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# Genetic variability and partial integrative revision of *Platypelis* frogs (Microhylidae) with red flash marks from eastern Madagascar

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## Abstract

We studied the genetic variability of *Platypelis* species (Anura: Microhylidae) with red flash marks from Madagascar based on mitochondrial (16S rRNA) and nuclear (RAG1) genes. Our molecular phylogenetic results suggest that the red colour evolved independently in the *Platypelis barbouri* complex and *P. milloti* and confirm the validity of a long-known but still undescribed new species from eastern Madagascar. *Platypelis ranjomena* sp. nov. is distinctly coloured with dark red to purple patches at the base of the forelimbs, in the inguinal region, and on the ventral parts of the hind limbs. It differs from most other arboreal cophylines by this red colour and from its sister species *P. barbouri* by smooth dorsal skin texture, iris colour, bioacoustics (much longer note duration in advertisement calls), and genetics (strong differences in mitochondrial and nuclear markers). The new species is widespread at low elevations along the east coast from Marojejy in the north to Manombo in the south. However, genetic evidence indicates substantial intraspecific variability among populations, suggesting that the taxonomy of *P. ranjomena* and the other species in this complex is still incompletely resolved. An individual of *P. barbouri* from Mahasoa shared a nuclear allele with *P. ranjomena*, and its call was to some degree intermediate between these species, suggesting a possible case of hybridization in an area that we hypothesize could be a contact zone between the two species. Individuals from Madagascar's northeast hitherto assigned to *P. barbouri* represent a lineage that is sister to the clade of *P. barbouri* + *P. ranjomena*. It is herein identified as a new candidate species (*Platypelis* sp. Ca11), which occurs in syntopy with *P. ranjomena*.

## Key words

Amphibia; Anura; colouration; Cophylinae; Madagascar; molecular genetics; new species; *Platypelis ranjomena*; systematics.

## Introduction

Within the microhylid subfamily Cophylinae, which is endemic to Madagascar, two major ecological groups can be distinguished: arboreal species with expanded fingertips and non-arboreal species without expanded fingertips (Andreone *et al*., 2005; Wollenberg *et al*., 2008). Neither of these two ecogroups is monophyletic (Scherz *et al*., 2016; Tu *et al*., 2018), and molecular evidence indicates repeated instances of ecological transition (Andreone *et al*., 2005; Scherz *et al*., 2019a). Arboreal cophylines include the genera *Anodonthyla*, *Cophyla*, *Platypelis* and a few species of the genus *Plethodontohyla*; a few species of *Stumpffia* and *Anilany* are rupicolous or scansorial, and also have expanded toe tips. Among arboreal microhylids in Madagascar, *Anodonthyla* are relatively

well characterized by the presence of a distinct prepollex in males and the absence of vomerine teeth, and some *Plethodontohyla* have a distinctive body shape with a strongly pronounced canthus rostralis and dorsolateral colour border. The non-genetic distinction of *Cophyla* and *Platypelis* is less clear; it has been debated controversially (see Peloso *et al*., 2016, 2017; Scherz *et al*., 2016, 2017; Tu *et al*., 2018), and the two genera remain separated mainly based on osteological characters (Guibé 1978; Blommers-Schlösser & Blanc, 1991; Rakotoarison *et al*., 2015; Scherz *et al*., 2016). These two genera are, however, diverse, with a total of 20 described species plus several recognised but undescribed candidate species, and represent the greatest portion of arboreal microhylids in Madagascar.

An increasing portion of newly discovered reptile and amphibian species cannot be distinguished from their closest relatives by simple morphological characters and require detailed integrative studies, using different and independent lines of evidence, to resolve their taxonomy (e.g. Padial *et al*., 2010; Miralles *et al*., 2011; Hawlitschek *et al*., 2012). For 25 years there has been growing evidence that the *Platypelis barbouri* complex – small arboreal frogs with reddish areas on their arms, inguinal regions, and legs – might include more than one species (e.g. Glaw & Vences, 2007; Vieites *et al*., 2009), but previous unpublished morphological and genetic data led to ambiguous results and were considered insufficient to clarify their taxonomy. In the meantime new individuals have been collected and their integrative study now reveals a more complex, but largely resolved picture for this group of frogs. In this paper, we study the genetic variability of *Platypelis* species with rather sharply delimited, bright orange or red markings on the posterodorsal and ventral thigh, shank, and posterior venter, which we here refer to as flash marks (following and slightly expanding the definition of this character by Vences *et al*., 1999), and provide a partial revision based on an integrative species delimitation. We also describe a new species, which was first collected by us in 1995, and subsequently discovered in a number of other locations across eastern Madagascar.

## Materials and Methods

Specimens were euthanised using chlorobutanol or MS222 solutions, fixed in concentrated ethanol and preserved in 70% ethanol. Morphological measurements were taken to the nearest 0.1 mm by MV with calipers. Abbreviations used are as follows: SVL, snout–vent length; HW, head width at widest point; HL, head length, measured as the diagonal from the rictus to the anterior-most point of the head; TD, horizontal tympanum diameter; ED, horizontal eye diameter; END, eye–nostril distance (anterior corner of eye to centre of nostril); NSD, nostril–snout tip distance (centre of nostril); NND, nostril–nostril distance (from the centres of the nostrils); FORL, forelimb length, measured from the axilla to the tip of the longest (third) finger with the forelimb extended; HAL, hand length, measured from the base of the hand to the tip of the third finger; HIL, hind limb length, measured from the cloaca to the tip of the longest (fourth) toe with the foot extended laterally outward from the body; FOTL, foot and tarsus length, measured from the back face of the tibiotarsal articulation to the tip of the longest toe; FOL, foot length, measured from the back face of the tarsal-metatarsal articulation to the tip of the longest toe; TIBL, tibia length, from the back face of the tibiotarsal articulation to the knee. The webbing formula is given according to Blommers-Schlösser (1979).

Institutional abbreviations: MRSN (Museo Regionale di Scienze Naturali, Torino), UADBA (Université d'Antananarivo, Mention Zoologie et Biodiversité Animale, Antananarivo), ZFMK (Zoologisches Forschungsmuseum Alexander Koenig, Bonn), and ZSM (Zoologische Staatssammlung, München). Acronyms of field numbers are ACZCV and ACP (Angelica Crottini), APR (Achille P. Raselimanana), CRH (Carl R. Hutter), DLR (Dina Lydie Ramamonjisoa), FAZC (Franco Andreone), FGZC (Frank Glaw), MSTIS (Mark D. Scherz), RAN (Ronald A. Nussbaum), and ZCMV (Miguel Vences).

Vocalizations were recorded using a Tensai RCR-3222 portable tape recorder with external Vivanco EM 238 microphone or a Tascam DR-07 digital recorder with built-in microphone (Ambodivoangy) and saved as uncompressed files at a sampling rate of 44.1 kHz. Recordings were digitized/re-sampled at 22.05 kHz and 32-bit resolution and computer-analysed using Adobe Audition 1.5. Frequency information was obtained through Fast Fourier Transformation (FFT; width 1024 points). Spectrograms were obtained with Hanning window function with 256 bands resolution. Temporal and spectral measurements are given as range with mean ± standard deviation in parentheses. Terminology in call descriptions follows the call-centred approach of Köhler *et al*. (2017).

We excised muscle tissue samples from the euthanised animals before fixation, and preserved them separately in 99% ethanol. Genomic DNA was extracted using a standard salt extraction protocol (Bruford *et al*., 1992), and a segment of the 5' end of the mitochondrial 16S rRNA gene (16S) amplified with primers 16SL3 (AGCAAAGAHYWWACCTCGTACCTTTTGCAT) and 16SAH (ATGTTTTTGATAAACAGGCG) from Vences *et al*. (2003) using the following PCR protocol: 90 s at 94 °C followed by 33 cycles of 45 s at 94 °C, 45 s at 52 °C, 90 s at 72 °C, and a final extension step of 300 s at 72 °C. Furthermore, for a selection of specimens, a segment of the nuclear recombination-activating gene 1 (RAG1) was amplified with primers Rag1_Coph_F1 (CGTGATCGGGTAAAAGGTGT) and Rag1_Coph_R1 (TCGATGATCTCTGGAACGTG) from Rakotoarison *et al*. (2015), with 120 s at 94 °C followed by 35 cycles of 20 s at 94 °C, 50 s at 53 °C, 180 s at 72 °C, and a final extension step of 600 s at 72 °C. PCR products were purified with 0.15 units of Shrimp Alkaline Phosphatase

(SAP) together with 1 unit of Exonuclease I (New England Biolabs, Frankfurt am Main, Germany), incubating first for 15 min at 37 °C and subsequently for 15 min at 80 °C. PCR products were sequenced on automated DNA sequencers at LGC Genomics (Berlin), and chromatograms were checked with CodonCode Aligner 3.7.1 (Codon Code Corporation, Dedham, MA, USA). Newly obtained sequences were submitted to GenBank (accession numbers MT196007–MT196029 and MT209906–MT209929).

