhombophryne serratopalpebrosa* group consists of small to medium-sized, cryptically colored, leaf-litter-dwelling frogs of the microhylid subfamily Cophylinae, found in rainforests across northern and eastern Madagascar, possessing small, fleshy, superciliary spines above the eyes. At present, this group is comprised of *R. serratopalpebrosa*, *R. coronata*, *R. vaventy*, and the herein described species *R. ornata* and *R. tany*.

SPECIES DESCRIPTIONS

Rhombophryne ***ornata*** sp. nov.

(Table 2; Figs. 2A–C, 3, 4; Online Supplementary Fig. S1–3)

Holotype.—ZSM 1816/2010 (ZCMV 12384), adult male, collected on the Tsaratanana Massif, Camp 2 (Matsabory Maiky), 14.15256°S, 48.95728°E (all datum = WGS84), 2021 m above sea level (asl), on the border between the

Table 1.—Uncorrected pairwise divergences of the sequenced 16S rRNA gene fragment in the *Rhombophryne serratopalpebrosa* group. *Rhombophryne* sp. Ca7 has uncertain affinities and is therefore conservatively included here. The diagonal reflects intraspecific variation.

Taxon	1	2	3	4	5	6
1 *R. tany*	NA[a]	3.9–4.3%	6.6%	9.8%	8.5%	7.4%
2 *R. ornata*		0.2–0.4%	5.8–5.9%	8.3–8.7%	8.5–8.6%	4.8–5.1%
3 *R.* sp. “Ambolokopatrika”			NA	10.1%	7.7%	6.4%
4 *R.* sp. Ca7				NA	10.1%	9.6%
5 *R. coronata*					NA	9.1%
6 *R. vaventy*						NA

[a] Abbreviation: NA, not applicable.

Antsiranana and Mahajanga provinces, northern Madagascar, by M. Vences, D.R. Vieites, R.D. Randrianiaina, F. Ratsoavina, S. Rasamison, A. Rakotoarison, E. Rajeriarison, and T. Rajoafiarison, on 13 June 2010.

Paratypes.—ZSM 1815/2010 (ZCMV 12382), UADBA-A 60834 (ZCMV 12381), UADBA-A 60835 (ZCMV 12383), UADBA-A 60734 (ZCMV 12455), and ZSM 2859/2010 (DRV 06156), five juvenile or subadult specimens with same collecting data as holotype (except ZCMV 12455, collected 15–20 June 2010).

Referred specimens.—ZCMV 12385 and 12386 (UADBA-A uncatalogued), two specimens with the same collecting data as the holotype, but not examined by the authors.

Diagnosis.—A microhylid frog assigned to the genus *Rhombophryne* on the basis of molecular phylogenetic affinities (see Fig. 1; Table 1; and Perl et al. 2014), as there are no known characters to distinguish between *Rhombophryne* and *Plethodontohyla*. A member of the *R. serratopalpebrosa* group on the basis of the possession of superciliary spines and molecular phylogenetic affinities.

Rhombophryne ornata is characterized by the following combination of characters: TDH 48–61% of ED; two superciliary spines above each eye; a weak or absent supratympanic fold; tibiotarsal articulation reaching between the tympanum and the eye; tibia 38–46% of SVL; fifth toe distinctly shorter than third; reddish coloration on anterior and posterior surface of thighs and the inguinal region; clavicle curving parallel to anterior edge of coracoid; and poorly ossified skeleton including unossified pubis, carpals, centrale, and bone epiphyses. Additionally it is separated from all sequenced Malagasy microhylid frogs including *R. coronata*, *R. vaventy*, and *R. tany* (described below) by uncorrected pairwise genetic divergences of ≥8.4% for COX1 (Perl et al. 2014) and ≥3.9% for 16S.

Rhombophryne ornata may be distinguished from all other *Rhombophryne* species and all *Plethodontohyla* species by the possession of reddish coloration on the anterior and posterior surfaces of the thighs and the inguinal region. Additionally, it may be distinguished from all *Rhombophryne* and *Plethodontohyla* species except *R. serratopalpebrosa*, *R. coronata*, *R. vaventy*, and *R. tany* sp. nov. by the possession of superciliary spines.

Within the *Rhombophryne serratopalpebrosa* group, *R. ornata* may, in addition to its reddish thighs and inguinal region, be distinguished as follows: from *R. vaventy* by much

Table 2.—Some measurements from the type material of *Rhombophryne ornata* and *R. tany*. UADBA animals could not be measured for ED, END, NSD, TDH, TDV, IMTL, or TTA. See Methods for descriptions of character acronyms.

Character[a]	ZSM 1816/2010 *ornata* holotype	ZSM 2859/2010 *ornata* paratype	ZSM 1815/2010 *ornata* paratype	UADBA-A 60834 *ornata* paratype	UADBA-A 60835 *ornata* paratype	UADBA-A 60734 *ornata* paratype	ZSM 1814/2010 *tany* holotype
SVL	33.0	21.7	19.4	18.8	20.5	24.4	24.6
HW	14.6	9.0	7.7	8.8	9.1	11.2	10.6
HL	9.7	6.5	6.1	5.9	6.0	6.9	6.9
ED	3.9	2.9	2.6				2.5
END	2.3	1.8	1.2				1.5
NSD	2.3	1.8	1.6				1.6
NND	3.4	2.1	2.1	3.0	2.7	2.5	2.6
TDH	2.4	1.4	1.2				1.5
TDV	2.4	1.4	1.1				1.4
HAL	8.7	5.1	4.7	4.9	4.6	6.0	5.8
UAL	6.4	3.8	3.3	2.9	3.5	3.1	4.7
LAL	8.3	4.7	3.7	3.4	4.4	4.5	5.0
FORL	23.4	13.6	11.7	11.2	12.5	13.6	15.6
FOL	15.3	9.2	9.1	7.4	9.6	10.8	10.9
TARL	8.4	5.3	4.7	4.5	5.0	6.1	6.3
FOTL	23.6	14.4	13.9	11.9	14.6	16.8	17.2
TIBL	15.1	8.2	8.3	7.4	8.9	10.3	10.5
THIL	15.3	8.9	8.7	8.3	9.6	10.9	10.8
HIL	23.6	14.4	13.9	11.9	14.6	16.8	17.2
IMCL	1.6	1.0	0.9	0.8	0.9	0.9	0.8
IMTL	1.3	1.0	1.1				0.8

