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A rapid amphibian survey at Itremo-Ambatofinandrahana, central Madagascar, with confirmed absence of chytrid fungus and recommendations for future monitoring activities

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Abstract. We conducted a rapid amphibian survey of the Atsirakambiaty relict altitude rainforest on Itremo-Ambatofinandrahana Massif, central Madagascar at an altitude of around 1550 m a.s.l. We detected a total of 12 amphibian species, whose taxonomic attributions were confirmed by both morphological and molecular data. Tissue samples were also screened for presence of a lethal chytrid fungus, *Batrachochytrium dendrobatidis* (Bd), which turned out to be negative. We encourage the herpetological community working in Madagascar to screen regularly amphibian tissue samples for Bd as a tool within the early detection plan recently launched for Madagascar.

Key words: Madagascar, Amphibians, *Batrachochytrium dendrobatidis*, Itremo, DNA Barcoding.

Madagascar is renewed for the high degree of biodiversity and concurrent habitat alteration (Meyers et al. 2000). Large sectors of its territory are currently threatened with deforestation since the first human settlements occurred around 2000 years ago. This is especially true when looking at the frog fauna of this large island (Andreone et al. 2005). It is well known that amphibians are among the most threatened vertebrates of the world, with about one third of species under threats due to habitat loss, alteration, trade and emerging pathologies (Stuart et al. 2004). Madagascar is not an exception. Recently, some leading experts on the endemic Malagasy amphibian fauna estimated that the island possesses at least 450 species, with many awaiting formal classification and description (Vieites et al. 2009). However, this stunning array of often remarkable and endemic amphibians is up against a catalogue of increasingly overwhelming threats (Andreone et al. 2008). These are principally habitat loss, forest degradation due to illegal logging, climate change, and pet trade (Andreone et al. 2006).

The eastern rainforest belt, once largely continuous, is currently severely fragmented (Vallan 2000). Anyhow, it still maintains a certain degree of connectivity, especially in north-eastern sectors, where large protected areas such as Masoala, Marojejy, and Tsaratanana constitute the last rainforest strongholds (Andreone 2004). The situation of the central highlands is more serious, as they are almost totally spoiled, eroded and virtually without any forest coverage, with exception of residual areas where relict altitude rainforests still persist (Andreone et al. 2007). Frequent fires and slash-and-burn practices at these montane sites shape composition and structure of the vegetation, and the herpetofaunal communities appear to withstand these predictable events, indicating that most probably the original vegetation type of the highlands of Madagascar was a mosaic of forests, grasslands and marshlands (Raxworthy & Nussbaum 1996, Klein 2002). However, small forest fragments still represent remarkable biodiversity and sometimes host in a few square meters species that have disappeared elsewhere (Rabemananjara et al. 2008). This happened for one of the most threatened amphibians of Madagascar, *Mantella cowanii* (Andreone & Randrianirina 2003). Indeed, this sharply coloured terrestrial frog is currently very rare and has a scattered distribution: apart from a well-known site next to Antoetra, only a few other sites have been recently confirmed, including a new record on the Itremo-Ambatofi-

nandrahana Massif in the Amoron'i Mania Region (Rabibisoa 2008, Rabibisoa et al. 2009). This site is a small altitude rainforest patch, and one of the last remnants of the likely original habitat for the species (Andreone et al. 2007). The study site is the Atsirakambiaty Forest, elevation 1550 m, 20°35'36"S, 046°33'48"E. The whole area has been severely deforested, which led to serious erosion of crystalline hills. The surface extension of this small forest fragment is extremely reduced and corresponds to a few hectares (< 5) (Randrianantoandro et al. 2009) (Fig. 1). To characterize the amphibian diversity of Atsirakambiaty, we carried out (25-30 November 2008) a rapid survey coupled with a screening for presence of a lethal chytrid fungus, *Batrachochytrium dendrobatidis* (Bd).

Figure 1. Localisation of the Atsirakambiaty Forest (from Google Earth®).

