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Molecular systematics of Malagasy poison frogs in the *Mantella betsileo* and *M. laevigata* species groups

FALITIANA C.E. RABEMANANJARA[1,2], ANGELICA CROTTINI[4,5,8], YLENIA CHIARI[3], FRANCO ANDREONE[5], FRANK GLAW[6], REMÍ DUGUET[7], PARFAIT BORA[2], OLGA RAVOAHANGIMALALA RAMILIJAONA[2] & MIGUEL VENCES[8,9]

[1] *Institute for Biodiversity and Ecosystem Dynamics, Zoological Museum University of Amsterdam, Mauritskade 61, 1092 AD Amsterdam, The Netherlands. E-mail: frabeman@science.uva.nl*
[2] *Département de Biologie Animale, Université d'Antananarivo, BP, 906, 101 Antananarivo, Madagascar*
[3] *Department of Ecology and Evolutionary Biology, YIBS-Molecular Systematics and Conservation Genetics Lab., Yale University, 21, Sachem Str., New Haven, CT, 06520-8105 USA*
E-mail: ylenia.chiari@yale.edu
[4] *Universitá degli Studi di Milano, Dipartimento di Biologia, Sezione di Zoologia e Citologia, Via Celoria 26, 20133 Milano, Italy.*
[5] *Museo Regionale di Scienze Naturali, Sezione di Zoologia, Via G. Giolitti, 36, 10123 Torino, Italy.*
[6] *Zoologische Staatssammlung München, Münchhausenstr. 21, 81247 München*
[7] *Biotope, Agence Océan Indien, 969 ch. cent Gaulettes, 97440 Saint-André, La Réunion*
[8] *Zoological Institute, Technical University of Braunschweig, Spielmannstr. 8, 38106, Braunschweig, Germany.*
E-mail: m.vences@tu-bs.de
[9] *Corresponding author*

Abstract

Malagasy poison frogs of the genus *Mantella* with its 16 species are currently sub-divided into 5 major groups. Of these, the *Mantella betsileo* group is traditionally understood as containing four species, *Mantella betsileo, M. expectata, M. viridis* and *M. manery*, while the *M. laevigata* group is considered to be monospecific. A phylogenetic analysis of samples from multiple localities of all species in these two groups, based on sequences of the mitochondrial cytochrome *b* gene, shows the existence of several well-distinct clades in what is currently considered to be *Mantella betsileo*: (1) central-western populations from Kirindy, Isalo, and near Antsirabe close to the Betsileo region, to which the name *M. betsileo* is to be applied, (2) populations of the north-east and north-west, which are closely related to *M. viridis* and to which the name *M. ebenaui* is to be applied, and (3) a clade from southernmost Madagascar and from the Tsingy de Bemaraha, which is sister to *M. expectata* and furthermore includes important intra-clade variation, therefore probably representing one or two undescribed species. Our data also support a large genetic distance of *M. manery* to all other species and its probable sister-group relationship to the sympatric *M. laevigata; M. manery* is consequently transferred from the *M. betsileo* group to the *M. laevigata* group.

Key words: Anura, Mantellidae, *Mantella betsileo, Mantella ebenaui, Mantella expectata, Mantella laevigata, Mantella manery, Mantella viridis,* Madagascar

Introduction

Vis-à-vis of the world amphibian decline (Kiesecker *et al.* 2001; Pounds *et al.* 2006; Stuart *et al.* 2004), an increased knowledge on genetic data and their interpretations are needed for conservation purposes (e.g. Moritz & Faith 1998; Frankham *et al*. 2002; DeSalle & Amato 2004). Mainly due to fast deforestation, the amphibian biodiversity of the fourth largest island of the world, Madagascar, is heavily threatened (Andreone

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& Luiselli 2003; Andreone *et al.* 2005; Vallan 2000). As a result of this ongoing process, numerous Malagasy species are actually classified in the Red List of IUCN (Andreone & Luiselli 2003; Andreone *et al.* 2005; IUCN 2006) as threatened species, and Madagascar is considered to be a hotspot for biodiversity conservation (Myers *et al*. 2000).