[a] Abbreviations: SVL, snout–vent length; HW, maximum head width; HL, head length, from the maxillary commissure to the snout tip; ED, horizontal eye diameter; END, eye–nostril distance; NSD, nostril–snout tip distance; NND, internarial distance;TDH, horizontal tympanum diameter; TDV, vertical tympanum diameter; HAL, hand length, from the metacarpal–radioulnar articulation to the tip of the longest finger; LAL, lower arm length, from the metacarpal–radioulnar articulation to the radioulna–humeral articulation; UAL, upper arm length, from the radioulna–humeral articulation to the trunk; FORL, forelimb length, given by the sum of HAL, LAL, and UAL; FOL, foot length, from the tarsal–metatarsal articulation to the tip of the longest toe; TARL tarsal length, from the tarsal–metatarsal articulation to the tarsal–tibiofibular articulation; FOTL, foot length including tarsus, from the tibiotarsal articulation to the tip of the longest toe; TIBL, tibiofibula length; THIL, thigh length, from the cloaca to the femur–tibiofibular articulation; HIL, hindlimb length, given by the sum of FOL, TARL, TIBL, and THIL; IMCL, maximum length of inner metacarpal tubercle; IMTL, maximum length of the inner metatarsal tubercle.

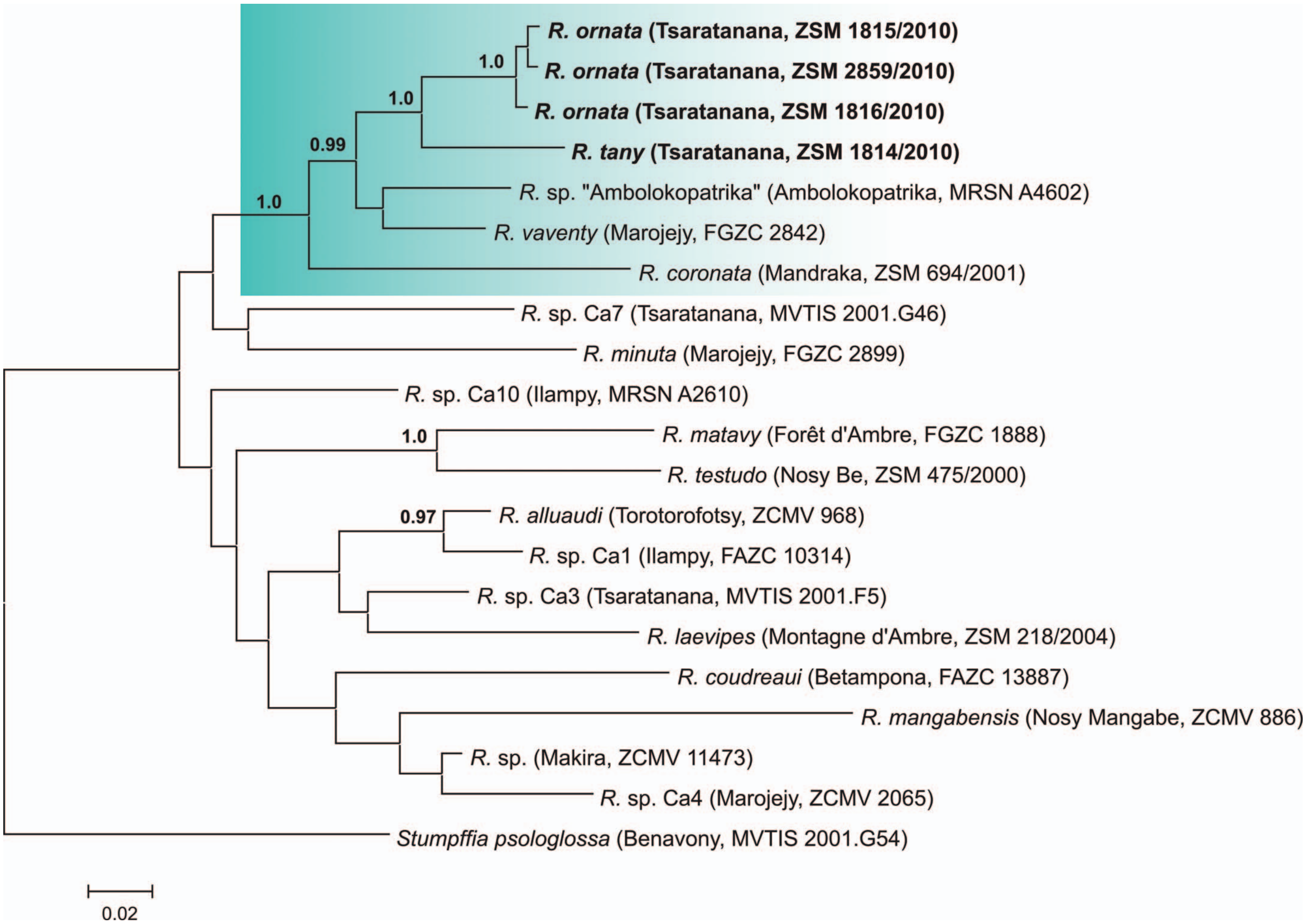

FIG. 1.—Majority-rule consensus tree derived from a Bayesian inference analysis of concatenated cytochrome oxidase subunit I and 16S rRNA sequences (total alignment length 1221 nucleotides). Numbers are posterior probabilities (only shown on nodes with values ≥90%). Samples corresponding to the two new species described herein are set in bold; the shaded area shows the *Rhombophryne serratopalpebrosa* group.

smaller body size (max. known SVL 33.0 mm vs. 52.9 mm), weak or absent supratympanic fold (vs. strong, curved over and behind the tympanum), possession of two distinct superciliary spines (vs. four, the anterior two of which are most distinct), tibiotarsal articulation reaching between the tympanum and the eye (vs. reaching beyond the snout), shorter relative tibia length (TIBL 38–46% of SVL vs. 56%), and medial arm of pterygoid projecting posteromedially (vs. projecting medially perpendicular to the anteroposterior axis); from *R. serratopalpebrosa* by smaller relative tympanum size (TDH 48–61% of ED vs. 78%), weak or absent supratympanic fold (vs. a strong, almost straight supratympanic fold extending to the supraocular region), two distinct superciliary spines (vs. four, the posterior-most of which is indistinct), and broader skull (skull length/width 76.7–79.4% vs. 74.1%); and from *R. coronata* by larger size (SVL up to 33.0 mm vs. 21–23 mm), larger relative tympanum size (TDH/ED 48–61% vs. 51%), and two distinct superciliary spines (vs. three). For distinction from *R. tany* sp. nov., see description of that species below.