Sequences were combined with those available from previous studies, and alignment performed using the ClustalW algorithm in MEGA7 (Kumar *et al*., 2016). For analysis, three different sets of molecular data were used: (1) An alignment of all available sequences of the 5' segment of the 16S gene for the focal group, combined with representative sequences of other species of *Platypelis* and *Cophyla*. (2) A concatenated alignment of the 5' segment and the 3' segment of the 16S rRNA gene, to include multiple samples of the focal taxa that had been previously sequenced only for the 3' segment of the gene. However, due to the very uneven distribution of missing data in this alignment (only 3' segment available for some and only 5' segment available for other samples), we consider this tree as less reliable. (3) An alignment of RAG1 sequences that were analysed separately from the mitochondrial DNA to obtain evidence from unlinked loci (mitochondrial versus nuclear) for genetic differentiation of lineages, adding further support to their status as distinct species, under the genealogical concordance species criterion (Avise & Ball, 1990).

For the two 16S data sets (5' segment only, and concatenated 3' and 5' segments) we calculated maximum likelihood (ML) trees under substitution models determined by model testing in MEGA7 based on the Akaike Information Criterion (TN93+G and GTR+G, respectively). Pairwise distances between sequences (uncorrected p-distances) were calculated in MEGA7. For the RAG1 data set, we inferred alleles (haplotypes) using the PHASE algorithm (Stephens *et al*., 2001) in DnaSP (version 5.10.3; Librado & Rozas, 2009). We then constructed a ML tree from the phased and unpartitioned RAG1 sequences using the Jukes-Cantor substitution model in MEGA7 (Kumar *et al*., 2016), 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).

## Results

### Molecular phylogenetics

The ML phylogenetic trees built based on the mitochondrial 16S rRNA gene (658 and 1096 bp alignment length, respectively; Fig. 1 and Suppl. Fig. S1) in the current study mainly serve as a visual representation of genetic divergences among lineages, and a means to assign samples to main mitochondrial lineages. Inter-species relationships in these phylogenies will need to be reinforced in future phylogenomic studies. Our analyses placed the focal lineages with red flash marks in a monophyletic group together with *P. pollicaris*, a species that lacks red pigmentation. Within this group, and leaving aside the morphologically distinct *P. pollicaris*, three main lineages could be distinguished (see phylogenetic tree in Fig. 1A and Fig. 2 for the geographical distribution of the clades): (i) one lineage containing samples assigned to *Platypelis barbouri* based on the current morphological definition of this species (small-sized, rugose skin, mostly from mid-elevation localities); (ii) a second lineage containing samples assignable to the usually large-sized new species *P. ranjomena* described below (defined as candidate species *P.* sp. Ca5 by Vieites *et al*., 2009 and Perl *et al*., 2014), occurring mostly at low-elevation sites along the east coast; and (iii) a third lineage of large-sized specimens with rugose dorsal skin from several localities of northern Madagascar, not assigned to a candidate species in previous studies—we here dub this new candidate species *P.* sp. Ca11. Another species with red colour, *P. milloti*, was not placed together with the focal lineages in the mitochondrial trees (Fig. 1A and Suppl. Fig. S1).

The three red-flanked lineages and *P. pollicaris* were highly divergent from each other. Pairwise uncorrected distances (p-distances) in the 5' 16S segment for *P. barbouri* were 7.1–9.0% to *P.* sp. Ca5 and 10.5–16.0% to *P.* sp. Ca11, while *P.* sp. Ca11 and *P.* sp. Ca5 differed by 10.7–16.7%, and *P. pollicaris* differed from the three red-flanked lineages by 11.3–16.3%. Substantial differentiation was also detected within lineages: up to 4.6% in *P. barbouri*, up to 5.6% in *P.* sp. Ca5, and up to 7.2% in *P.* sp. Ca11. In the 16S 3' segment typically used for DNA barcoding of Madagascar frogs (Vieites *et al*., 2009), the divergences between *P. barbouri* and *P.* sp. Ca5 were 4.9%.

The RAG1 haplotype network (based on 361 base pairs for 25 individuals; Fig. 1B) suggested that these lineages do not regularly share alleles of this nuclear gene. All included *P. pollicaris* sequences differed from those of the other red-flanked forms, confirming the genetic divergence of this species. Although the three red-flanked forms had mostly private alleles, a single allele was shared among all three of them. The individuals involved in this sharing were (i) an individual from Mahasoa (ZCMV 8801) assigned by mtDNA to the *P. barbouri* lineage, (ii) an individual of *P.* sp. Ca5 from Manombo, and (iii) one individual (by one allele only) of *P.* sp. Ca11 from Ambodivoangy (FGZC 4275).

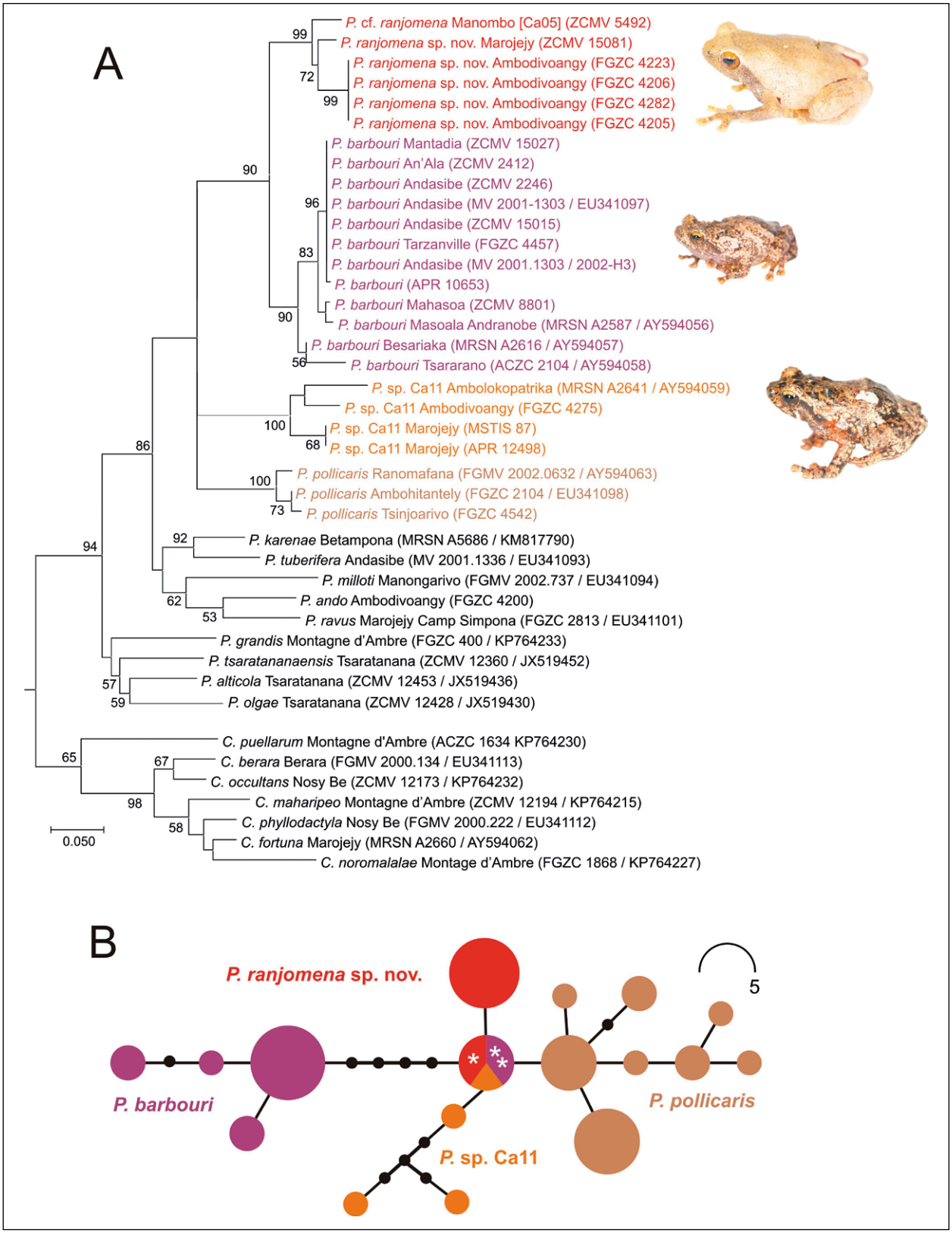


**Fig. 1.** Molecular differentiation of species of the *Platypelis barbouri* complex compared to other species of *Platypelis* and *Cophyla*. (A) Maximum Likelihood tree calculated from a 658 bp alignment of the mitochondrial 16S rRNA gene (5' segment of the gene). Numbers at nodes are support values from a bootstrap analysis (2000 replicates) in percent (not shown if below 50%). A sequence of *Stumpffia gimmeli* was included as outgroup (graphically excluded from figure to better illustrate branch lengths among *Platypelis*). See Suppl. Fig. S1 for a phylogenetic tree with individuals from additional localities. (B) Haplotype network based on 361 bp of the nuclear RAG1 gene (phased sequences, hence each individual represented with two alleles in the network). One asterisk marks the individual of *P.* cf. *ranjomena* from Manombo; two asterisks mark the putative hybrid individual from Mahasoa. Inset photos show *P. barbouri* specimen ZSM 506/2016 (ZCMV 15015), *P.* sp. Ca11 specimen CRH 1630/MSTIS 87, and *P. ranjomena* specimen ZSM 508/2016 (ZCMV 15081).