The genus *Mantella* is composed of 16 recognized species, and is probably the most prominent group of Madagascar frogs (Vences *et al*. 1999; Glaw & Vences 2006). Frogs in the unrelated family Dendrobatidae, which inhabit Central and South America, are among the ecological equivalents of the genus *Mantella*, and both groups, because of their bright colouration and diurnal habits, have been intensively exploited by the international pet trade (Andreone *et al*. 2005; Rabemananjara *et al*. *in press*). At the same time, the presence of numerous alkaloid compounds in their skin makes these groups of poisonous frogs particularly interesting from a pharmacological point of view, and as in many other animals the presence of these substances goes along with an aposematic colouration that acts as a warning signal for predators (Servedio 2000). The source of these alkaloids is basically determined by the frogs' diet, largely consisting of ants and mites (Daly *et al*. 1994, 1997, 2002; Jones *et al*. 1999; Clark *et al*. 2005). Currently all species in the genus *Mantella* are included in the Appendix II of the Convention on the International Trade of Endangered Species (CITES).

The descriptions and systematics of the Madagascan batrachofauna have been pioneered by the explorer A. Grandidier in the second part of the 19th century (e.g., Grandidier 1869, 1872), and the species of *Mantella* firstly described were *Mantella madagascariensis,* and *M. betsileo* from the Betsileo region (Grandidier 1872). Until now, over 230 amphibians have been described from Madagascar, and the increasing use of molecular biological methods to detect genetic differences and to reconstruct phylogenies, in concert with intensive field explorations, is leading to an accelerated rate of taxonomic revisions and discoveries of new species (Köhler *et al*. 2005).

Recent molecular work on mantellid frogs led to a largely revised classification at the genus and subgenus level (Glaw & Vences 2006; Glaw *et al.* 2006). For the genus *Mantella*, the analysis of the full mitochondrial genome has been completed, which will help to speed up phylogenetic and taxonomical studies (Kurabayashi *et al*. 2006). The subdivision of the genus into five monophyletic groups as well as the evidence of homoplastic evolution of colour pattern of the different species was first demonstrated by Schaefer *et al.* (2002) and later corroborated by Vences *et al.* (2004a) and Chiari *et al.* (2004). Some species of the genus show evidence of hybridization, with shared haplotypes and a weakly expressed phylogeographic structure: e.g., *M. aurantiaca*, *M. crocea* and *M. milotympanum* (Chiari *et al*. 2004), *M. cowani* and *M. baroni* (Chiari *et al*. 2005), and *M. baroni* and *M. nigricans* (Rabemananjara *et al*. 2007). In contrast, other species such as *M. bernhardi* show a strong genetic differentiation among geographically close populations (Vieites *et al*. 2006). In general, however, it is remarkable that the genetic differentiation among and within species of *Mantella* is relatively small as compared to other species of frogs in Madagascar which often show remarkable intraspecific genetic distances of up to 5% in the 16S rRNA gene and above 10% in the coxI gene (Vences *et al*. 2005), and even much higher interspecific genetic distances. This indicates that divergences among current species of *Mantella* are relatively recent in age, although thorough molecular clock calculations are so far missing.

Two species groups in the genus *Mantella* have so far remained unstudied from a molecular systematic perspective: the *Mantella betsileo* and *Mantella laevigata* groups. While the *M. laevigata* group contains a single species, *M. laevigata*, the *M. betsileo* group is usually considered to contain four described species (Vences *et al*. 1996, 1999): *Mantella betsileo, M. manery, M. viridis* and *M. expectata*. Of these, *M. expectata* and *M. viridis* are considered as Critically Endangered, *M. laevigata* as Near Threatened, *M. manery* as Data Deficient, and *M. betsileo* as Least Concern, following IUCN categories (Andreone *et al*. 2005). The "brown" populations, considered to be *Mantella betsileo,* are widespread throughout much of Madagascar. Preliminary data have indicated that probably more than one species is included in *M. betsileo*, both from a morphological and a genetic perspective (Vences *et al*. 1999; Schaefer *et al*. 2002). Large-sized specimens both from Ankarana in the north-west, and from the west and south-west of Madagascar, were considered to be a different species ("*Mantella* sp. 1") by Vences *et al*. (1999). Recently, Glaw & Vences (2006) argued that based on the finding of a "brown" *Mantella* population near Antsirabe, close to the Betsileo region of Madagascar, the western populations of "brown" *Mantella* are to be seen as the true *M. betsileo*, and consequently the name *Mantella ebenaui*, originally described by Boettger (1880), was resurrected for populations from the northern part of the island.

