A new narrow-mouthed frog of the genus *Paradoxophyla* (Microhylidae: Scaphiophryninae) from Masoala rainforest, northeastern Madagascar

**Franco Andreone** 1, **Gennaro Aprea** 2, **Gaetano Odierna** 2, and **Miguel Vences** 3

1 Museo Regionale di Scienze Naturali, Via G. Giolitti, 36, I-10123 Torino, Italy
Corresponding author. E-mail:f.andreone@libero.it
2 Università di Napoli Federico II, Dipartimento di Biologia Strutturale e Funzionale, Via Cinthia, Napoli, Italy
3 Institute for Biodiversity and Ecosystem Dynamics, Zoological Museum, Mauritskade 61, 1092 AD Amsterdam, The Netherlands [Current address: Zoological Institute, Technical University of Braunschweig, Spielmanstr. 8, 38106 Braunschweig, Germany]

**Abstract.** A new microhylid frog of the genus *Paradoxophyla* is described from the rainforests of northeastern Madagascar (Masoala). *Paradoxophyla tiarano* n. sp. was found in still waters within rainforest. In external morphology it is similar to *P. palmata*, hitherto the only known species belonging to this genus, but it differs by a much less extended foot webbing. We also provide a description of the probable tadpole of the species, which is a specialized suspension feeder of the typical microhylid type. The new species also differs from *P. palmata* in karyology and mitochondrial DNA sequences. *Paradoxophyla tiarano* n. sp. is currently known from Masoala, but we suspect that its distribution might extend over a wider area in northeastern Madagascar.

**Key Words.** Amphibia, Anura, Microhylidae, *Paradoxophyla*, New species, Madagascar.

**INTRODUCTION**

Most of the amphibians of Madagascar, currently estimated to consist of over 220 species (Andreone et al., 2005), breed in or along forest streams or are independent from water. In contrast, a few species prefer stagnant pools for reproduction. These frogs are usually linked to desert and savannah habitats where streams and ponds are rare, and they exhibit highly seasonal breeding habits depending on the availability of free water. The few pond breeders occurring in rainforest belong to the genera *Scaphiophryne*, *Dyscophus* and *Boophis* (Vences et al., 2002 b, 2003). However, these species appear much less diverse compared to their rainforest.
relatives that have stream-breeding and other derived reproductive modes (i.e., some clades of *Mantidactylus*, *Boophis*, and cophyline microhylids; Vences et al., 2002 a).

All pond-breeding Madagascan frogs go to water only (or mainly) during the rainy season, and during the rest of the year they carry out a terrestrial or arboreal life. One genus, *Paradoxophyla* Blommers-Schlösser & Blanc, 1991, is an exception in two respects. This genus, so far represented by the single species *Paradoxophyla palmata* (Guibé, 1974), is the only deep Madagascan lineage restricted to rainforests and at the same time specialized to pond breeding. It is a pond breeding species with a hydrodynamic and flattened body, small eyes, and extensive foot webbing that resembles that of the African *Xenopus* and *Hymenochirus* and suggests a strongly aquatic preference. However, this small microhylid, included in the subfamily Scaphiophryninae (Blommers-Schlösser & Blanc, 1991), is probably fully aquatic only during its relatively explosive reproduction and is not found in water except when breeding (Glaw & Vences, 1994).

In the course of a study at sites in the Masoala Forest, northeastern Madagascar, we found a *Paradoxophyla* with much less extensive webbing that also was different from *P. palmata* in molecular and karyological characters. We here describe it and its probable tadpole and provide data on its mitochondrial and karyological differentiation.

**MATERIALS AND METHODS**

*Capture, preservation, and drawing techniques*

Frogs and tadpoles were captured in a pool close to a forest stream. After photography, they were euthanized with chlorobutanol, fixed in 4% formalin or 90% ethanol, and preserved in 70% ethanol. Voucher specimens were deposited in the Museo Regionale di Scienze Naturali, Torino under the acronym MRSN. Drawings were made by the senior author from original photographs along with comparisons with the preserved adults and tadpoles.