**Table 1.** Morphometric measurements (all in mm) of available type specimens and comparative individuals of *Platypelis ranjomena* and *P. barbouri*. For abbreviations of measured variables, see Materials and methods; further abbreviations used: M (male); F (female); HT (holotype); PT (paratype). Tympanum diameter (TD) values in brackets indicate that the tympanum was not distinct. RHL (relative hind limb length) is coded as follows: Tibiotarsal articulation 1, reaches between forelimb insertion and tympanum, or 2, reaches tympanum. In all specimens, third and fifth toe were of equal length, except for MRSN A2616 and ZSM 170/2019 (ACZCV 0049) in which the third toe was slightly shorter than fifth toe.

| ***Platypelis ranjomena*** | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Voucher** | **Field number** | **Status** | **Sex** | **Locality** | **SVL** | **HW** | **HL** | **TD** | **ED** | **END** | **NSD** | **NND** | **HAL** | **FORL** | **HIL** | **FOTL** | **FOL** | **TIBL** | **RHL** |
| ZSM 406/2010 | FGZC 4206 | PT | M? | Ambodivoangy | 25.5 | 9.1 | 8.4 | 1.6 | 3.2 | 2.0 | 1.7 | 3.4 | 7.6 | 16.2 | 34.3 | 15.6 | 10.3 | 10.4 | 2 |
| ZSM 407/2010 | FGZC 4282 | HT | M | Ambodivoangy | 24.1 | 8.5 | 7.4 | [1.3] | 3.0 | 1.7 | 1.5 | 2.9 | 7.6 | 15.4 | 33.4 | 15.2 | 9.8 | 10.3 | 2 |
| ZFMK 59908 | — | PT | M | Marojejy | 28.8 | 10.0 | 8.6 | 1.2 | 3.1 | 2.0 | 1.8 | 2.7 | 8.6 | 17.8 | 39.8 | 17.5 | 11.3 | 12.2 | 2 |
| ZSM 508/2016 | ZCMV 15081 | PT | ? | Marojejy | 24.8 | 9.3 | 7.5 | 1.7 | 2.9 | 1.3 | 1.4 | 1.9 | 13.8 | 11.6 | 32.0 | 14.8 | 8.9 | 10.4 | 2 |
| ZSM 170/2019 | ACZCV 0049 | | ? | Betampona: Sahaindrana | 28.8 | 9.8 | 9.1 | 1.7 | 3.2 | 1.3 | 1.6 | 2.4 | 7.6 | 15.9 | 37.4 | 16.2 | 11.2 | 11.3 | 2 |
| | | | | | | | | | | | | | | | | | | | |
| ***Platypelis* cf. *ranjomena*** | | | | | | | | | | | | | | | | | | | |
| ZSM 2421/2007 | ZCMV 5500 | | M | Manombo | 24.1 | 8.4 | 7.9 | [1.3] | 3.0 | 1.9 | 1.3 | 2.5 | 7.7 | 16.2 | 33.3 | 15.0 | 9.3 | 10.6 | 2 |
| | | | | | | | | | | | | | | | | | | | |
| ***Platypelis barbouri*** | | | | | | | | | | | | | | | | | | | |
| ZSM 323/2000 | — | | M | Vohidrazana | 17.9 | 6.6 | 6.2 | [1.2] | 2.4 | 1.3 | 1.4 | 2.3 | 6.1 | 13.1 | 27.2 | 12.2 | 8.1 | 8.0 | 2 |
| ZSM 465/2005 | ZCMV 2246 | | M? | Andasibe | 20.6 | 6.5 | 6.7 | 1.5 | 2.6 | 1.7 | 1.4 | 2.1 | 6.5 | 14.3 | 29.5 | 13.2 | 8.7 | 9.1 | 2 |
| ZSM 388/2010 | FGZC 4457 | | M | Tarzanville | 17.5 | 6.4 | 5.9 | 1.3 | 2.2 | 1.3 | 1.3 | 2.0 | 5.9 | 12.6 | 25.7 | 12.0 | 7.5 | 7.7 | 2 |
| ZSM 765/2009 | ZCMV 11186 | | M | Befanjana | 25.0 | 8.4 | 8.0 | [0.9] | 3.7 | 2.1 | 1.7 | 2.5 | 7.3 | 17.1 | 34.4 | 14.9 | 9.0 | 10.5 | 2 |
| MRSN A2587 | — | | F | Masoala | 28.9 | 10.0 | 9.3 | 1.8 | 3.8 | 2.1 | 1.7 | 3.0 | 9.2 | 20.5 | 42.3 | 18.7 | 12.2 | 12.4 | 2 |
| MRSN A2616 | — | | F | Besariaka | 28.9 | 9.4 | 8.5 | 2.1 | 3.3 | 2.1 | 1.7 | 2.9 | 9.2 | 18.8 | 41.6 | 18.7 | 12.4 | 11.8 | 1 |
| ZSM 171/2019 | ACZCV 0219 | | M | Betampona: Vohitsivalana | 23.9 | 8.6 | 6.7 | 1.2 | 3.0 | 1.3 | 1.1 | 2.9 | 6.5 | 14.9 | 29.6 | 14.3 | 9.0 | 9.8 | 2 |

## Description of *Platypelis ranjomena* sp. nov.

Figs 3–4, Table 1

Zoobank: urn:lsid:zoobank.org:act:7D285383-05D3-4541-9956-8060F3E510D5

**Remarks:** This species has previously been referred to as *Platypelis* sp. "ranjomena" by Glaw & Vences (2007), as *Platypelis* sp. 5 by Vieites *et al*. (2009) and Tu *et al*. (2018), as *Platypelis* sp. [Ca FJ559289] by Rosa *et al*. (2012), and as *Platypelis* sp. Ca5 Manombo by Perl *et al*. (2014) and Scherz *et al*. (2016). It was most likely referred to as '*Platypelis* sp. Masoala' by Glaw & Vences (1994: Fig. 358).

**Holotype:** ZSM 407/2010 (field number FGZC 4282), adult male, collected at Ambodivoangy (Makira area), 15.2899°S, 49.6203°E, ca. 100 m a.s.l., Sava Region, eastern Madagascar, on 3 April 2010 by F. Glaw, J. Köhler, P.-S. Gehring, M. Pabijan, and F. M. Ratsoavina (Figs 3–4).

**Paratypes:** ZSM 406/2010 (field number FGZC 4206), UADBA uncatalogued (FGZC 4205, and FGZC 4223), three adult specimens, all collected from the same locality as the holotype on 31 March 2010 by F. Glaw, J. Köhler, P.-S. Gehring, M. Pabijan, and F. M. Ratsoavina; ZSM 508/2016 (ZCMV 15081), unsexed adult, collected in Marojejy National Park, Sava Region, northeastern Madagascar at an unofficial site called 'Camp 0', located at ca. 14.446°S, 49.785°E, ca. 310 m a.s.l., on 15 November 2016 by A. Rakotoarison, M. D. Scherz, M. C. Bletz, J. H. Razafindraibe, and A. Razafimanantsoa; ZFMK 59908, adult male, collected in the Marojejy National Park, Sava Region, northeastern Madagascar at ca. 300 m a.s.l., about halfway between the Park entrance and the campsite now locally named 'Camp Mantella' (which is located at 14.4377°S, 49.7756°E), on 24 February 1995 by F. Glaw and O. Ramilison.

**Referred material:** ZSM 170/2019 (ACZCV 0049), for measurements see Table 1. The following samples/specimens are referred to this species based on mitochondrial DNA sequences (Suppl. Fig. 1), but most of the corresponding individuals were not examined by us: UADBA uncatalogued (ACZCV 0045, ACZCV 0279), DLR 539, FAZC 13691, MRSN A6194, MRSN A6356, MRSN A6597, and MRSN A6195 from Betampona; RAN 42521 from Antalaha; and ZSM 2421/2007 (ZCMV 5500) and ZCMV 5492 from Manombo.

