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Short communication

Amphibian survey and current absence of *Batrachochytrium dendrobatidis* (*Bd*) in Ivoloina Park, Toamasina (eastern Madagascar)

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Abstract.—Amphibians are threatened globally by the chytridiomycete fungus *Batrachochytrium dendrobatidis* (Bd), which is still expanding in range. Madagascar, rich in amphibian diversity, remains one of the few places that the fungus has not invaded. Herein, we present results from a pilot survey for Bd in conjunction with a rapid amphibian survey conducted at Ivoloina Park, a forestry station near Toamasina, eastern Madagascar. This park is located on the south-western side of the Ivoloina River in a former lowland rainforest now predominantly covered by plantations of exotic trees. Our amphibian survey confirmed the presence of 12 anuran species identified by both morphology and molecular barcoding and revealed the presence of four candidate species. Real-time polymerase chain reaction screening for the presence of Bd showed that all 59 samples tested negative, thus confirming the absence of the pathogen in this area. Our survey of Ivoloina provides the first species list for a suburban park in Madagascar complemented with chytridiomycosis screening.

Key words.—Madagascar, Tamatave, amphibians, chytridiomycosis, DNA barcoding

For the past few decades, amphibians have been facing an extinction crisis that threatens up to 50% of all species (Stuart et al. 2004; Gewin 2008). Amphibian extinctions are caused by the synergy of multiple factors, including habitat loss, climate change, allochtonous invasive species and emerging infectious diseases (Beebee & Griffiths 2005; Blaustein & Dobson 2006). *Batrachochytrium dendrobatidis* (Bd) is known to be the agent responsible for chytridiomycosis (Berger et al. 1998; Lips et al. 2006), infamous for the number of species that have been impacted and its propensity to drive them to extinction (ACAP 2005).

Most of the extant terrestrial vertebrate fauna of Madagascar colonised the island 60–70 million years ago when Madagascar was already separated from other landmasses (Vences et al. 2003; Crottini et al. 2012; Samonds et al. 2012), and as a

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result Madagascar now hosts a high concentration of endemic, diverse and threatened animals and plants (Myers et al. 2000; Wilmé et al. 2006). The degree of endemicity in Malagasy amphibians is surprisingly high for vertebrates, where undescribed species diversity is also exceptionally high with about 150 candidate species currently awaiting formal description (Glaw & Vences 2007; Vieites et al. 2009).

Madagascar has a well-developed network of protected areas (Conservation International 2003), but ongoing habitat destruction and political instability have weakened protection in many Reserves and put others at risk. Therefore, the listing of Madagascar's endemic species should be a priority. Moreover, the government of Madagascar is responsible for evaluating species richness and taking any necessary conservation actions as part of the Convention on Biological Diversity. In response, the number of species inventory surveys has increased over the last two decades, and the application of an integrative taxonomic approach using comparative morphology, bioacoustics and molecular genetics has increased the number of described amphibian species (Mercurio et al. 2008; Gehring et al. 2010; Crottini et al. 2011a, 2011b; Rosa et al. 2012).

Madagascar is currently considered to be free of Bd (Weldon et al. 2008; Crottini et al. 2011a; Vredenburg et al. 2012), despite areas of high habitat suitability within the island (Lötters et al. 2008). This, coupled with a recent unconfirmed finding of Bd at Makay Massif (Rabemananjara et al. 2011; Fig. 1), provides cause for alarm. The importance of monitoring the status of Bd within Madagascar was highlighted in 'A Conservation Strategy for the Amphibians of Madagascar (ACSAM)' launched in 2006 (Andreone & Randriamahazo 2008; Andreone et al. 2008; Andreone et al. 2012), an initiative under the umbrella of the International Union for Conservation of Nature/Species Survival Commission (IUCN/SSC) Amphibian Specialist Group – Madagascar. Accordingly, in 2010 the National Monitoring Plan (NMP) for early detection of Bd was launched. Eight sites will be monitored for the presence of Bd twice a year for three years (Fig. 1).