*Morphometric measurements*

Measurements were made with calipers (precision: 0.1 mm): SVL (snout-vent length), HW (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 (inter-narial distance), TD (horizontal tympanum diameter), HAL (hand length, from the carpal-metacarpal articulations to the tip of the longest finger), FORL (forelimb length, from the axilla to the tip of the longest finger), HIL (hind limb length, from the cloaca to the tip of the longest toe), FOL (foot length, from the tarsal-metatarsal articulations to the tip of the longest toe), FotL (foot length including tarsus, from the tibiotarsal articulation to the tip of the longest toe), and TIBL (tibia length). The toe webbing formula is given as in Blommers-Schlösser & Blanc (1991) and Glaw & Vences (1994). For the tadpoles, we measured the following parameters: BL (body length), TAL (tail length), TMH (tail muscle height), TH (tail height including the dorsal and ventral fins), and HW (head width).

*Comparative examined specimens*

We compared the adult specimens of the new species with the holotype and paratype of *Paradoxophyla palmata* from Ambana (south-eastern Madagascar), and with a further series from
eastern-central Madagascar (Andasibe and Ranomafana). Tadpoles were compared with the description given by Blommers-Schlösser & Blanc (1991). Used acronyms were as follows: MRSN, Museo Regionale di Scienze Naturali, Torino; MNHN, Muséum national d’Histoire naturelle, Paris; ZFMK, Zoologisches Forschungsinstitut und Museum Alexander König, Bonn; ZSM, Zoologische Staatssammlung, München.

DNA analysis

Muscle tissue samples taken from freshly killed specimens were preserved in 98% ethanol. DNA was extracted with different standard protocols and a fragment of the mitochondrial 16S rRNA gene was amplified with the primers 16Sa-L and 16Sb-H of Palumbi et al. (1991). After purification with Qiaquick kits (Qiagen), the fragments were resolved on an automated DNA sequencers (ABI 377 and ABI 3100). Sequences were validated and aligned with the software Sequence Navigator (Applied Biosystems) and deposited in Genbank (accession numbers of newly obtained sequences AY834186-AY834198). The alignment required inclusion of gaps to account for indels in only a few cases in one hypervariable region. Data analysis was carried out with PAUP, version 4b10 (Swofford, 2002). Because of the low number of informative sites in *Scaphiophryne* (Vences et al. 2004), we refrained from performing phylogenetic analyses and report genetic divergences among sequences only. Exploratory maximum parsimony and maximum likelihood analyses in all cases recovered *Paradoxophyla* as highly supported monophyletic group, with the northeastern specimen placed as a sister to a clade containing the eastern specimens. Altogether 103 sites were parsimony informative and 416 were constant.

Karyology

The chromosomes were obtained from intestine, spleen, gonads and lungs with the air-drying method of Odierna et al. (2001). We compared the karyology of one adult specimen of the new species from Masoala (MRSN A2525) and one adult *P. palmata* from Andasibe (MRSN A2518). Besides conventional staining (5% Giemsa at pH 7), the following techniques were applied: (1) Ag-NOR banding of the nucleolar organizer regions following Howell and Black (1980); (2) the C+G specific fluorochrome chromomycin A3 (CMA3) /methyl green staining according to Sahar & Latt (1980) with exposure to the non-fluorescent dye, methyl green reduced to a few seconds; and (3) C-banding according to Sumner (1972) by incubating the slides for 5 min at 45°C in Ba(OH)2. Good results were achieved by staining, either separately or sequentially, with CMA3 and DAPI after hydrolysis in Ba(OH)2 (Odierna et al., 2001). From each taxon, at least four Giemsa-stained metaphases and two metaphases stained with each of the banding methods were studied.

RESULTS

Description of *Paradoxophyla tiarano* sp. nov.
(Figures 1-6)

Type series

Holotype MRSN A2498 (male) and paratypes MRSN A2499 and A2525, adult male and adult female, all from Masoala Peninsula, Campsite 5 (locally known as Menamalo-
Additional material

MRSN A2522, 70 tadpoles, with the same locality and collector. They are assigned to *P. tiarano* because due to its similarity to the tadpole of *P. palmata*, as shown by Blommers-Schlösser & Blanc (1991) (see below). Furthermore, the tadpoles show a typical microhylid morphology, which in the study area is not known to be shared by other frog species.