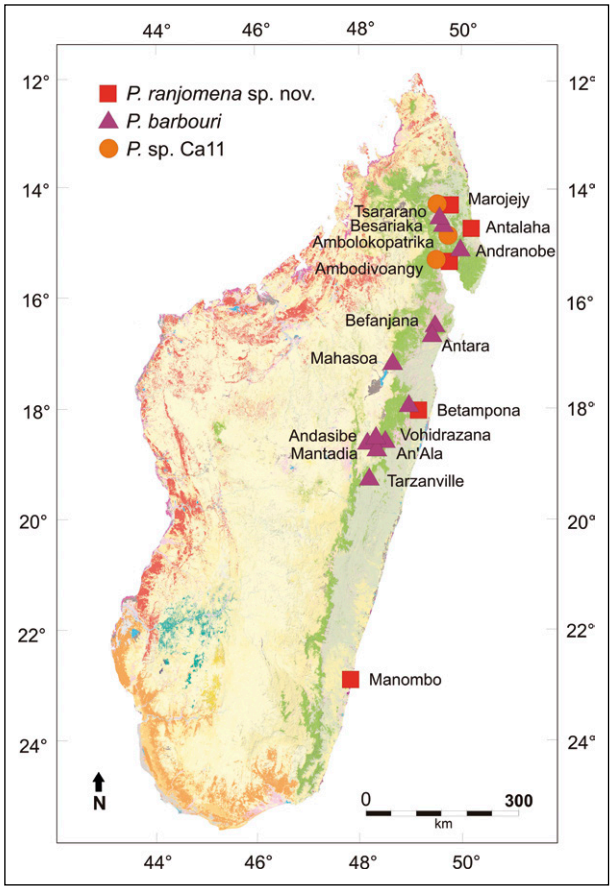


**Fig. 2.** Map showing genetically confirmed locality records of *Platypelis ranjomena* sp. nov., *P. barbouri*, and *P.* sp. Ca11 (based on Fig. 1 and Suppl. Fig. S1, except for the unlocated Ambatomandondona). Note that position of localities is only approximate, especially in cases where two symbols indicate syntopic occurrences. The map shows the remaining primary vegetation of Madagascar (layers formerly openly available from vegmad.org), green colours indicating rainforest, reddish colours deciduous dry forest, and orange colour arid spiny forest.

**Diagnosis:** A medium-sized arboreal cophyline frog, SVL 24.1–28.8 mm, tips of fingers and toes well expanded, third toe slightly shorter than fifth, hind limbs relatively short (tibiotarsal articulation reaching the tympanum), vomerine teeth absent (not recognisable when examining the oral cavity), tympanum small and poorly recognisable, dorsal skin smooth, belly reddish and yellowish with intensely dark red areas in the axilla, inguinal region, and on ventral parts of shanks.

*Platypelis ranjomena* can be distinguished from all other arboreal cophylines (and indeed all other frogs in Madagascar) by the intensely dark red (sometimes purple) patches in its axilla and inguinal regions; similar colouration is present only in *P. milloti* and *P. barbouri*, but in the former the colour is much brighter and more orange, while in the latter the colour is usually paler; a detailed distinction from *P. barbouri* is given below. We are confident in using this as a distinguishing feature not only in life but also in preservation, because the red colouration is apparently robust to preservation in ethanol and is still vibrantly visible in specimens preserved for over 20 years. From *P. milloti*, the species can be distinguished by its rounded snout in dorsal and lateral view (versus rather pointed, compare photographs in Fig. 3 with those of *P. milloti* in Glaw & Vences 2007 and the drawing in Guibé 1978, Fig. 318), and absence of the very distinctive dorsal colouration of that species. From *P. barbouri* (Fig. 5) the new species can be distinguished based on its smooth skin (versus presence of moderate to large prominent tubercles on the dorsum), a more or less solid red to gold iris (versus brown to yellowish with darker brown areas anterior, posterior, and ventral to the pupil), and a generally darker red colouration in the axilla and inguinal region and on the ventral limbs.

Morphologically, *P. ranjomena* differs from all other arboreal cophyline species with expanded finger tips as follows: from all male *Anodonthyla* by the absence of a distinct prepollex; from *Plethodontohyla inguinalis* (SVL 55–100 mm), *P. guentheri* (SVL ca. 33 mm), *P. notosticta* (SVL 29–43 mm), and *P. mihanika* (SVL 25–34 mm) by the absence of lateral folds and contrasting colours along a sharp border on the flanks (versus presence), rounded snout (versus pointed), colouration, absence of vomerine teeth (versus presence), and smaller size (except *P. mihanika*).

Bioacoustically, *P. ranjomena* can be distinguished from its sister species *P. barbouri* by significantly longer note duration, with notes having approximately twice the length of those of *P. barbouri* (303–379 versus 142–160 ms). Calls of *P. alticola*, *P. ando*, and *P. ravus* consist of longer notes (411–466, 424–441, and 384–443 ms, respectively) and the latter two species show a higher mean dominant frequency at 5402 Hz and 4010 Hz, respectively, whereas *P. alticola* shows a lower dominant frequency of 1894 Hz (Glaw *et al.*, 2012; Rakotoarison *et al.*, 2012; Scherz *et al.*, 2019b). Note duration of *P. ranjomena* calls is longer compared to calls of *P. karenae* (131–145 ms), *P. milloti* (55–65 ms), *P. pollicaris* (160–180 ms), *P. tsaratananaensis* (79–145 ms), and *P. tuberifera* (280 ms) (Glaw & Vences, 1994; Rakotoarison *et al.*, 2012; Rosa *et al.*, 2014). Note duration in calls of *Cophyla berara* (774–824 ms), *C. maharipeo* (1166–1346 ms), *C. noromalalae* (662–821 ms), and *C. occultans* (500–550 ms) is longer (Glaw & Vences, 1994; Vences *et al.*, 2005; Rakotoarison *et al.*, 2015). Note duration in calls of *C. phyllodactyla* (360–450 ms) is in the same range, but inter-note intervals (555–605 ms) are much shorter, whereas calls of *C. puellarum* show broad overlap in temporal parameters with those of *P. ranjomena* (Rakotoarison *et al.*, 2015), but differ by lower dominant frequency.

**Description of the holotype:** Adult male specimen in good state of preservation, SVL 24.1 mm; for other measurements, see Table 1. Body moderately stout; head wider than long, not wider than body; snout bluntly rounded in dorsal and lateral views; nostrils directed laterally, not protuberant, nearer to tip of snout than to eye; canthus rostralis indistinct; loreal region straight; tympa-


**Fig. 3.** *Platypelis ranjomena* sp. nov. in life in dorsolateral (left) and ventral (right) views: (a, b) adult male holotype ZSM 407/2010 from Ambodivoangy; (c, d) adult male paratype ZSM 406/2010 from Ambodivoangy; (e, f) adult male paratype ZFMK 59908 from Marojejy; (g, h) adult male specimen ZSM 2421/2007 from Manombo, provisionally assigned to this species.