To address the requirements of contributing to the listing of Madagascar's endemic amphibian species and to test amphibian communities for the occurrence of Bd, we used a molecular taxonomic approach coupled with a Bd screening of the amphibian community inhabiting Ivoloina Park in eastern Madagascar.

Sampling area

Ivoloina Park is located 15 km north of Toamasina (18°02′-18°04′S, 49°20′-49°21′E, 10-100 m.a.s.l.) on the south-western side of the Ivoloina River in an area originally comprised of lowland rainforest (Fig. 1). It consists of a 282 ha forestry station, created in the 1920s, where researchers have carried out fisheries research and evaluated the performance of exotic tree species in Madagascar's eastern climate. The habitat is dominated by exotic tree plantations (e.g. *Eucalyptus* spp. and *Pinus* spp.) and interposed with marshlands and associated rice paddies, lakes and a small patch of native lowland forest (Ramasindrazana 2009). This station also has a small botanical garden and a holding facility/rehabilitation centre for confiscated lemurs and tortoises, which was developed into a zoo in 1963. More recently, activities in Ivoloina Park have focused on forest restoration with native plant species, *ex-situ* conservation, agroforestry practices/trials, environmental education as well as training activities.

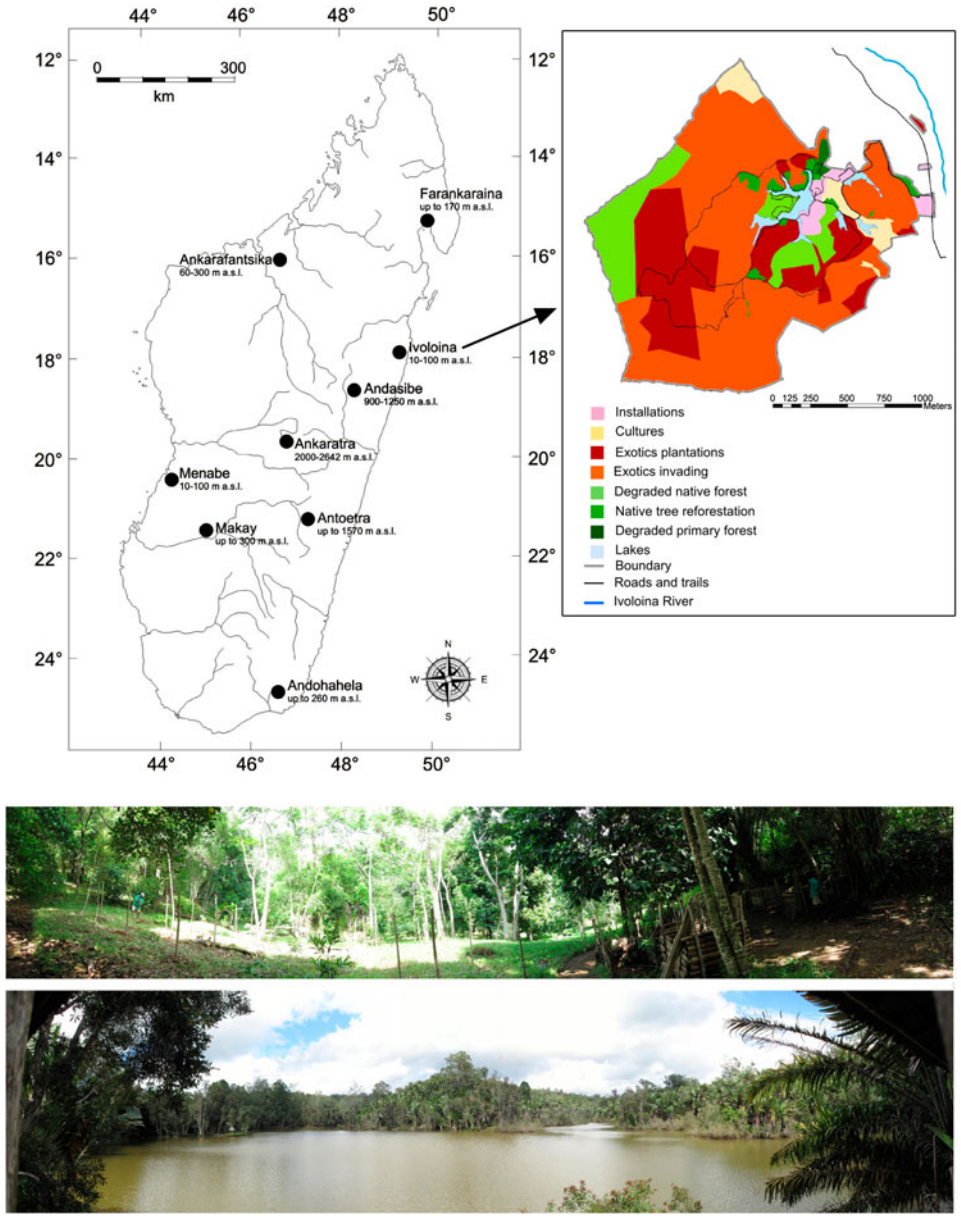

Figure 1. Distribution of survey sites of the National Monitoring Plan (NMP) for early detection of in Madagascar (including the position of Ivoloina Park), and position of the Makay Massif, where an unconfirmed positive record for Bd has been reported. The arrow points to a use map of the Ivoloina Forestry station, where the present survey took place. At the bottom are two pictures of the Ivoloina Park.

Sampling strategy