Description of the holotype.—Specimen in a good state of preservation. Tissue sample taken from right thigh for genetic sequencing. An incision running around the posterior of the body made to check the sex. Left eye slightly depressed.

Body robust. Head wider than long. Pupil large and round. Snout rounded in dorsal view, and blunt in lateral view. Canthus rostralis slightly concave. Loreal region slightly concave. Nostrils closer to tip of snout than to eyes, directed laterally, slightly protuberant. END smaller than NND. Tympanum indistinct, slightly oval in shape, 61% of eye diameter. A pair of distinct soft superciliary spines above each eye (<1 mm long), clearly visible in the external surface view of the specimen (Fig. S1 in Supplementary Material available online). Indistinct, weak supratympanic fold directly above the tympanum. Approximately 10 distinct vomerine teeth present, five on each side, forming a row on either side of the midline of the head, separated medially by ~1.5 mm. Tongue broad and disc-like, not lobed, posteriorly free.

Arms stout. Fingers without webbing; relative lengths I < II < IV < III; second and fourth fingers almost equal in length. Finger tips not enlarged. Nuptial pads absent; prepollex not externally visible; inner metacarpal tubercle present; outer metacarpal tubercle absent. Hindlimbs slender but strongly built; tibiotarsal articulation reaches between the tympanum and the eye; tibial length 46% of SVL. Small inner metatarsal tubercle present; outer metatarsal tubercle absent. No webbing between toes; relative toe

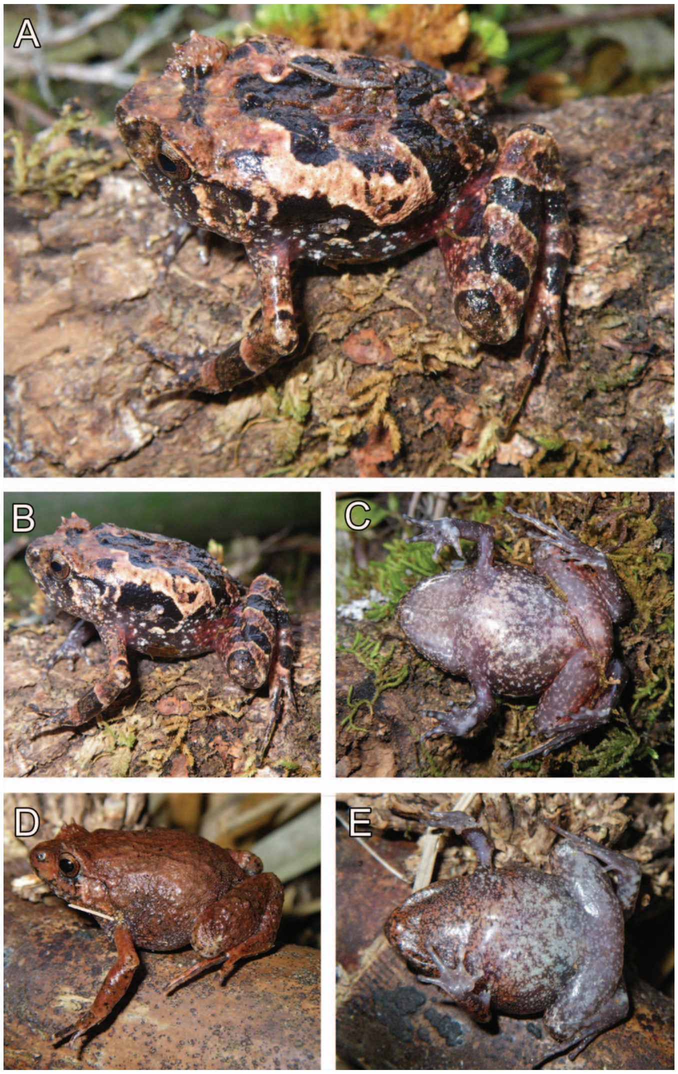

FIG. 2.—Holotypes of *Rhombophryne ornata* (ZSM 1816/2010; A–C) and *R. tany* (ZSM 1814/2010; D,E) in life. A color version of this figure is available online.

lengths I < II < V < III < IV; fifth toe distinctly shorter than third. Dorsal and lateral skin textured in life (see Fig. 2A,B) but smooth in preservative. Glandular formations in the dorsolateral skin (based on micro-CT images) porous in life (see Fig. 2A). Ventral skin smooth. Dorsolateral folds absent.

Coloration.—After 4 yr in preservative, the specimen has a grayish brown base color. The dorsolateral regions are distinctly purplish. Dorsally, two large dark markings are present: between the arms, a large, almost triangular marking, and toward the posterior of the frog a large chevron, extending anteriorly from the inguinal region and converging on the midline. A dark horizontal bar is present between the eyes. Laterally, there is another large dark marking in the axial region, and a small one directly posterodorsal to the tympanum. Venter light brown flecked with cream, fading to cream over the sternum, and then becoming increasingly dark brown again posteriorly. A subtle cream midline extends from the mental region to the sternum. Arms with one dark cross-band just behind the hands. Legs show a series of dark dorsal cross-bands: three on the thighs, two on the shank, and two on the foot. A round dark spot is present on both heels. A strong reddish orange coloration is present on the posterior and anterior of the legs, and in the inguinal region where it meets the chevron, but not extending to the ventral surface of the legs, which are dorsally brown and ventrally flecked with cream.

Color in life similar to in preservative, but with much stronger contrast (Fig. 2A–C). The dull brown base color in preservative is an earthy brown in life, and the purple tinge of the dorsolateral regions is light brown. The red of the inguinal region and anterior and posterior thighs is less orange and much darker, but no less distinct.

Variation.—Intraspecific variation in the 16S region is 0.2–0.4% (Table 1). Paratypes agree well in morphology with the holotype. ZSM 1815/2010 has a less distinct supratympanic fold and an almost indistinguishable tympanum. All paratypes are smaller than the holotype (18–24 mm). Paratypes from which the full suite of measurements could be taken (ZSM 1815/2010 and 2859/2010) had smaller relative tympanum sizes (TDH/ED 48% vs. 68%) and shorter relative tibial lengths (TIBL/SVL 38–43% vs. 46%; Table 2).