The presence of Bd on Madagascar has not been reported (Weldon et al. 2008, Weldon & du Preez 2008), except for an unconfirmed report from the Makay Massif (Rabemananjara et al. 2011). However, screening across the island is important in order to detect Bd as early as possible. Indeed, laboratory tests confirm that several Malagasy species are sensitive to it and potentially subject to mass mortality (C. Weldon, pers. comm. 2010); therefore an early detection plan is crucial to safeguard Malagasy amphibian fauna. As stressed elsewhere (Fisher et al. 2009), incidence of the chytrid is more likely at high altitude and where intense human activity potentially could introduce fungal spores, and sudden arrival of the chytrid in Madagascar could be extremely lethal for all its frog species. Although international amphibian trade is possibly driving spread of Bd (Kriger & Hero 2009), a human-mediated introduction of Bd to Madagascar, for example by ecotourists, is more likely (Mazzoni et al. 2003, Weldon et al. 2004, Garner et al. 2005, Fisher & Garner 2007, Skerratt et al. 2007, St-Hilaire et al. 2009).

Recent examples indicate potential sensitivity of insular amphibian populations. The recent arrival of the chytrid on Montserrat caused rapid extinction of a local mountain chicken (*Leptodactylus fallax*) (Martin et al. 2007). Recent surveys in Sardinia (Italy) also showed mass mortality in *Discoglossus sardus* (Bielby et al. 2009).

We searched for diurnal and nocturnal frog species using standard survey methodologies: opportunistic search and following male calls during the day and with the use of hand-held lights and headlamps during the night. Individuals were photographed, and, when possible, calls were recorded. Single toes of adults and fin tips of tadpoles were sampled and stored in 96% ethanol for molecular analyses. A few whole specimens were collected, anaesthetized, fixed in ethanol and subsequently deposited in the herpetological collections of Museo Regionale di Scienze Naturali, Torino, Italy (acronym MRSN), and Parc Botanique et Zoologique de Tsimbazaza, Antananarivo, Madagascar (PBZT-FAZC) (see Table 1 for details). *Mantella cowanii* was not collected as voucher material due to concern for its conservation, but only toe-clipped. A voucher collected previously is conserved at Antananarivo University (C. Randrianantoandro, pers. comm.).

Total genomic DNA was extracted from tissue samples using 5 PRIME, ArchivePure DNA Purification Kit. A fragment of ca. 550 bp from the 3' terminus of the mitochondrial 16S rRNA gene, suitable for amphibian identification through DNA barcoding (Vences et al. 2005), was amplified for 56 individuals of all identified taxa. Standard cycling protocols and primers (Vences et al. 2003) were used for amplification. Chromatographs were checked and sequences were edited when necessary using CodonCode Aligner (v. 2.0.6, Codon Code Corporation). The alignment required inclusion of gaps to account for indels in hyper-variable regions of the analyzed fragment. To assess species attribution and genetic distinctiveness of candidate new species, each sequence was compared using the BLAST algorithm in GenBank, taking into special account recently published wide screening of Malagasy amphibian di-

versity (Vieites et al. 2009). Working names of the candidate new species follow Vieites et al. (2009), but we also provide working names that candidate new species will have following recent suggestions of Padial et al. (2010) (see Tab. I).

The presence of the pathogen was detected using Bd-specific primers: Bd1a (5′-CAGTGTGCCATATGTCACG-3′) and Bd2a (5′-CATGGTTCATATCTGTCCAG-3′) (Annis et al. 2004), designed from ITS1 and ITS2 regions, respectively. PCR was performed in 20µl reactions using 0.2µl of Bd1a (0.2 mM), 0.2µl of Bd2a (0.2 mM), 2µl of total dNTPs (0.2 mM), 0.1µl (0.5U) of MasterTaq Eppendorf®, 2µl 1x buffer including $MgCl_2$ at 1.5 mM and 14.5µl of water. PCR conditions followed a sequence of an initial denaturation step at 93°C for 60s, 30 cycles of denaturation at 93°C (45s), annealing at 58°C (45s) and extension at 72°C (60s), followed by 10 minutes at 72°C of final elongation.

Twelve species of amphibians were identified in the forest fragment of Itremo (Table 1). The faunal assemblage found here is typical of Madagascar's highlands, and we confirm the presence of two previously identified but still undescribed species: *Mantidactylus* sp. 48 and *Mantidactylus* sp. 20 (*sensu* Vieites et al. 2009).