**Diagnosis**

An aquatic *Paradoxophyla* characterized by a small-medium size, dorso-ventrally flattened body, a sharply pointed and longish snout, small and circular eyes, fingers without evident expansions, toes slightly enlarged, absence of hand webbing and foot webbing nearly absent.

**Fig. 1.** *Paradoxophyla tiarano*. The paratype MRSN A2499 in nature. Menamalona Forest, Masoala Peninsula, NE Madagascar.
A new *Paradoxophyla* from NE Madagascar

Table 1. Morphometric measurements (to 0.1 mm) of specimens of *Paradoxophyla tiarano* sp. nov. and *Paradoxophyla palmata*. M = male, F = female, HT = holotype, PT = paratype. For other abbreviations see the text.

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Morphology

Measurements of the three adult specimen are given in Table I. Head almost as long as wide; snout longish, protruding beyond margin of lip; pointed in dorsal view and in lateral profile; END bigger than ED; END 27–33% of HL; eye small in size; ED 22–27%

Fig. 2. *Paradoxophyla tiarano*. Drawing of the male holotype (MRSN A2498), dorsal side (a), and ventral side (b) SVL = 17.5 mm.

Fig. 3. *Paradoxophyla tiarano*. Drawing of the female paratype (MRSN A2499), dorsal side (a), ventral side (b). SVL = 28.1 mm.
of HL; upper eye lid smooth. Top of head flat; canthus rostralis indistinct; internarial area not depressed; nostril circular; protruding laterally at a point above margin of lower jaw. Slight supratympanic fold usually visible, tympanum not visible. Choanae relatively small, round, separated, partially obscured by palatal shelf of maxillary arch. Vomerine teeth present and posterior to choanae. Tongue trapezoid in shape with rounded edges, widest at the free margin, no groove or notch, free behind for about two-thirds of its length. Pupil circular. Skin on dorsum of head, body, and limbs smooth in the female, and with rounded warts and tubercles in the male. Ventral surfaces smooth. Cloacal opening elliptical, unmodified. Fingers moderate in length, with small, moderately sized discs; disc on first digit smaller than others: 1<2=4<3; subarticular tubercles flattened, ovoidal, not elevated; supernumerary tubercles absent; palmar tubercle semidistinct, not very elevated. The 3rd finger is thicker than the other three in both sexes. No webbing between digits of hands. Feet without outer metatarsal tubercle; inner metatarsal tubercle present, although weakly evident. Small enlarged pads on digits and toes, ovoid in shape. The feet have only traces of web. Toe length: 1<2<5<3<4.

Colouration and variation

After about five years of preservation, the type specimens show a colouration that is quite similar to that observed in life (Fig. 1). The dorsal colour shades from light brown to tan, with small and irregular markings. Legs have darker transverse bands. The belly is whitish in its central part, with a darker network and spots, which in some cases assume the aspect of a reticulation. The throat is darker than the surrounding areas. The two females (MRSN A2499 and A2525) are larger than male (MRSN A2498).

Tadpoles

Tadpoles assigned to *P. tiarano* correspond to the microhylid type 1, suspension feeder, sensu Altig & Johnston (1989), Altig & McDiarmid (1999), and McDiarmid & Altig (1999). They have a roundish body, a little bit longer than wide. The snout is flattened and quite horizontal, subcircular when seen from above, and rounded in profile. The eyes are distant and project dorsolaterally. The location of the closed external nares are dorsal, closer to snout tip than to eyes, and positioned in a slight light-coloured furrow. The ventral fin extends parallel to the tail musculature, the dorsal fin has a maximum height at about three-fourth to a third of the tail. The dorsal fin originates at the tail-body junction, and the ventral fin originates at the posteroverentral terminus of the body. The spiracle opens ventrally and has a fringed border. The mouth is wide, with the marginal edges linearly arranged. The lower jaw projects upwards. The mouth is therefore visible in dorsal view. At stage 38 morphometric measurements are (data for a sample from MRSN A2618, given as means ± standard deviation): body length = 11.2 ± 1.4 mm (n = 20, range: 9.0-13.9), TAL = 16.2 ± 2.3 mm (n = 17; 12.0-21.1); TMH = 2.6 ± 0.3 mm (n = 19; 2.0-3.5), TH = 6.1 ± 0.9 mm (n = 15; 4.3-7.9), HW = 8.5 ± 0.8 mm (n = 20; 7.00-9.80). In life, the body of the tadpoles is transparent and translucent without a distinct colour, although the muscular part of the tail and the dorsal wall of the body are light brownish, shading to
pinkish (Fig. 4). The belly is whitish, while the blackish intestines are seen through the ventral, lateral and dorsal body walls. The dorsal and ventral fins are transparent, but the margins are more opaque than the rest. In preservative the specimens maintained their natural colour pattern.