All specimens except ZSM 1815/2010 possess the same general color pattern characteristics as the holotype. ZSM 1815/2010 differs strongly in its coloration. The base color in preservative is darker than that of the holotype, and the dark dorsal markings are absent altogether. Instead, this specimen has a large pink marking extending from in front of the eyes over them, narrowing to become a midline that terminates at the vent. Gray markings extend up from the inguinal region to the midline at almost the midpoint of the specimen. The legs have strong cross-bands, but there are just two on the thigh, one on the shank, and one on the foot. The heel spot and the single cross-band on the arms are shared with the holotype, as is the reddish orange coloration of the posterior and anterior thighs and inguinal region (although this coloration extends onto the ventral surface of the leg, where it dominates). The ventral color is cream with dark flecks. A light midline runs from the mental region to the sternum, beyond which it fuses with the cream of the trunk.

The two micro-CT scanned paratypes (ZSM 1815/2010 and 2859/2010) are practically identical with the holotype in all aspects of their osteology, including the lack of ossification among their small bones and long bone epiphyses, except the order of their relative transverse process widths, which varies somewhat. These are likely influenced, however, by the extent of ossification of the skeleton.

Etymology.—The specific epithet is the Latin adjective *ornata*, meaning ornate or decorated, and refers to the ornate color pattern of the dorsum of this frog, especially in comparison to its congeners.

Natural history.—All specimens were collected in the cool and dry season of Madagascar, during the day, hidden under logs and stones in the dried-up headwater spring of a small stream that drains part of the Matsabory Maiky plateau, running through rainforest. Along with the *Rhombophryne* specimens, numerous individuals of an undescribed species of *Stumpffia* were also collected.

Stomach contents of ZSM 1816/2010 consisted of a more or less digested frog (suspected family Mantellidae) with an estimated SVL of 9 to 10 mm (its skeleton partly visible in Fig. S1), a small piece of plant material, and a minute stone. The colon contained remains of insects and a nematode worm (5 mm long).

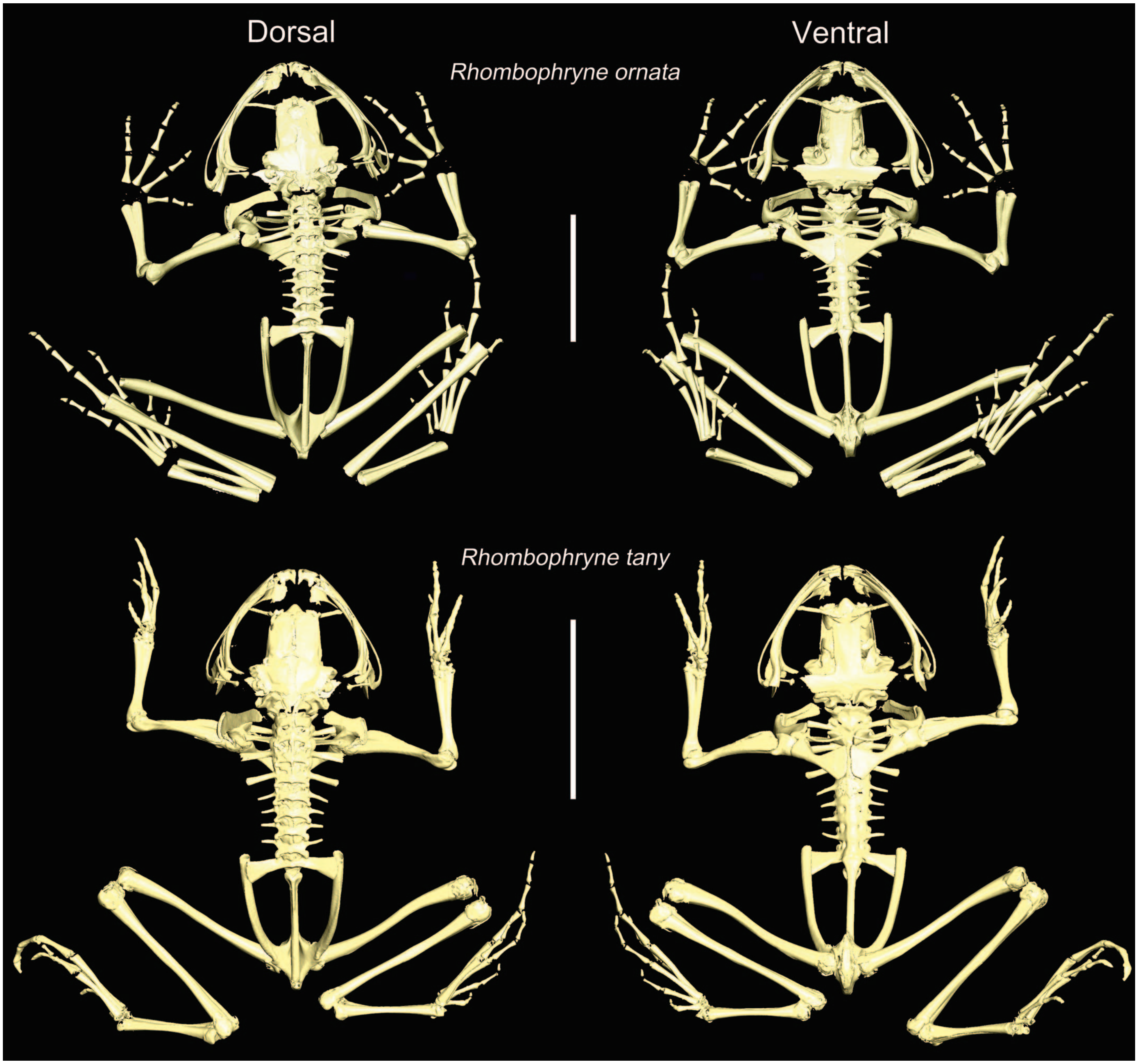

FIG. 3.—Skeletons (derived from micro-CT scans) of *Rhombophryne ornata* (holotype, ZSM 1816/2010) and *R. tany* (holotype ZSM 1814/2010) in dorsal and ventral view. Scale bars indicate 10 mm. PDF-embedded 3D models of the skeletons of these specimens, and two additional paratypes of *R. ornata*, are available in the Supplementary Material online.