Ivoloina Park was one of eight sites from the National Monitoring Plan, which also included Andasibe, Ankarafantsika, Ankaratra, Antoetra, Fohisokina, Mandena and Menabe (see Fig. 1 for more details). Our Bd survey here at Ivoloina doubled as disease surveillance training for members of the NMP, which was provided for partnered non-governmental organisations (NGOs), academia and other institutions. For this work we followed the sampling method outlined in the survey protocol for the NMP. The two aspects of the protocol that we deviated from were the choice of species for compiling the sample and the diagnostic assay. Because the national survey had not officially commenced, we were non-selective with regards to species. Rather, we sampled all the 59 frogs that were encountered during three consecutive nocturnal searches that took place between 20:00 and 22:00 on 12, 13 and 14 October 2010. During these three nocturnal searches all participants of the course 'Disease screening in amphibians' (around 30 people) plus six instructors investigated an area of approximately 20 ha, for a total of about 216 survey hours (36 people $\times 2$ hours $\times 3$ days). We searched for frogs with head-torches and by following the calls of reproductively active males in suitable habitat such as forest leaf litter, fallen logs and the edges of ponds and brooks. We used a rigorous pathogen hygiene protocol to minimise accidental anthropogenic spread of Bd between sites and specimens. This involved the disinfection of footwear before and following fieldwork (5 min soak in 2% sodium hypochlorite solution), a pair of disposable gloves for each specimen handled, and individual housing of frogs in clear plastic bags (Declining Amphibian Population Task Force [DAPTF] 1998). We disposed of all waste materials through incineration.

Chytrid survey

To detect Bd on the collected specimens, we took a skin sample using a sterile fine-tip swab that was streaked five times over the ventral surface of the hind legs, feet and drink patch. We stored the swabs in dry and cool conditions and sent them to the National Zoological Gardens (NZG) of South Africa. We isolated Bd DNA following the PrepMan® Ultra protocol (Applied Biosystems™, Foster City, CA).

Table 1. Frog species per family and outcome of *Bd* screening at Park Ivoloina.