Apart from critically endangered *Mantella cowanii*, which is thus confirmed for the area and represents an important finding, the presence of two still undescribed *Mantidactylus* candidate species is remarkable due to the precarious habitat of the studied area. *Mantidactylus* sp. aff. *cowanii* [Ca FJ559273] (= *Mantidactylus* sp. 48, *sensu* Vieites et al. 2009) is morphologically similar to *M.* (*Hylobatrachus*) *cowanii* by the presence of small white spots on dorsum and flanks, although it is smaller and less uniformly blackish (Fig. 2). *Mantidactylus* sp. aff. *pauliani* [Ca AY848219] (= *Mantidactylus* sp. 20, *sensu* Vieites et al. 2009) is phenetically similar to *M.* (*Brygoomantis*) *pauliani*, which is restricted to the Ankaratra Massif (Glaw & Vences 2007) (Fig. 3). These two candidate species are likely endemic to Itremo, and, taking into account their very restricted extent of occurrence, severe habitat fragmentation, and increasing habitat alteration, they would qualify as Critically Endangered candidates (according to IUCN 2010), worthy of special conservation actions.

At Atsirakambiaty, we did not detect dead metamorphs or other episodes of illness or mortality associated with chytridiomycosis (Bosch et al. 2001). Indeed, all examined tissue samples were negative for Bd.

Our brief field study at Itremo is the first carried out in this highland area of Madagascar and represents the first published study in which a combination of taxonomic identification and chytrid detection screening has been applied. Although those techniques are comparatively easy and economical, there is still a lack of this kind of survey across the island, despite of the importance of monitoring for Bd presence. For such a reason, the present study is a blueprint for future activities

Table I. List of voucher specimens and tissue samples collected in Atsirakambiaty, and currently conserved in the Museo Regionale di Scienze Naturali (Torino, Italy; MRSN-FAZC), Parc Botanique et Zoologique de Tsimbazaza (Antananarivo, Madagascar; PBZT). Accession numbers for GenBank are provided between square brackets).

HYPEROLIIDAE - *Heterixalus rutenbergi,* MRSN A6742 [JF903869], MRSN A6771[JF903868], FAZC 14050 [JF903870]; MANTELLIDAE BOOPHINAE - *Boophis (Boophis) ankaratra*, MRSN A6754 [JF903877], MRSN A6755 [JF903879], FAZC 13999 [JF903874], FAZC 14001 [JF903876], FAZC 14036 [JF903878], FAZC 14060 [JF903880]; *Boophis (Boophis) goudotii,* MRSN A6745 [JF903884], MRSN A6746 [JF903883], FAZC 14024 [JF903881], FAZC 14030 [JF903882], *Boophis (Boophis) microtympanum*, MRSN A6769 [JF903885], FAZC 14064 [JF903886]; MANTELLIDAE MANTELLINAE - *Mantella cowanii,* PBZT 14068 [JF903923], FAZC 14069 [JF903924]; *Mantidactylus (Brygoomantis) curtus,* MRSN A6748 [JF903908], MRSN A6750 [JF903897], MRSN A6757 [JF903896], MRSN A6758 [JF903901], MRSN A6760 [JF903898], MRSN A6761 [JF903907], MRSN A6764 [JF903902], MRSN A6766 [JF903899], MRSN A6775 [JF903895], FAZC 13997 [JF903900], FAZC 14004 [JF903906], FAZC 14016 [JF903909], FAZC 14019 [JF903910], FAZC 14022 [JF903903], FAZC 14041 [JF903904], FAZC 14067 [JF903905]; *Mantidactylus (Chonomantis) brevipalmatus,* MRSN A6762 [JF903894], MRSN A6776 [JF903893]; FAZC 14009 [JF903892]; *Mantidactylus (Brygoomantis) betsileanus,* MRSN A6756 [JF903887], MRSN A6767 [JF903888], FAZC 14005 [JF903889], FAZC 14058 [JF903890], FAZC 14062 [JF903891]; *Mantidactylus (Ochthomantis) femoralis,* MRSN A6743 [JF903914], MRSN A6753 [JF903913], FAZC 14006 [JF903911], FAZC 14007 [JF903912], FAZC 14029 [JF903915], FAZC 14033 [JF903916], FAZC 14040 [JF903917]; *Mantidactylus (Hylobatrachus)* sp. aff. *cowanii* [Ca FJ559273], MRSN A6759 [JF903918], MRSN A6763 [JF903920], FAZC 14035 [JF903919]; *Mantidactylus (Brygoomantis)* sp. aff. *pauliani* [Ca AY848219], FAZC 14066 [JF903922]; PTYCHADENIDAE - *Ptychadena mascareniensis,* MRSN A6747 [JF903871], MRSN A6749 [JF903872].