**Etymology**

The specific epithet “tiarano” (pronounced: tee-how-row-noo) is a Malagasy term composed by two words: “tia”, which means “love” and “to love,” “to like,” and “rano,” meaning “water.” The name means “that one loving the water,” and is used as a noun in apposition, underlining the aquatic habits of this frog.

**Fig. 4.** Tadpole assigned to *Paradoxophyla tiarano* derived from some individuals of the sampe MRSN A2166, dorsal (a), ventral (b), lateral (c) view. Total length of about 28 mm.
A new *Paradoxophyla* from NE Madagascar

Natural history and distribution

The adults and tadpoles were collected in a small pool (about 3.0 x 1.5 m; 1 m deep) a short distance from a forest stream. We believe that the adults carry out an aquatic life at least during the wet season (from November to April). Tadpoles were found in December suggesting that reproduction likely occurred some days or weeks before. We suppose that the distribution extends at least over most of the Masoala rainforest, at least at low and mid-elevations.

Morphological comparisons with other species

*Paradoxophyla tiarano* appears externally similar to *P. palmata*. Of course, comparisons are limited by the small number of available specimens. The new species has a body size 17-30 mm long, while the measured specimens of *P. palmata* ranged 19-26 mm. When compared to specimens of *P. palmata*, the male of *P. tiarano* appears a little bit wartier and with a somewhat darker colouration. The most important distinction between the two species is that in *P. palmata* the feet are extensively webbed, while the foot webbing is lacking or scarcely present in the new species. The foot webbing in *P. palmata* is usually given as “total” (see Guibé, 1974; Blommers-Schlösser & Blanc, 1991; Glaw & Vences, 1994). Our comparative analyses of the specimens from Ambana extended types), Ranomafana and Andasibe confirmed the webbing character and revealed the following webbing formula:

**Table 2.** Genetic differentiation among species of *Paradoxophyla*, *Scaphiophryne* and *Dyscophus*. The table shows pairwise divergences among sequences (535 base pairs) of the mitochondrial 16S rRNA gene. Above diagonal are total number of substitutions, below diagonal are uncorrected p-distances. The shaded parts of the matrix show intragenic comparisons.

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In *Paradoxophyla palmata* the foot has only traces of webbing, both in the examined male or females (see Fig. 5 for a better comprehension of this character).

**Fig. 5.** Particular of the foot in *Paradoxophyla palmata* (ZFMK 60009) and *P. tiarano* (MRSN A2499). Note the state of the webbing, present and complete in *P. palmata* (left), and rudimentary to absent in *P. tiarano* (right).

**Fig. 6.** Karyotypes of *P. palmata* (A) and *P. tiarano* (C), and relative metaphasic plates, with C-banding (B and D). The square borders include the pairs with NORs.

1(0), 2i(1), 2e(0), 3i(1), 3e(1), 4i(1.5), 4e(2), 5(0). In *P. tiarano* the foot has only traces of webbing, both in the examined male or females (see Fig. 5 for a better comprehension of this character).
Mitochondrial differentiation

In the mitochondrial 16S rRNA gene, the uncorrected pairwise DNA sequence divergence of the *Paradoxophyla tiarano* sequence to those of *P. palmata* from east-central and southeastern Madagascar (7.8%, corresponding to 41 mutations) is about twice that observed between many species of *Scaphiophryne* (Tab. 2). Such high divergences have so far not been found within species of Malagasy amphibians, and the molecular data therefore corroborate the hypothesis of reproductive isolation and species status of *Paradoxophyla tiarano*.