Suggestion for International Union for Conservation of Nature (IUCN) Red List status.—So far this species has only been found from a single location, albeit from a relatively poorly surveyed part of Madagascar (Andreone et al. 2009). The surface area of the forested portions of the Tsaratanana Massif and contiguous forests is ~4300 km^2 (calculated in Google Earth® Pro v6.1.0, Google Inc., Mountain View, CA). The extent of occurrence (EOO) of this species, given by a minimum convex polygon drawn around this forested area is ~6640 km^2. This species is likely restricted to a limited altitude range and particular microhabitats, but we cannot accommodate these into a Red List assessment without more distributional and ecological data. Although parts of the Tsaratanana Massif are protected as a Strict Nature Reserve, and areas to the east are included in the Corridor Marojejy Tsaratanana New Protected Area (NPA), deforestation continues to affect these forests (Andreone et al. 2005b, 2009). Indeed, the type locality is 2.6 km from the edge of the forest. Currently available data regarding the exact boundaries of these reserves is to some extent ambiguous, but according to information obtained from local staff of Madagascar National Parks, the type locality of *R. ornata* is located within Tsaratanana Strict Nature Reserve.

In summary, the new species has an EOO $< 20{,}000$ km^2, it is known from a single location, and the condition and extent of its habitat are continuing to decline. It therefore

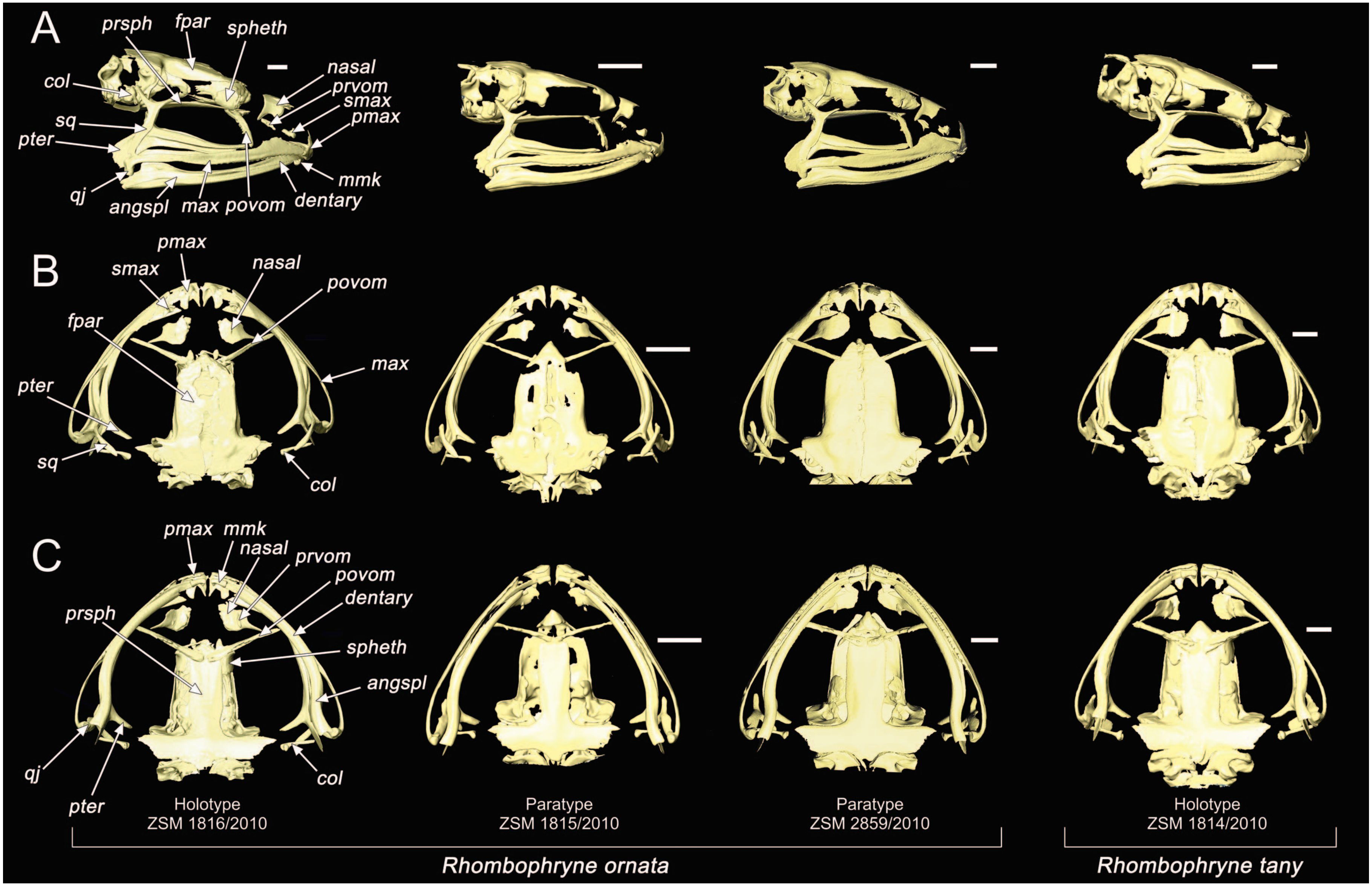

Fig. 4.—Comparative skull osteology of *Rhombophryne ornata* (holotype ZSM 1816/2010 and paratypes ZSM 1815/2010 and ZSM 2896/2010) and *R. tany* (holotype ZSM 1814/2010), in (A) lateral view, (B) dorsal view, and (C) ventral view. Scale bars indicate 1 mm. Abbreviations: angspl = angulosplenial, col = columella, fpar = frontoparietal, max = maxillary, mmk = mentomeckelian bone, pmax = premaxilla, prsph = parasphenoid, pter = pterygoid, povom = postchoanal portion of vomer, prvom = prechoanal portion of vomer, qj = quadratojugal, spheth = sphenethmoid, smax = septomaxilla, sq = squamosal. PDF-embedded 3D models of the skeletons are available in the Supplementary Material online.

qualifies to be listed as Vulnerable B1ab(iii) under the IUCN Red List Criteria (IUCN 2012).

Remarks.—This species was listed in the genetic study of Perl et al. (2014) as *Rhombophryne* sp. Ca11. A further specimen from Tsaratanana, photographed by A. Raselimanana, was shown by Glaw and Vences (2007:118, upper small photo) with uncertain identity.

Rhombophryne ***tany*** sp. nov.