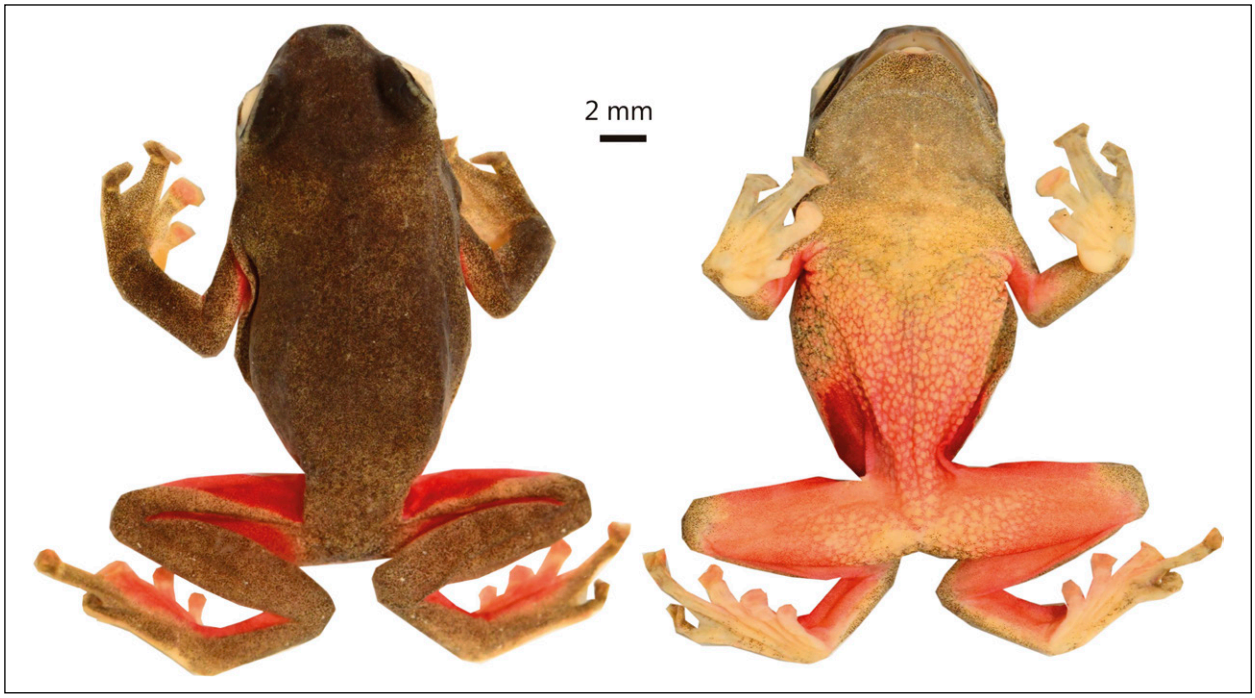


**Fig. 4.** Preserved male holotype of *Platypelis ranjomena* sp. nov. (ZSM 407/2010) from Ambodivoangy in dorsal (left) and ventral (right) views.

num indistinct, 43% of eye diameter; supratympanic fold distinct, straight; tongue partially removed as tissue sample; maxillary teeth present; vomerine teeth absent (not recognisable); choanae rounded. Forelimb slender; subarticular tubercles single, flat, and hardly recognisable; outer metacarpal tubercle large but flat (not prominent); large inner metacarpal tubercle, forming distinct protuberance at base of first finger; hand with some webbing between fingers 2, 3, and 4; fingers distinctly flattened and relatively broad along entire length; relative length of fingers $1<2\leq4<3$, fourth finger similar to second in length; terminal finger discs distinctly expanded, slightly triangular; nuptial pads absent. Hind limbs slender; tibiotarsal articulation reaching tympanum when hind limb adpressed along body; tibia length, 43% of SVL; lateral metatarsalia strongly connected; inner metatarsal tubercle small and indistinct; outer metatarsal tubercle absent; webbing between toes moderately developed, webbing formula 1(1), 2i(1.5), 2e(1), 3i(2), 3e(1), 4i(2), 4e(2), 5(1), but poorly recognisable; toes flattened and relatively broad along their entire length; relative length of toes $1<2<3\leq5<4$; third toe only slightly shorter than fifth. Dorsal skin smooth, without dorsolateral folds. Ventral skin smooth on throat, moderately granular on chest and belly.

**Colouration in preservative** (Fig. 4)**:** After nine years in 70% ethanol, dorsal surfaces uniformly grey-brown: Hind limbs with one indistinct dark crossband. Ventral side brown on throat, fading to cream on belly. Posterior parts of the venter, thigh, shank, and tarsus with fine reddish pigment. Intense, dark red blotches in the axil of the arm and the inguinal region. Cloacal region brown.

Colouration in life (Fig. 3a,b): Dorsum yellowish-brown with some yellowish pigment on flanks. Belly generally dirty reddish becoming gradually yellowish toward the chest, dark brown on throat and chest. Iris reddish-brown; outer iris area bluish. Greyish brown markings on dorsum. Distinct red colour posteriorly at the base of the forelimbs, in the inguinal region, and on the lateral and ventral parts of the shanks.

**Variation:** Measurements are provided in Table 1 and the spectrum of colour variation of four individuals in life is shown in Fig. 3. The dorsal colouration can range from uniformly beige (Fig. 3c) to beige or brown with indistinct or distinct and largely symmetrical dark (grey or blackish) pattern (Figs 3e,g). A beige or brown band can be present between the eyes (Figs 3e,g). We did not observe any changes between nighttime and daytime colouration, but cannot exclude that they exist. Belly yellowish to light brown with or without small dark pigmentations. The extension and distinctness of the red or even purple colour on the ventral surface is rather variable. The throat of calling males can be blackish (Figs 3b,f).

**Etymology:** The specific epithet is derived from the Malagasy words 'ranjo' (= leg) and 'mena' (= red). It is used as a noun in the nominative singular, standing in apposition to the generic name.

**Advertisement call:** Calls recorded at Ambodivoangy from the holotype (ZSM 407/2010) in the evening on 3 April 2010 (estimated air temperature 26 °C) consist of a single tonal note that is repeated at regular intervals (Fig. 6a). Note duration (= call duration) ranges from


**Fig. 5.** *Platypelis barbouri* in life in dorsolateral (left) and ventral (right) views: (a, b) individual from Andasibe photographed in 1991 (not assignable to collection number); (c, d) individual from Tarzanville (ZSM 388/2010); (e, f) the putative hybrid individual from Mahasoa (ZCMV 8801; deposited in UADBA).

303–356 ms (332 ± 15; n = 15), inter-note interval ranges between 2592–3485 ms (3042 ± 261; n = 14). Note repetition rate (= call repetition rate) is approximately 19 notes/minute. The dominant frequency is at 2741–2874 Hz (2820 ± 45; n = 15). Weak harmonic frequency bands are recognisable at around 4600 and 7000 Hz. The prevalent bandwidth is within a narrow band of ca 2680–3000 Hz. Obvious frequency modulation is absent. Calls recorded in Marojejy National Park on 24 February 1995, 21:50 h, at 24 °C air temperature (call voucher ZFMK 59908) have the following numerical parameters: Note duration is 321–379 ms (351 ± 13 ms, n = 27), inter-note interval varies between 2713–4279 ms (3062 ± 362 ms, n = 25). Note repetition rate is approximately 17 notes per minute. The dominant frequency is at 2200–2400 Hz, with weak harmonics in some calls at 4500–4700 and 6850–7000 Hz. Calls from an individual from the same locality (recorded on 28 March 1994 after 21:00 h at 22.5 °C air temperature) agree with this description (note duration 352–407 ms, inter-note interval 2916–2963 ms). A short series of four calls from Manombo recorded on 24 February 2007 has note durations of 230–300 ms (255 ± 31; n = 4), inter-note intervals of 1934–3327 ms, and a dominant frequency of approximately 2540 Hz (Fig. 6b).