Karyological comparison

Both the species show a karyotype of $2n = 26$ chromosomes, all biarmed and with the first five pairs much larger than the remaining 6-13 pairs. The pairs 2, 3, 10 and 11 are submetacentric, and the others are metacentric (Fig. 6 A, C). Loci of NORs in both the species are close to the centromere on the short arm of the 2nd chromosome pair (Fig. 6 A, C). The chromosomal formula and NOR location are basal, as is typical for the subfamily Scaphiopryninae and were found in 8 out of the 9 *Scaphiophryne* species (unpublished data). In the anurans it is common to observe the NOR location shared in several groups of closely related species (Mahony & Robison 1986, Aprea et al. 2004). The two *Paradoxophyla* species differ distinctly for the distribution of the heterochromatic material (Fig. 6 B, D). In *P. palmata* centromeric C-bands are visible only on the centromeric regions of the 2nd chromosome pair, corresponding to the NOR-associated heterochromatin. In *P. palmata* all the chromosomes have abundant telomeric heterochromatin, while in *Paradoxophyla tiarano* the telomeric heterochromatin is scarce. The centromeric heterochromatin, beside the nucleolus-associated material in the 2nd pair, is present in all the chromosomes of 6-13 pairs.

Justification for the new species

The difference in webbing between the two *Paradoxophyla* species is remarkably diagnostic and indicates a substantial morphological split. Furthermore, the DNA differentiation of the northern *Paradoxophyla* (7.8%, 41 mutations) to those from central, eastern and southeastern Madagascar is about twice as great as that between many species of *Scaphiophryne* (Tab. 2). Concerning the karyological aspects, there is a remarkable repartition of the heterochromatic component. The heterochromatin is mainly constituted by families of highly repeated DNA. Such a fraction of DNA rapidly accumulates mutations and diversifies quickly during the evolutionary speciation processes (Singer, 1982). C-banding staining carried out in different amphibian species, including the congeneric ones, shows karyotypes almost identical in number, shape and size. In all cases, the different species, and also the closely-related ones, showed differences in the distribution and/or composition of the C-bands (Morescalchi, 1983). Thus, the differences found in the C-banding pattern between the two *Paradoxophyla* species appear to be taxonomically relevant and support the morphological and molecular conclusion of distinctness of *P. tiarano*.
DISCUSSION

The discovery of a new *Paradoxophyla* in northeastern Madagascar confirms that the herpetofauna of this area is still incompletely known and home of peculiar endemics (Andreone, 2004). The application of two non-morphological techniques (karyology and mitochondrial DNA analysis) also detected a relevant differentiation between the populations from northeastern and eastern Madagascar. These differences are at the same level of those observed in other well-differentiated and undisputed species of microhylids, such as *Scaphiophryne* and *Dyscophus* (see Tab. 2). This confirms the validity of these methods that have been used in previous taxonomic surveys of Madagascan frogs (e.g., Andreone et al., 2003). The morphology and colouration of the two *Paradoxophyla* species appear similar and rather conservative, and the new species does not exhibit important and diagnostic morphological differences except for the webbing. We lack call recordings of the new species.

Although living within rainforest, *Paradoxophyla palmata* shows an explosive reproductive behaviour, and we predict that this might also apply to *P. tiarano*. Such a breeding strategy is typical for frogs from ephemeral waters in xeric areas, although it is also exhibited by species which frequent seasonal habitats. In general, these species are characterised by a great number of eggs. It might be questioned whether the explosive reproductive habitats is a plesiomorphic character or a phylogenetic apomorphy (as considered by Blommers-Schlösser & Blanc, 1993). The habitat choice in *Paradoxophyla* is considered as an adaptation to temporary pools in the forest. However, because the sister group of scaphiophrynines is not reliably known (Van der Meijden et al., 2004), phylogenetic data are currently insufficient and premature to speculate whether the presence of *Paradoxophyla* may be a secondary adaptation.

In terms of conservation, *P. tiarano* as well as *P. palmata* have so far only been found in aquatic environments within or very close to quite unaltered rainforests. Therefore, they appear to be critically tied to mature rainforests and to high humidity levels, especially outside of the breeding season. Given the fast deforestation rate in Madagascar, the safeguard of as many fragments as possible of pristine rainforests is almost certainly the most effective strategy to conserve Madagascar’s peculiar amphibian fauna. The presence of a large National Park at Masoala (ANGAP, 2003) is indeed a favourable factor for the conservation of *P. tiarano* and the many described or yet undiscovered frogs inhabiting this large forest block.

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