(Figs. 2D,E, 3, 4; Online Supplementary Fig. S4)

Holotype.—ZSM 1814/2010 (field number ZCMV 12359), male, collected on the Tsaratanana Massif, Camp 2 (Matsabory Maiky), 14.15256°S, 48.95728°E, 2021 m asl, on the border between the Antsiranana and Mahajanga provinces, northern Madagascar, by M. Vences, D. Vieites, R.D. Randrianiaina, F. Ratsoavina, S. Rasamison, A. Rakotoarison, E. Rajeriarison, and T. Rajoafiarison, on 13 June 2010.

Diagnosis.—A microhylid frog assigned to the genus *Rhombophryne* based on molecular phylogenetic affinities (see Perl et al. 2014), on account of the current absence of diagnostic characters to distinguish between *Rhombophryne* and *Plethodontohyla*. A member of the *R. serratopalpebrosa* group on the basis of the possession of superciliary spines and molecular phylogenetic affinities (Fig. 1).

Rhombophryne tany is characterized by the following combination of characters: TDH 60% of the ED; two superciliary spines above each eye; a supratympanic fold extending above and behind the tympanum down to the axillary pit; tibiotarsal articulation reaching between the tympanum and the eye; tibia 43% of SVL; second and fourth finger equal in length; fifth toe distinctly shorter than third; finely granular dorsal and ventral skin; and an unossified pubis. It is further distinguished from all sequenced Malagasy microhylid frogs including *R. coronata*, *R. vaventy*, and *R. ornata* by a pairwise genetic divergence of ≥8.4% for COX1 (Perl et al. 2014) and ≥3.9% for 16S.

Rhombophryne tany is distinguished from all *Plethodontohyla* species and all other *Rhombophryne* species except members of the *R. serratopalpebrosa* group by the possession of superciliary spines. Among the members of the *R. serratopalpebrosa* group, *R. tany* may be distinguished from all other species except *R. ornata* by the possession of two superciliary spines (vs. three or four), and an unossified pubis. Furthermore, it may be distinguished from *R. vaventy* by its smaller size (max. known SVL 24.6 mm vs. 52.9 mm), larger relative tympanum size (TDH 60% of ED vs. 41%), tibiotarsal articulation reaching between the tympanum and the eye (vs. extending beyond the snout), smaller relative tibia length (TIBL 43% of SVL, vs. 56%), plain brown dorsal coloration with speckled legs (vs. flecks of darker coloring, with leg cross-bands), and finely granular dorsal skin (vs. rough and tubercular); from *R. serratopalpebrosa* by its smaller relative tympanum size (TDH 60% of ED vs. 78%), supratympanic fold not extending anterior to the tympanum (vs. extending to the supraocular region), and fifth toe distinctly longer than third (vs. fifth toe of roughly equal size to third); from *R. coronata* by possession of a supratympanic fold above and behind the tympanum extending down to the axillary pit (vs. indistinct supratympanic fold), shorter and broader head (HL/HW 1.5 vs. 1.1–1.2), longer relative leg length (HIL/SVL 1.6 vs. 1.3), and absence of an hourglass-shaped marking on the dorsum (vs. presence); and from *R. ornata* by its smaller size (male SVL 24.6 mm vs. 33.0 mm), supratympanic fold above and behind the tympanum extending down to the axillary pit (vs. weak or absent supratympanic fold), absence of red pigment in the inguinal region (vs. presence), ossified bone epiphyses and carpals (vs. poorly ossified; cf. Figs S1–3 and S4 of the Supplementary Material), pterygoid in broad anterolateral contact with the maxilla (vs. almost without contact to maxilla), otic ramus of squamosal dorsoventrally flattened (vs. slim), and clavicle not parallel to anterior edge of coracoid (vs. parallel).

Description of the holotype.—Specimen in a good state of preservation. A tissue sample was taken from the right thigh for genetic sequencing. An incision running around the posterior of the body was made to check the sex.

Body robust. In life (Fig. 2D,E), possessing a weakly raised middorsal ridge beginning between the nares. Head wider than long. Pupils round. Snout rounded in dorsal view, blunt in lateral view. Canthus rostralis slightly concave. Loreal region concave. Nostrils closer to tip of snout than to eyes, directed laterally, slightly protuberant. END longer than NND. Tympanum distinct, round, 60% of ED. A pair of distinct soft superciliary spines above each eye (<1 mm; see Fig. S4 of the Supplementary Material). A supratympanic fold runs over and behind the tympanum, down to the axillary pit. Vomerine teeth small, medially separated by a small gap (~1 mm). Tongue broad and disc-like, attached anteriorly for half of its length.

Arms stout. Fingers without webbing; relative lengths I < II = IV < III. Finger tips not enlarged. Nuptial pads absent; inner metacarpal tubercle present; outer metacarpal tubercle absent. Hindlimbs strongly built. Tibiotarsal articulation reaches between the tympanum and the eye; TIBL 43% of SVL. Inner metatarsal tubercle present; outer metatarsal tubercle absent. Toes with weak traces of webbing; relative toe lengths I < II < V < III < IV; fifth toe distinctly shorter than third. Dorsal and ventral skin finely granular. Dorsolateral folds absent.

Coloration.—After 4 yr in alcohol, the specimen is uniformly brown in color. The sides of the head are lighter in color than the dorsum, with a few fine dark spots beneath the eyes. A light marking is present on the anterior edge of the tympanum. Venter yellowish cream, with fine dark speckles beneath the chin that become less dense posteriorly. Arms lighter brown than dorsum with a few dark speckles and a hint of a single cross-band on the lower arm. Dorsal surface of legs slightly paler brown than the dorsum, and the lower leg has small dark speckles. Inguinal region pale with brown vermiculations. Ventral surface of thighs cream.

Dorsal color in life similar to that in preservative, but a richer brown (Fig. 2D,E). The ventral surface is anteriorly brown with dark flecks, becoming cream posteriorly.

Etymology.—The specific epithet, *tany*, is a Malagasy word meaning “earth,” “soil,” or “land,” and refers both to the earthy color and the terrestrial habit of this species. It is used as a noun in apposition.

Natural history.—The single specimen was collected in the dry and cool season, during the day, moving along a recently set-up pitfall line. The specimen was found in dense bamboo forest that is typical for much of the Matsabory Maiky area (Rakotoarison et al. 2012).

Suggestion for IUCN Red List status.—We recommend this species be listed as Vulnerable B1ab(iii), for the same reasons given above for *R. ornata*.