Family	Species	No. *Bd* infected / no. frogs collected
Hyperolidae	*Heterixalus madagascariensis*	0/19
Mantellidae	*Blommersia dejongi*	0/2
	Boophis tephraeomystax	0/7
	Guibemantis timidus	0/2
	Mantidactylus sp. 36 MV2009/*Mantidactylus* (*Brygoomantis*) sp. aff. *betsileanus* [Ca AY848260]	0/21
Microhylidae	*Anodonthyla* sp. aff. *boulengeri* [Ca JX101750]	0/3
Ptychadenidae	*Ptychadena mascareniensis*	0/5
Total		**0/59**

We determined the presence and concentration of the fungus using a real-time Polymerase Chain Reaction (rt-PCR) TaqMan standard curve assay according to Boyle et al. (2004). We used the StepOnePlus™ real-time PCR system from Applied Biosystems™ for the TaqMan assay. As a positive control, we used Bd isolate CW36 that was isolated from an *Amietia fuscigula* from Van Staden's Bridge, Eastern Cape, South Africa in 2004. In addition, we included no-template controls to ensure absence of contamination. All samples and controls were analysed in duplicate.

We analysed 59 swab samples from seven species (Table 1) and all were Bd negative. Among the tested species, both *Mantidactylus* sp. 36 MV-2009 and *P. mascareniensis* proved to be susceptible to chytridiomycosis when exposed to the pathogen through *ex-situ* challenge experiments (C. Weldon, pers. comm.). Because Bd shows little to no host specificity (Berger et al. 1998; Daszak et al. 1999), it is likely that frogs from Madagascar will display susceptibility traits similar to other naïve populations after initial exposure to the pathogen (Berger et al. 1998; Lips et al. 2005; Vredenburg et al. 2010).

AMPHIBIAN SURVEY

A total of 31 samples, consisting of single toes of adults, were stored in 96% ethanol for molecular analyses (see Online Supplementary Material). Additionally, we collected 33 voucher specimens, which were anaesthetised by immersion in chloretone, fixed in 90% ethanol and stored in 70% ethanol (details in Online Supplementary Material). We deposited prepared specimens in the herpetological collections of the Museo Regionale di Scienze Naturali, Torino (MRSN), Italy, and in the Université d'Antananarivo, Département de Biologie Animale (UADBA). We used proteinase K digestion (10 mg/ml concentration) to digest the toe-clips followed by a standard salt protocol to extract total genomic DNA (Bruford et al. 1992).

For amphibian identification we amplified a ~550 bp fragment from the 3' terminus of the mitochondrial 16S rRNA gene using standard primers and cycling protocols as in Rosa et al. (2012). We used CodonCode Aligner (v. 2.0.6, Codon Code Corporation) to visualise chromatographs and edit sequences. The alignment required inclusion of gaps to account for indels only in the hyper-variable regions of the analysed fragment. To assign species and to test for genetic distinctiveness of new candidate species, we compared each sequence using the BLAST algorithm in GenBank and an almost complete Malagasy amphibian inventory as a reference database (Vieites et al. 2009). Confirmed candidate species are generally identified based on a 5% minimum divergence threshold for the 16S rRNA gene (Vences et al. 2005; Fouquet et al. 2007; Vieites et al. 2009; Padial et al. 2010), and a divergence in morphology, bioacoustics or ecological features from other similar taxa. Amphibians that could not be assigned to any described or identified candidate species were considered Unconfirmed Candidate species (sensu Vieites et al. 2009) and were recognised as potentially new taxa that will likely be described in the future. We submitted our newly generated sequences to GenBank (see Online Supplementary Material).