**Call variation and comparisons:** Advertisement calls of all recordings assigned to *Platypelis ranjomena* are tonal

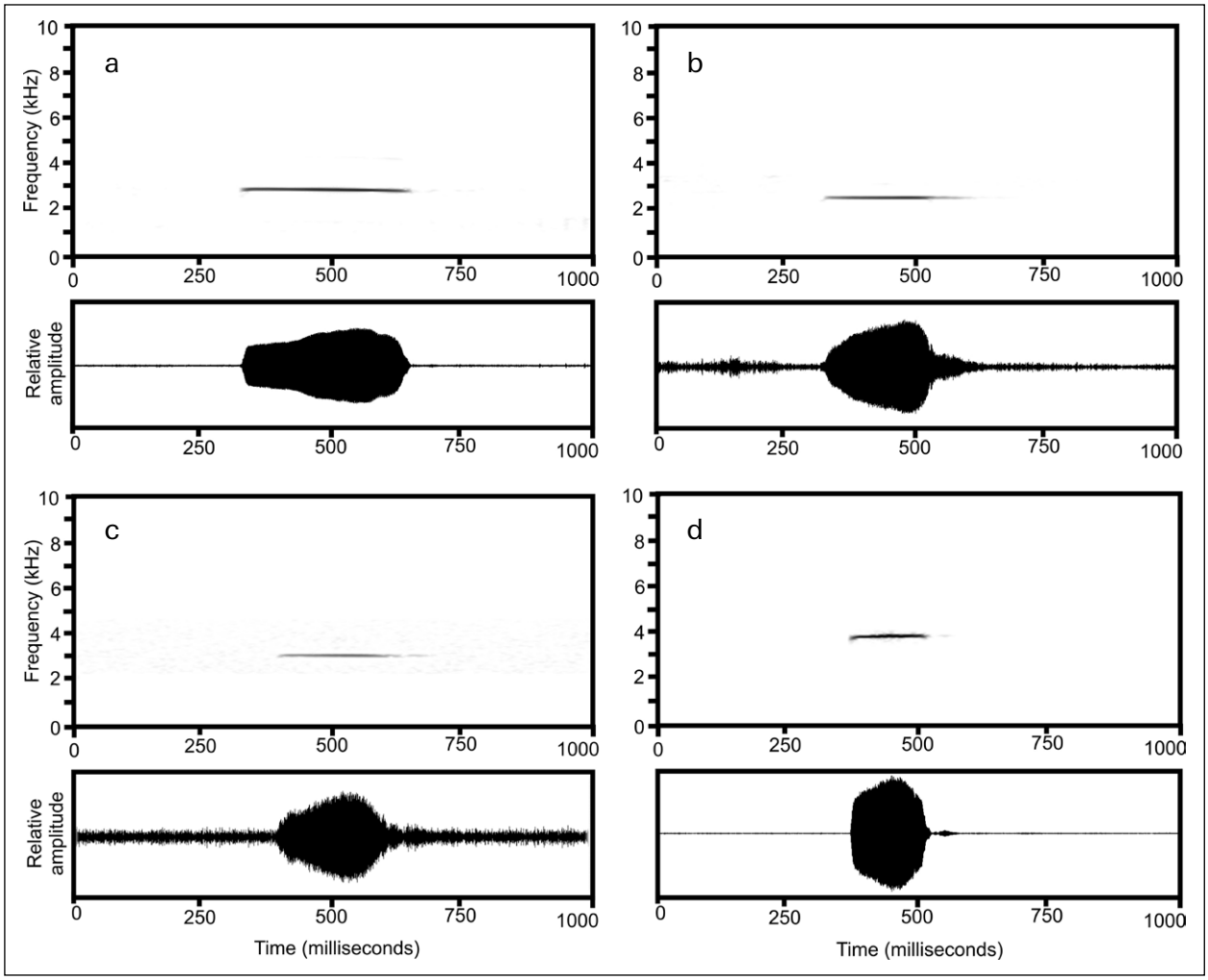


**Fig. 6.** Audiospectrograms and corresponding oscillograms of advertisement calls of *Platypelis* species (Hanning window function, 256 FFT width): (a) *Platypelis ranjomena* sp. nov. (holotype, ZSM 407/2010), recorded on 03 April 2010 at Ambodivoangy; (b) *Platypelis* cf. *ranjomena* (unvouchered), recorded on 23 February 2007 at Manombo; (c) *Platypelis* sp. (ZCMV 8801, in UADBA), recorded on 14 February 2008 at Mahasoa (band-pass filtered at 2000–5000 Hz); (d) *Platypelis barbouri* (unvouchered), recorded on 10 January 1992 at Andasibe.

notes of moderate length (Fig. 6) repeated at more or less regular intervals, forming longer call series. The inter-note intervals are always much longer than the note duration. Prevalent bandwidth in all analysed recordings is very narrow and obvious frequency modulation in notes is absent. Calls of *P. ranjomena* from the type locality and those from Marojejy largely agree in all parameters with broad overlap, apart from slight differences in dominant frequency likely explainable with differences in body size of calling males. Calls from Manombo, here tentatively referred to *P.* cf. *ranjomena*, are slightly shorter in duration, but generally agree with those of *P. ranjomena* from the north and could be considered as intra-specific variation. The calls from the type locality and Marojejy differ from calls of *P. barbouri* from Andasibe (Fig. 6d) by longer note duration (303–407 ms versus 142–160 ms) and lower dominant frequency (2200–2874 Hz versus 3820–3840 Hz). Remarkably, the call of a male *P. barbouri* from Mahasoa (ZMCV 8801, in UADBA), a specimen with an allele of *P. ranjomena* and possibly a hybrid, is intermediate in call characters between *P. ranjomena* and *P. barbouri* in both note duration and dominant frequency. Its call (Fig. 6c) showed the following parameters (n = 6): note duration 233–256 ms (243 ± 8.6 ms), inter-note interval 3730–7301 ms (4964 ± 1423 ms) and a dominant frequency at 3160–3204 Hz (3190 ± 18 Hz). For advertisement call differences to other species of *Platypelis* and *Cophyla* see Diagnosis.

**Natural history:** The holotype was found calling after dusk on the leaf of a ginger-like plant, ca. 2 m above the ground, in secondary vegetation close to the border of primary forest. Three paratypes (FGZC 4205, 4206 and 4223) were found together during the day swimming in the same water-filled leaf axil of a banana-like plant, together with several single whitish eggs (Fig. 7). The male from Marojejy (ZFMK 59908) was captured while calling in the vegetation only ca. 0.5 m above the forest floor on a leaf with the head directed upwards. The single subgular vocal sac was strongly inflated during the emission

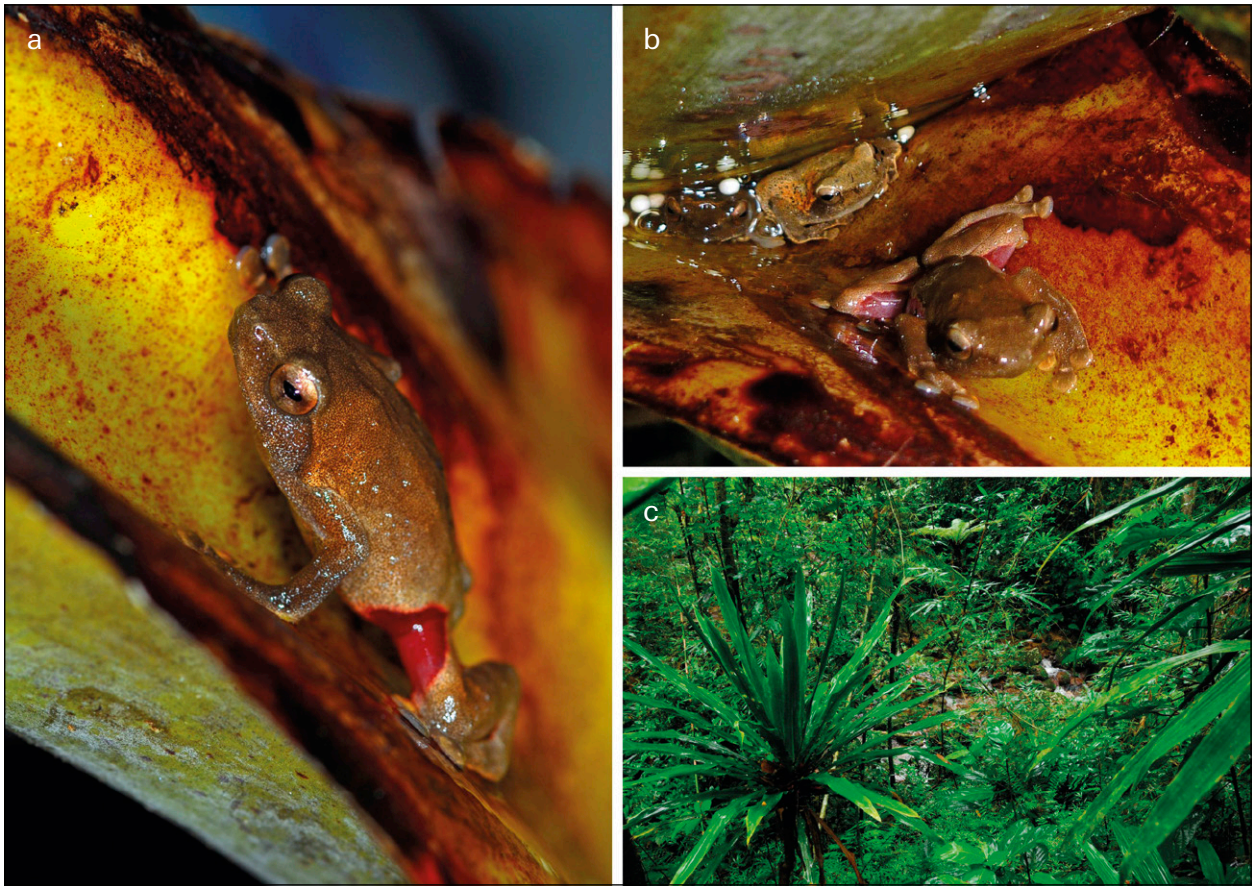


**Fig. 7.** *Platypelis ranjomena* sp. nov. in situ in Ambodivoangy, the type locality. (a) Individual of *Platypelis ranjomena* sp. nov. in life, as discovered in leaf axil of a banana-like plant at the type locality; (b) same leaf axil showing three adult individuals of *Platypelis ranjomena* sp. nov. together with freshly laid eggs; (c) semi-open habitat within rainforest in Ambodivoangy.

of the calls. A calling individual recorded at the same locality in 1994 was sitting in a tree several metres above the forest floor, and we therefore were unable to catch it. During surveys in Marojejy (27–31 March 1994, 22–25 February and 2–4 March 1995) we only heard single individuals, indicating that the species either is rare locally or that its calling activity is not continuous during the rainy season. A further survey in the Marojejy National Park in February 2005 did not reveal any new individuals or call records, whereas in November 2016 a single, non-calling individual was found. At the type locality Ambodivoangy, the new species occurs in syntopy with *Platypelis* sp. Ca11, *P. ando* (see Scherz *et al*., 2019b) and *P.* sp. aff. *tetra*. In Marojejy it apparently is parapatric with *Platypelis ravus*, which occurs at higher elevation (Glaw *et al*., 2012), but sympatric with *P.* sp. Ca11.