Remarks.—This species was listed in the genetic study of Perl et al. (2014) as *Rhombophryne* sp. Ca12. A specimen from Tsaratanana possibly assignable to this species was photographed by A. Raselimanana and shown by Glaw and Vences (2007:118, lower small photo) with uncertain identity.

OSTEOLOGY

We describe here the osteology of both *R. ornata* and *R. tany* simultaneously to allow for better comparisons. All descriptions pertain to both species except where explicitly stated otherwise. For *R. ornata*, intraspecific variation was assessed by scanning, in addition to the holotype ZSM 1816/2010, the paratypes ZSM 1816/2010 and ZSM 2859/2010. In general they agree strongly with the osteology of the holotype. PDF-embedded 3D models of *R. ornata* and *R. tany* are depicted in Figs. S1–3 and S4, respectively, of the Supplementary Material.

Skull.—Fig 4. Vomer paired, divided into pre- and postchoanal portions; prechoanal part small, longer than broad, triradiate in *R. ornata* with a thin lateral ramus extending from the middle of its lateral edge, lacking this ramus in *R. tany*; postchoanal part overlapping neopalatine, bearing ventral serrations (vomerine teeth). Neopalatine + postchoanal vomer in contact with parasphenoid dorsally, and through it variably with the sphenethmoid; not in lateral contact with the maxilla. Teeth present on the maxilla and premaxilla. Premaxilla paired, medially unfused, anterodorsal alary processes rising in parallel (*R. ornata* ZSM 1816/2010 and 1815/2010) or weakly diverging from the midline (*R. ornata* ZSM 2859/2010, *R. tany* ZSM 1814/2010), pars palatina with two well-defined processes, the medial (palatine) process thin, the lateral process thicker, the pars dentalis bearing teeth. Septomaxilla spiraled, medial ramus extending posterodorsal to the posterior ramus and parallel to the lateral ramus, anterior ramus thick, possessing ventral and dorsal rami toward its lateral edge, the dorsal ramus particularly strong in *R. ornata*, lateral ramus not as thick as anterior ramus, narrowing to a point toward its posterior end, posterior ramus extending from the middle of the lateral ramus ventromedially, broad and dorsoventrally flattened in *R. ornata*; tapering rapidly in *R. tany* (Figs. S1–4 of the Supplementary Material). Nasals paired, isolated, not approaching one another medially, situated directly dorsal to prechoanal portion of vomer, possessing a pointed posterolateral maxillary process extending ventrolaterally toward the maxilla. Maxilla long, bearing teeth, possessing a horizontal pars palatina along its lingual margin, in broad lateral contact with ptyergoid in *R. tany* but only approached by the pterygoid in *R. ornata*; posteriorly fused to quadratojugal. Pterygoid broad and triradiate with anterior, medial, and posterior rami, the lateral face of its anterior ramus and posterior face of its medial ramus strongly sculpted; medial ramus much shorter than posterior ramus. Quadratojugal L-shaped, anterior process without clear distinction from posterior of maxilla, possessing a bulbous posteroventral process, dorsally with a thin connection to the squamosal in *R. ornata* and broad connection with the squamosal in *R. tany*. Squamosal dorsally bifurcated, broad and flattened, broader in *R. tany* than in *R. ornata*, extending anterodorsomedially from quadratojugal to level of the otic capsule, passing anterior to columella; otic (posterodorsal) ramus slim in *R. ornata*, broad and dorsoventrally flattened in *R. tany*, longer than zygomatic (anterodorsal) ramus. Columella with a long stem and curved footplate in *R. tany*; a somewhat bilobed curved footplate in *R. ornata* (Figs. S1–S4 of the Supplementary Material) subparallel and curved ventrally to form the dorsolateral border of the braincase, their posterolateral contact with prootics and exoccipitals not clear from micro-CT scans; anterior contact of frontoparietals and sphenethmoid obscured by mineralization or fused. Parasphenoid T-shaped, almost not in contact with any other bones in *R. ornata*, anteriorly in broad contact with sphenethmoid and neopalatine + vomer in *R. tany* (possibly fused, but obscured by mineralization); parasphenoid alae perpendicular to cultriform process, in weak contact to the prootics or exoccipitals in both species, their posterior edge straight but for an emarginated posterior medial process.

Mandible slim, without teeth. Mentomeckelian bones paired, small, not in medial contact, anterolaterally fused to the anterior process of the dentary. Dentary long and thin, overlapping angulosplenial for much of its length. Angulosplenial with a sculpted lateral surface, broadening posteriorly, with an enlarged coronoid process.

Postcranial skeleton.—In *R. ornata*, considerable parts of the postcranial skeleton are unossified, including the epiphyses of the femur, tibia, tarsals, carpal bones except the phalanges and metacarpals, and the pubis (see Figs. 3 and S1–3). In consequence, most articulations are not visible in the scans.

Posterolateral processes of the hyoid shovel-like, a medial crista running along the posteromedial process, the base of which is broad and flat with a rounded anteromedial edge and sharp anterolateral and posteromedial corners; parahyoid absent.

Humerus broad, ventral and lateral cristae present, medial crista absent, crista ventralis broadening proximally from midpoint, ending abruptly before reaching the unossified caput humeri in *R. ornata*, extending to the caput humeri in *R. tany*. Radioulna broad. Carpals unossified in *R. ornata*, well ossified in *R. tany*. Finger phalangeal formula 2–2–3–3. Terminal phalanges of Fingers III and IV with small distal knobs in *R. ornata*; fingers without distal knobs in *R. tany*. Prepollex not visible.

Omosternum not visible (possibly absent). Sternum unossified. Pectoral girdle composed of paired coracoids, clavicles, scapulae, cleithra, and suprascapulae. Coracoids broadly separated in *R. ornata*, approximating one another in *R. tany*, medially dorsoventrally flattened, laterally dorsoventrally thickened with a flattened cap; anterior and posterior edges curved inward, anteriorly more strongly than posteriorly, more strongly in *R. tany* than in *R. ornata*. Clavicle thin and curved, parallel to anterior edge of coracoid in *R. ornata*, not parallel in *R. tany*; lateral surface curved parallel to anterior medioventral scapular surface, in *R. tany* making contact with coracoid at its junction with the scapula. Scapula curved, hourglass-shaped, medioventrally bifurcated; pars acromialis distally rounded, approaching lateral end of clavicle, its anterior surface indented; pars glenoidalis curved ventrally, approaching lateral face of coracoid; dorsolateral end broad. Cleithrum thin and long, anteriorly thicker, thinning posteriorly; somewhat broader at the scapular border of the suprascapula. Suprascapula unossified in *R. ornata*, weakly ossified ventrally and posteriorly in *R. tany*.