We identified 12 species of amphibians in Ivoloina: *Blommersia dejongi*, *Blommersia* sp. aff. *blommersae* [Ca JX101724] (where [Ca ABXXXXXX] represents Candidate species 'Ca', and the following code and number refer to the GenBank

accession number of a reference sequence deposited in a public database together with all information on the sample and its origin), *Boophis tephraeomystax*, *Gephyromantis* sp. 25 MV-2009 (or *Gephyromantis* (*Duboimantis*) sp. aff. *boulengeri* [Ca FJ559196]), *Guibemantis bicalcaratus*, *Heterixalus madagascariensis*, *Mantidactylus* sp. 36 MV-2009 (or *Mantidactylus* (*Brygoomantis*) sp. aff. *betsileanus* [Ca AY848260]), *Guibemantis timidus*, *Ptychadena mascareniensis*, *Plethodontohyla mihanika*, *Anodonthyla* sp. aff. *boulengeri* [Ca JX101750], *Aglyptodactylus madagascariensis*. The latter was the only species for which we could not obtain a 16s rRNA gene sequence.

The presence of four undescribed species (two of which are candidate new species and two are unconfirmed candidate species) is particularly noteworthy. *Mantidactylus* sp. 36 MV-2009 (or *Mantidactylus* (*Brygoomantis*) sp. aff. *betsileanus* [Ca AY848260]) and *Gephyromantis* sp. 25 MV-2009 (or *Gephyromantis* (*Duboimantis*) sp. aff. *boulengeri* [Ca FJ559196]) were already identified by Vieites et al. (2009) and classified therein as confirmed candidate species worthy of being described in the future. In addition to high genetic divergence *Mantidactylus* sp. 36 MV-2009 (or *Mantidactylus* (*Brygoomantis*) sp. aff. *betsileanus* [Ca AY848260]) differs from *Mantidactylus betsileanus* by quantitative call differences, slight morphological differences and occurs in the region of Toamasina; while *Gephyromantis* sp. 25 MV-2009 (or *Gephyromantis* (*Duboimantis*) sp. aff. *boulengeri* [Ca FJ559196]) in addition to high genetic divergence has qualitative call differences, although a morphological assessment with regard to *G. boulengeri* has not yet been performed. This taxon was already known from the nearby Mahasoa Forest. *Blommersia* sp. aff. *blommersae* [Ca JX101724] is morphologically similar to the sympatric *Blommersia dejongi*, but differs from it by greater than 5% genetic divergence; however, its status still needs to be verified, we therefore propose to considered it an Unconfirmed Candidate Species (UCS). *Anodonthyla* sp. aff. *boulengeri* [Ca JX101750] is phenotypically similar to *Anodonthyla boulengeri*, although they are genetically highly differentiated ($>7\%$); however, comparative morphological analyses are still needed and call recordings are missing, therefore in the meantime we propose to consider it a UCS.

Conclusions

The lack of Bd in Ivoloina Park is encouraging, especially because the park is close to Toamasina, a large city with the largest seaport in Madagascar. High commercial trade can negatively impact the batrachofauna of Ivoloina by potentially introducing disease along with commercial cargo. Further threats come from tourist access to the zoological park and intensive agricultural activities. Despite these threats, we add Ivoloina Park to the list of regions in Madagascar that are currently Bd-free (Weldon et al. 2008; Crottini et al. 2011a; Vredenberg et al. 2012); however, Ivoloina will be screened twice a year for the next three years to monitor the disease status of its batrachofauna.

Our study on Bd screening and molecular identification of frog species is the first for a suburban park in Madagascar. Owing to the importance of Bd screening to the conservation of the amphibians inhabiting Madagascar, we encourage the herpetological community working there to regularly screen amphibian tissue samples, or at

least the tissue samples that are extracted to perform molecular taxonomic identification.

The previously described study and the parallel screening at eight selected sites during the three-year NMP will maximise the chances for early detection of chytrid and could act as a powerful tool for the conservation of this unique fauna. Indeed, this ongoing monitoring activity has encouraged coordination of the research activities on chytrid across the island and has gained support from all national authorities, such as the Ministère de l'Environnement et des Forêts and the University of Antananarivo, thus maximising the chances of counteracting the spread of this pathogen if it should arrive in Madagascar.

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