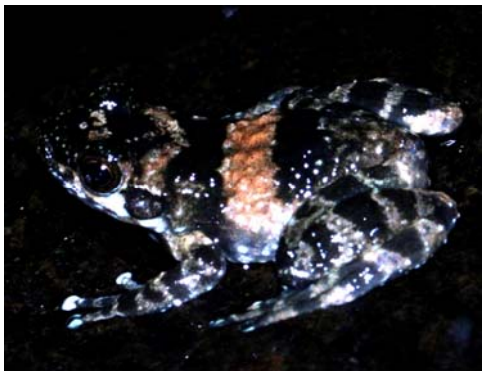

Figure 2. *Mantidactylus (Hylobatrachus)* sp. aff. *cowanii* [Ca FJ559273] = *Mantidactylus* sp. 48, *sensu* Vieites et al., 2009).

Figure 3. *Mantidactylus* (*Brygoomantis)* sp. aff. *pauliani* [Ca AY848219] = *Mantidactylus* sp. 20, *sensu* Vieites et al., 2009).

to identify and monitor the presence of chytrid fungus in Madagascar.

The current results for Itremo also provide further evidence that Bd is possibly yet localised in Madagascar, or, at least, not yet widely distributed. Considering the relevance of early detection of the pathogen, we stress the need to couple future taxonomic survey with rapid screening for Bd. It will be crucial investigate a wide range of sites, including high altitude sites with heavy anthropogenic pressure, although we also advocate Bd screening in areas much more sensitive to potential introductions and human transit, such as spots close to harbors, airports, etc. This is also valid for the most visited protected areas (national parks, special nature reserves, etc.), where tourist influx could introduce the Bd pathogen (Wollenberg et al. 2010).

Although this negative evidence for Bd is encouraging, it is also compulsory to continue research in Madagascar. During a workshop held at Ivoloina, Toamasina, Madagascar (13-18 October 2010), a working group of more than 30 participants established a protocol to systematically test the presence of Bd across the island during a trial period of three years (2011-2013). This protocol recommended experimental monitoring twice yearly at eight sites for 3 years, starting in 2011 (Garcia 2010). Moreover, a draft nationwide "Early Detection Plan" was also developed during this workshop, and a set of key sites and species to survey has been proposed. This plan is currently under revision with the Amphibian Specialist Group in Madagascar to determine how to turn the plan into action.

In many areas of the word, chytridiomycosis has been rapidly spread into naïve ecosystems, causing severe problem to native amphibian fauna (Berger et al. 1998, Lips et al. 2006). Therefore, we stress the importance of such a monitoring program. Similarly, we advocate the need to carry out screening for Bd whenever an amphibian survey is conducted on the island. This would be the best way to assure an early detection of chytrid all around Madagascar. Indeed, although it might also not be enough, an early detection of the pathogen is the first step in saving amphibian fauna of the island in case the chytrid should arrive to Madagascar. Since Bd is a major issue of amphibian conservation, distributed surveys can be extremely useful for early detection of new infections hotspots across wide, poorly investigated areas.

Similarly, due to the high number of candidate species we stress the importance of using an integrative taxonomy approach (e.g. bioacoustics, molecular) across Madagascar (Vieites et al. 2009, Padial et al. 2010), encouraging the publication of species accounts for investigated areas. In absence of an integrative taxonomic approach, the final list of taxa present in a certain studied area may be imprecise. So far, sampling toe-clips seems ideal for this purpose, since it allows taxonomic molecular identification and chytrid detection.

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