In Betampona *P. ranjomena* and *P. barbouri* occur sympatrically, and at least in Vohitsivalana they can be found syntopically. *Platypelis ranjomena* seems to be the species with the widest distribution across the reserve, having been found both in pristine (e.g. Sahambendrana and Sahabefotza) and more disturbed areas (e.g. Sahaïndrana, Vohitsivalana), while *P. barbouri* has been predominantly found in Vohitsivalana and along the Piste Fotsimavo. In Betampona, both species share a similar microhabitat, and they can generally be found in tree holes and leaf axils along the slopes and crests. Males of both species have been found calling from the leaves at about 1 to 2 meters above the ground, but call activity seems to be limited to the rainy season.

**Distribution:** The distribution based on genetically confirmed locality records is shown in Figure 2 and indicates that the species is widespread in the lowlands of eastern and north-eastern Madagascar. Additional unconfirmed locality records based on photographs and non-sequenced voucher specimens include the coast of Masoala (near 'Eco-Lodge chez Arol', ca. 15.712°S, 49.964°E, ca. 21 m a.s.l.), Ambodiriana (ca. 16.6746°S, 49.7028°E), and near Analalava (ca. 17.707°S, 49.460°E, ca. 30 m a.s.l.) close to Foulpointe (= Mahavelona). The Manombo population deserves further study as it may represent a deep conspecific lineage or (less likely) a more divergent form.

**Available names:** Several junior synonyms in the genus *Platypelis* must be excluded as available names for *P. ranjomena*. *Platyhyla verrucosa* Mocquard, 1901 and *Platyhyla voeltzkowi* Boettger, 1913 (both junior synonyms of *P. grandis* according to Noble & Parker, 1926) are larger, have a granular dorsal skin and completely lack any red ventral colour; *Cophyla tuberculata* Ahl,

1929, considered a further synonym of *P. grandis* based on the comparison of juveniles (Blommers-Schlösser & Blanc, 1991), has granular skin and differs in colour pattern (Ahl, 1929); *Paracophyla tuberculata* Millot & Guibé, 1951, currently considered a junior synonym of *P. barbouri* (Blommers-Schlösser & Blanc, 1991), has a similar dorsal pattern to *P. ranjomena* (see Guibé, 1978: Fig. 348) and no vomerine teeth but is very small (SVL 17 mm) and has distinct dorsal tubercles, which are absent in *P. ranjomena*. The holotype of *Platypelis barbouri* is a gravid female of 20 mm SVL with large ovary eggs of 1.5 mm diameter and rugose skin (Noble, 1940) and thus smaller and more rough-skinned than *P. ranjomena*.

### New candidate species (*Platypelis* sp. Ca11)

Our genetic analysis revealed a deep new lineage in this species group that was not previously recognised (Fig. 1): specimens hitherto assigned to *P. barbouri* from Marojejy closely resemble that species and in fact represent a lineage that is sister to the *P. barbouri* + *P. ranjomena* clade, herein named *P.* sp. Ca11 (Fig. 8). This lineage also occurs in Ambolokopatrika, and at Ambodivoangy in syntopy with *P. ranjomena* (Figs 1–2). We are confident that the assignment of the name *P. barbouri* should be attributed to the southern lineage, as the type locality of Fanovana Forest is located close to Andasibe in central eastern Madagascar. This therefore represents a new candidate species that should be taxonomically investigated in the future; we have here refrained from doing so, as insufficient material was available to us for examination.

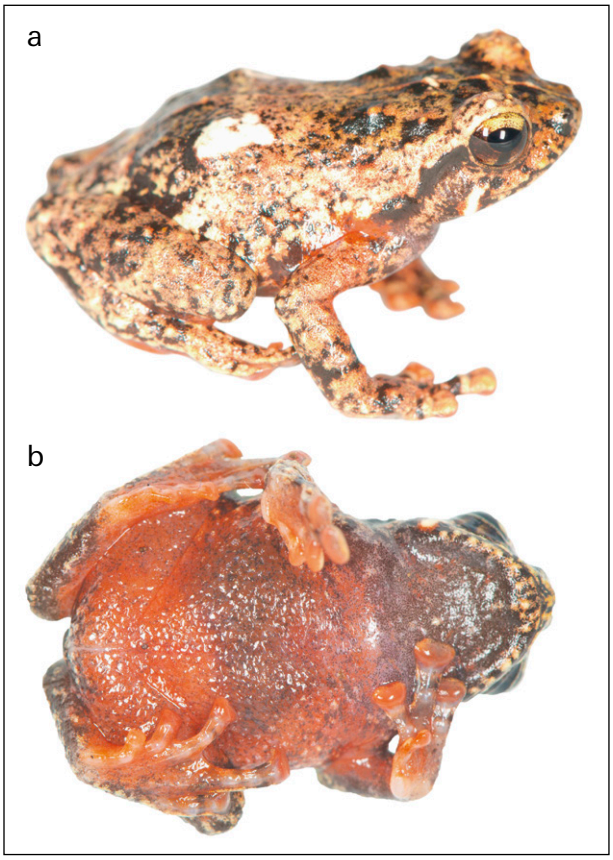


**Fig. 8.** Dorsolateral (a) and ventral (b) views of *Platypelis* sp. Ca11 from Marojejy in life (specimen CRH 1630 / MSTIS 87).

## Discussion

With the description of *P. ranjomena* we add a distinctive new species to the genus *Platypelis*. The new species is phylogenetically sister to *P. barbouri*, and is separated from that species by 7.1–9.0% in the analysed 5' segment of the mitochondrial 16S rRNA gene. Morphologically it closely resembles *P. barbouri:* both species have a small and rather indistinct tympanum, no (recognisable) vomerine teeth, at least some red colour on the ventral surface, and can have a similar dorsal pattern (see Noble, 1940). Individuals unambiguously assignable to *P. barbouri* differ, however, in calls (distinctly shorter note duration and higher frequency; see also Glaw & Vences, 1994) and granular vs smooth skin on the dorsum. Additionally, there appears to be a generally much darker red colouration in *P. ranjomena* than in *P. barbouri*, sometimes even reaching a purple hue. Individuals of *P. barbouri* from the Andasibe-Moramanga region are small-sized (SVL 18–22 mm; Table 1), and this is also true for the holotype of *P. barbouri* (see above; Noble, 1940). In contrast, specimens assigned to *P. ranjomena* reach body sizes of 24–29 mm. However, in the northern populations of the distribution range of *P. barbouri* (Fig. 2), large individuals are found (Table 1: 25–29 mm in Befanjana, Masoala, and Besariaka). These Masoala and Besariaka specimens are also genetically divergent, and were already considered by Vieites *et al*. (2009) as deep intraspecific genetic lineage. The population from Manombo, here considered to belong to *P. ranjomena*, is also genetically divergent from that at the type locality Ambodivoangy, with a high 16S p-distance of up to 5.6%. Most likely, the taxonomy of these frogs will require additional attention in the future, considering the high genetic divergences (> 4% in the 5' 16S segment) observed within both *P. barbouri* and *P. ranjomena*.

To understand the differentiation of these two species (as currently defined), it is important to note that *P. barbouri*, especially at the southern edge of its population, is generally found in mid-elevation forests > 600 m a.s.l., whereas *P. ranjomena* appears to be mostly distributed in coastal low-elevation forests < 500 m a.s.l. In accordance with this general pattern of distribution, we note that in Betampona, a low-elevation forest fragment that ranges from 275 to 650 m a.s.l., *P. ranjomena* is apparently considerably more common and widespread than *P. barbouri*, which seems to be restricted to the periphery of the reserve. In Marojejy, where *P.* sp. Ca11 occurs, it too seems to occur at elevations above those of *P. ranjomena*, although only very few records are available at present to corroborate this.