Toe phalangeal formula 2–2–3–4–3; terminal phalanges without distal knobs. Femur and tibiofibula approximately equal in length in *R. ornata*, tibiofibula slightly longer than femur in *R. tany*. Fusion of tibiale and fibulare in *R. ornata* is not assessable because their epiphyses are unossified; they are proximally and distally fused in *R. tany*, although their epiphyses are weakly ossified in this species.

Pelvic girdle consisting of paired ilia, ischia, and pubes. Iliac shafts passing ventral to and extending beyond the sacrum to a variable degree; almost cylindrical, with a dorsal crest extending nearly their full length, possessing a strong ridge dorsolateral to the acetabulum. Ilia posteriorly fused synchondrotically with the ischium in *R. ornata*, synostotically with ischium in *R. tany*; pubis unossified in both species. Iliosacral articulation type IIA sensu Emerson (1979).

Eight presacrals present. Posterior articular processes round. Atlas (Presacral I) not fused to Presacral II. Transverse processes of Presacrals II–IV thicker and broader than V–VIII, though their widths are variable because of the extent of ossification and thresholds in both *R. ornata* and *R. tany*. Neural spines weak. Sacrum wide, with broad diapophyses articulating with the ilia; anterior edge straight, posterior edge strongly curved. Urostyle with a dorsal ridge along a third of its length, beginning at its anterior end; with a bicondylar sacral articulation.

DISCUSSION

With the two new species described here, we have brought the total diversity of the *R. serratopalpebrosa* group to five nominal species, representing nearly 40% of the total named diversity of the genus *Rhombophryne*. We have so far extracted three species from the *R. serratopalpebrosa* complex (*R. vaventy* being the first), but examination of additional specimens and photographs leads us to conclude that several more still remain to be described. This group is highly diverse relative to the rest of the genus (Fig. 1), although numerous species of other affinities are still awaiting description.

Conclusions on the adaptive potential of the *R. serratopalpebrosa* group and investigations into its evolutionary history should be viewed as tentative without further taxonomic resolution and additional fieldwork. Nevertheless, we observed that the *R. serratopalpebrosa* group follows the typical pattern of endemism seen among several groups of cophyline microhylids (Wollenberg et al. 2008): the greatest diversity appears concentrated in the north, with the two new species described here being from the Tsaratanana Massif, and *R. vaventy* and *R. serratopalpebrosa* being from Marojejy, while just one species, *R. coronata*, is currently known from farther south, in the eastern rainforest between Mandraka and Andasibe (Vences and Glaw 2003). No species belonging to this group are known from the Manongarivo Massif to the west of the Tsaratanana Massif (Rakotomalala 2002), but this area has a similar herpetofaunal community composition to Tsaratanana (Andreone et al. 2009), and additional species of the *R. serratopalpebrosa* group might occur there.

The Tsaratanana Massif is thought to harbor one of the highest levels of local endemism in all of Madagascar (Raxworthy and Nussbaum 1996; Andreone et al. 2009), and was identified by Andreone et al. (2005b) as one of the areas of Madagascar with the highest extinction risk for frogs. Despite its status as a Complete Natural Reserve (IUCN Category I park), the forests of the massif, especially at its edges, are still threatened by habitat alteration and possibly by climate change (Raxworthy et al. 2008; Rakotoarison et al. 2012). As they have only been found in a single location in high-altitude rainforest on the Tsaratanana Massif, *R. ornata* and *R. tany* may face considerable threats, like other microhylids from the area (Rakotoarison et al. 2012). Because of their limited maximum EOO, and the threat of habitat alteration, emphasis should be placed on gathering data on their distribution and habitat requirements.

Rhombophryne now consists of 14 described species and at least 10 unnamed candidates. We suspect, however, that a total of 24 species is an underestimate of the diversity of this clade, because of the secretive, fossorial habits of most species. This can be surmised from what little we know of their ecology: Male calls consist of simple notes (Vences and Glaw 2003; D'Cruze et al. 2010; Glaw et al. 2010) that are often made during rainfall when they are difficult to triangulate. Calling is typically from within the leaf litter or at the mouth of a burrow, into which the frog can easily disappear upon detection. The burrows are also not associated with water, and the frogs are therefore not generally detected by riparian surveys (Vences et al. 2008). Finally, DNA barcoding studies on tadpoles are increasingly revealing new taxa (Vences et al. 2008; Randrianiaina et al. 2011), but the Cophylinae have nidicolous endotrophic (i.e., nonfeeding) tadpoles, which are raised within their burrows and not in streams where such tadpole studies might discover them (Glaw and Vences 2003; Andreone et al. 2005a; Vences et al. 2008). In short, their lifestyle makes them difficult animals to detect and study, and many are likely to have been overlooked.

Three micro-CT scanned specimens of *R. ornata* lacked ossification in the carpals, pubis, and epiphyses of the limb bones (see Figs. 3 and S1–3 of the Supplementary Material). We attempted a variety of different scanning settings with little success in resolving these features; their X-ray absorption is indistinguishable from that of the skin and flesh of the frogs. Although our sample size is small, this condition has not been observed in any other *Rhombophryne* (14 specimens from eight other species and candidate species), and we suspect it to be a characteristic, and not an artifact, of this species. It does not appear to be associated with developmental stage in these frogs, as it was observed from one adult, one subadult, and one juvenile. Interestingly, the closely related *R. tany*, collected with *R. ornata* at the same time, at the same locality, and fixed and preserved under identical conditions, has well-ossified carpals and epiphyses of the limb bones. However, *R. tany* also possesses an unossified pubis (Fig. S4 of the Supplementary Material) except *R. ornata*. More data are needed to assess the evolutionary implications of these observations.

Although we have begun to resolve the taxonomy of the *Rhombophryne serratopalpebrosa* group, aided by the implementation of data from micro-CT scans, our knowledge of the ecology of this apparently diverse and fairly widespread clade has remained practically nonexistent; of the entire group, calls are only known from *R. coronata*, and we know little of the breeding habit of any species. Our micro-CT scans have identified unique features of their skeletons that we cannot interpret without ecological information. Clearly, much more of the biology of cophyline frogs remains to be described.

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Supplementary Material

Supplementary material associated with this article can be found online at http://dx.doi.org/10.1655/Herpetologica-D-14-00048.S1

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