Here we provide an analysis of mitochondrial DNA sequences of multiple specimens from populations of all species of the *Mantella betsileo* and *M. laevigata* groups. We included samples of "brown" *Mantella* from all geographic regions of Madagascar and obtained a sample of *Mantella manery*, a species known from two specimens only and never studied from a genetic perspective thus far. Our results provide evidence for *M. manery* not being related to the *M. betsileo* group but instead being sister to the sympatric *M. laevigata*, while populations of "brown" *Mantella* belong to several highly divergent genetic lineages that may represent distinct species and need to be considered separately in conservation evaluations.

Methods

Sampling

Samples were collected from July 2003 to April 2006 at fifteen localities as summarized in Table 1 and in fig. 1 (Marojejy and Isalo containing two and three separate sampling sites, respectively), with samples of a total of 87 specimens. Tissues of many individuals were obtained by toe clipping and subsequent release of specimens, a method that in frogs is known to allow high survival rates for the released individuals (>98%; Hott *et al*. 1999). Samples were conserved in 99% ethanol. Representative *Mantella* individuals from the new localities were collected and preserved as voucher specimens in the collection of the Département de Biologie Animale of the University of Antananarivo (UADBA), in the Zoological Museum of Amsterdam (ZMA), and in the Zoologische Staatssammlung München (ZSM), partly after skinning them for analysis of alkaloids; from these specimens, larger tissue samples of femur muscle were taken. Further identifying acronyms used for tissue samples are FR (lab number of F. Rabemananjara), FGMV, FGZC (field number of F. Glaw), ZCMV (field number of M. Vences), and FA (samples collected by F. Andreone).

DNA extraction and sequencing

Total genomic DNA was extracted from the tissue samples using proteinase K digestion followed by a standard salt extraction protocol (Bruford *et al*. 1992). We used the primers CBJ10933 5’-TATGTTCTAC-CATGAGGACAAATATC-3’ (forward) and Cytb-c 5’-CTACTGGTTGTCCTCCGATTCATGT-3’(reverse) (Bossuyt & Milinkovitch 2000) to amplify one part of the mitochondrial cytochrome *b* gene of about 600 bp. PCRs were performed in 25.6 µl reactions using 1µl of genomic DNA, 0.8µl of each 10µM primer, 2µl of total dNTP 4mM, 1µl of 25mM $MgCl_2$, 2.5µl $10xNH_4$ superTaq PCR buffer (HT Biotechnology), 0.25µl of 5u/µl iTaq DNA Polymerase (HT Biotechnology) and 17.25µl of water. PCR conditions were performed with an initial denaturation step at 94°C for 90 seconds; 35 cycles at 94°C for 30 seconds, annealing temperature of 53°C for 45 seconds followed by an extension at 72°C for 30 seconds, final extension of 10 minutes at 72°C. PCR products were loaded on 1% agarose gels, stained with ethidium bromide, and visualised on a "Gel Doc" system (Syngene). Products were purified using QIAquick spin columns (Qiagen) prior to cycle sequencing. Each 10 µl sequencing reaction included 1µl of template, 1.75 µl of 5x sequencing buffer (BigDye Terminator Sequencing buffer, Applied Biosystems), 1 µl of 10 µM primer (Cytb-c or CBJ), 0.5 µl of ABI sequence mix (BigDye Terminator V1.1 Sequencing Standard, Applied Biosystems) and 5.75 µl of water. The sequence reaction was 3 minutes of denaturation at 90°C, 24 cycles of 30 seconds at 96°C, 15 seconds at 50°C and 4 minutes at 60°C, final extension 4 minutes at 60°C. Sequence data collection and visualisation were performed on an ABI 3100 or an ABI 3730 automated sequencer at the sequencing facility of the Medical Center of the University of Amsterdam. Sequences were submitted to Genbank (accession numbers EF179618-EF179704).