The single individual encountered in Mahasoa forest (ZCMV 8801; voucher deposited at UADBA and not available for detailed morphological study) was somewhat rough-skinned (Fig. 5) and small-sized according to our field notes, thus at first glance conforming more with *P. barbouri*; interestingly, it shared a nuclear allele with *P. ranjomena* (Fig. 1) and its call was somewhat intermediate between these species. The discordance between signals of mitochondrial and nuclear genes can be caused either by incomplete lineage sorting, which is more likely in the nuclear gene due to its 4-fold larger effective population size, or by hybridization. As pointed out by Leaché (2009), hybridization and gene tree incongruence is particularly to be expected close to the boundaries, i.e. contact zones, of phylogeographic clades. Unfortunately, the geographical sampling of amphibians in Madagascar is only rarely dense enough to allow for a precise identification of contact zones among closely related clades, and includes particularly large gaps along the east coast around the latitude where Mahasoa is located and where lowland rainforest has been largely destroyed (see Gehring *et al*., 2010); in fact, the collecting locality of ZCMV 8801 was under heavy pressure from logging and slash-and-burn agriculture when explored in 2008, and sadly, it is probable that no suitable *Platypelis* habitat remains in this area today. Yet, there are reasons to assume that this general area represents a contact zone between *P. barbouri* and *P. ranjomena*, because (i) *P. ranjomena* localities are known from scattered lowland localities along much of the east coast and the species was probably continuously distributed there earlier, and (ii) although the explored forest at Mahasoa is located at a relatively high elevation (17.29769°S, 48.70199°E, 1032 m a.s.l.), we also collected other species typical for low-elevation forests at this site (e.g. the leaf-tailed gecko *Uroplatus fimbriatus*: Ratsoavina *et al*., 2013), suggesting that this site may represent a contact zone between faunal assemblages of low- and mid-elevation. This leads us to hypothesize that ZCMV 8801 may possibly represent a hybrid of *P. barbouri* and *P. ranjomena*, but we are also convinced that such hybridisation between the two species will be occasional, given the overall rareness of haplotype sharing in the nuclear marker analysed here.

*Platypelis ranjomena* is remarkable by its blood-red flash markings on parts of the belly, on the ventral legs, in the inguinal region, and in the axilla. Partial red ventral surfaces of different colour intensity have evolved many times independently in anurans from Madagascar and elsewhere, and these colour patterns are often taxonomically diagnostic. Red flash markings and ventral colouration occurs in several mantellid frogs, in the genus *Mantella*, in the *Boophis majori* group, in the *B. goudotii* group, and in *Gephyromantis malagasius* (Vences *et al*., 1999, 2002; Glaw *et al*., 2001, 2010), but is even more common in cophyline microhylids, where it is known from *Platypelis ranjomena*, *P. milloti*, *P. barbouri*, *Stumpffia roseifemoralis*, *S. be*, *S. kibomena*, *S. miovaova*, *S. nigrorubra*, *S. meikeae*, *Rhombophryne ornata* and several undescribed species (Glaw & Vences, 2007; Köhler *et al*., 2010; Glaw *et al*., 2015; Scherz *et al*., 2015; Rakotoarison *et al*., 2017). As in mantellids, red colour can be found in both terrestrial and arboreal, diurnal and nocturnal species of different habits and body sizes. The function of this colouration is unknown; its location and distinctness is relatively consistent among both microhylids and mantellids, when present. It is apparently not related to diel activity, as *Mantella* and several *Stumpffia* species are diurnal, whereas other microhylids with red colour are nocturnal. Interestingly, in *P. barbouri* this reddish colouration seems to be more evident during the day than at night. It can also be highly stable, surviving years in ethanol, as in *P. ranjomena*, or apparently rather instable, disappearing in preserved specimens, as in *Stumpffia miovaova*. Whether this colouration has a function in antipredator behaviour or in intraspecific communication remains unknown, although several red-bellied toads from South America (*Melanophryniscus*) contain toxic alkaloids in their skin (Mebs *et al*., 2007).

During four surveys to the Marojejy massif we were not able to capture more than two individuals of *Platypelis ranjomena*. Extensive surveys by other teams (Raselimanana *et al*., 2000) apparently failed to record the species as well. This might indicate that the habits of *P. ranjomena* are either very seasonal and cryptic, or that the species is indeed rare, at least in Marojejy. Although this species is widespread in eastern Madagascar and relatively conspicuous in morphology and colouration, it is apparently not present in the historical collections of the Paris, London, or Amsterdam museums. On the other hand we found several adult individuals together in a phytotelmic plant at Ambodivoangy, and the species seems to be quite abundant in Betampona Strict Nature Reserve. The presence of *P. ranjomena* in the rather well-protected Marojejy National Park, the Strict Nature Reserve of Betampona, and its likely occurrence in the Makira Natural Park, the Masoala National Park, and other forests in its large distribution range might assure its survival for at least the near future.

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**File 1**: Suppl_Figure_1: Maximum Likelihood tree calculated from a 1096 bp alignment of two concatenated segments of the mitochondrial 16S rRNA gene in *Platypelis* and *Cophyla*.

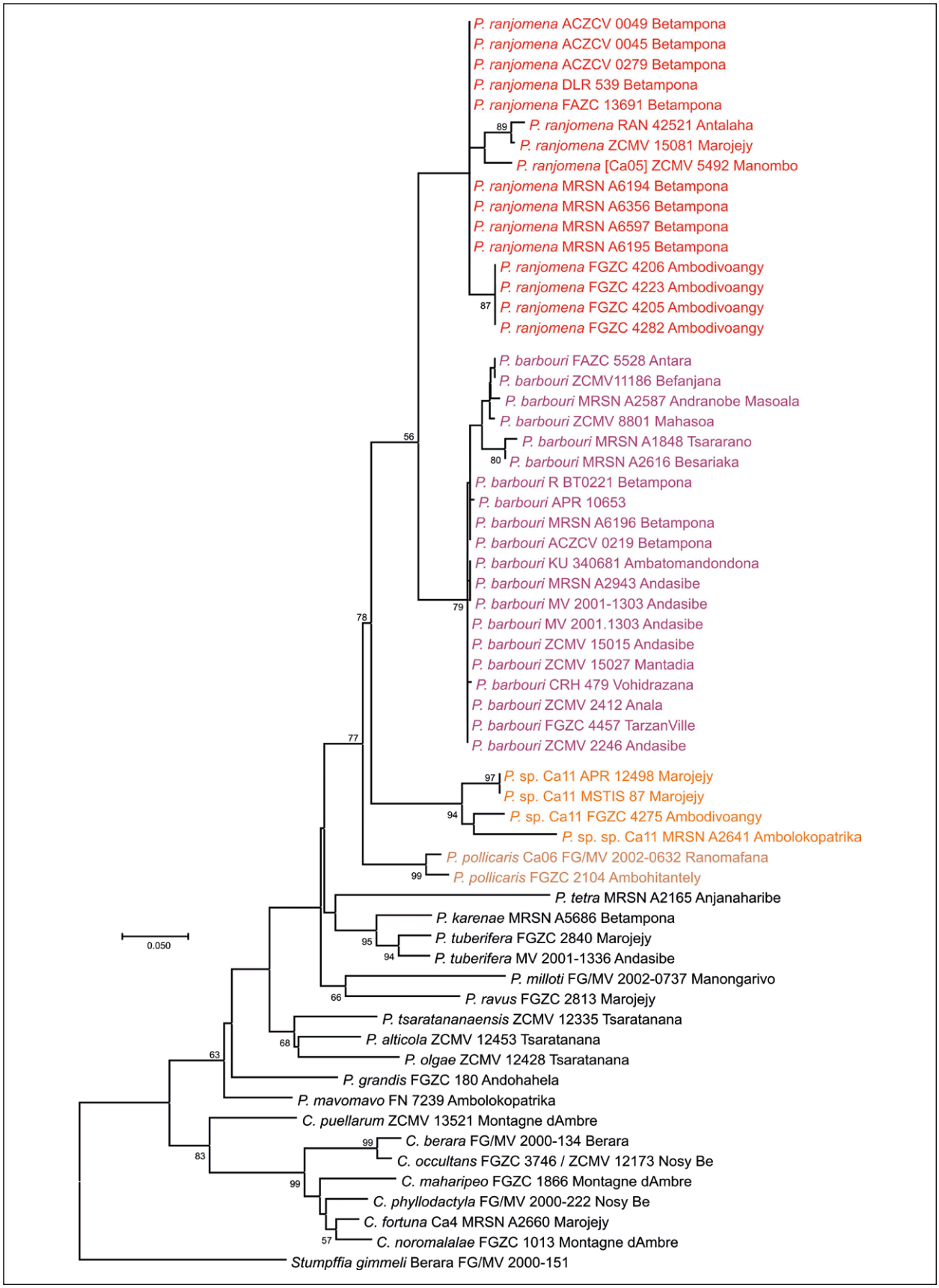


**Fig. S1.** Maximum Likelihood tree calculated from a 1096 bp alignment of two concatenated segments of the mitochondrial 16S rRNA gene in *Platypelis* and *Cophyla*. Numbers at nodes are support values from a bootstrap analysis (1000 replicates) in percent (not shown if below 50%). A sequence of *Stumpffia gimmeli* was included as outgroup. Note that due to an unbalanced amount of missing data (many individuals sequenced only for one of the two segments) the branch lengths in this tree do not reliably reflect the genetic divergence among samples, and node support is in many cases low.