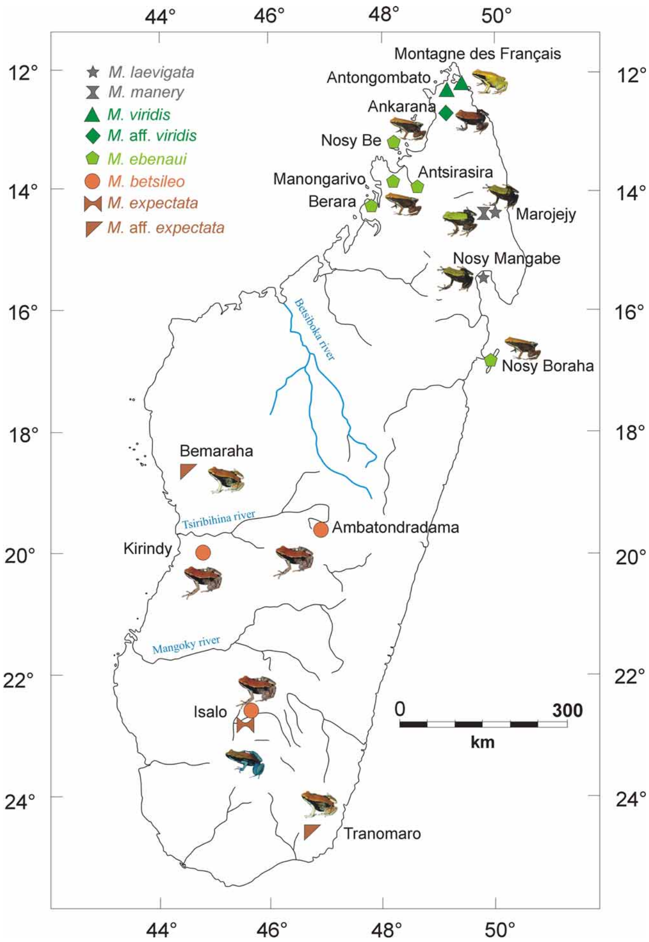

FIGURE 1. Localities of the collected samples included in the molecular analyses of the *Mantella betsileo* and *Mantella laevigata* groups.

Data analysis

Sequences were edited and aligned using Sequence Navigator software (Applied Biosystems). We did not detect stop codons or need to add indels to the alignment. A Neighbor-Joining tree based on uncorrected p-distances was constructed with PAUP*, version 4b10 (Swofford 2002) in order to gain a first overview of differentiation among sequences. Haplotypes were merged using the program Collapse, version 3.1 (Posada 2004). Phylogenetic analyses were performed using the programs PAUP* and MrBayes, version 3.1 (Ronquist & Huelsenbeck 2003). We performed both Bayesian and Maximum-Parsimony (MP) analyses in order to check for consistency in the results using different algorithms based on different assumptions of molecular evolution. MP bootstrap analysis was performed in PAUP* with 250 replicates for the distinct haplotypes. For the Bayesian analysis, we partitioned our data by codon position, as this partitioning strategy is known to often perform better with protein-coding mtDNA (Brandley *et al.* 2005). Modeltest version 3.06 (Posada & Crandall 1998) was used to choose the appropriate model of sequence evolution for each partition, which were (1) nst = 6, rates = gamma, with gamma distribution shape parameters of, respectively, 0.2314 and 2.1520, for the first and the third position, and (2) nst=1, rates=equal for the second codon position. The Bayesian analysis was run with four parallel chains for 1 million generations, sampling every tenth tree and discarding the first 10,000 trees (thus the first 100,000 generations) as burn-in based on empirical evaluation of the likelihood values.

In both analyses we used a homologous sequence of *Mantella bernhardi* as outgroup (Genbank accession number AB239569), based on previous studies that have shown this species to be the most basal *Mantella*

(Vences *et al*. 2004a; Chiari *et al*. 2004). One sequence of each *Mantella madagascariensis* and *M. cowani* were further included as hierarchical outgroups (Genbank accessions AY723691, AY862305).

Results

480 bp of 90 sequences (87 plus three outgroup sequences) were aligned and analysed (Table 1). The analysis of all sequences using Neighbor-Joining, as well as the analysis of the 59 distinct haplotypes (identified by Collapse) using Maximum Parsimony (MP) and Bayesian inference resulted in largely identical topologies. These are resumed here in the MP tree, with bootstrap and posterior probability values, shown in fig. 2.

TABLE 1. Coordinates, species and sample size for each locality.

Locality	Coordinates	Altitude	Species	Sample size
Montagne des Francais	12°19.09’S, 49°20.51’E	136 m	*M. viridis*	10
Antongombato	12°22.96’S, 49°13.50’E	110 m	*M. viridis*	4
Ankarana	12°58.48’S, 49°07.33’E	45 m	*M.* aff. *viridis*	9
Nosy-Be	13°25'S, 48°19'E	<50 m	*M. ebenaui*	1
Antsirasira	13°56'22'S, 48°33'16"E	<100 m	*M. ebenaui*	1
Manongarivo (Antanambao)	13°53'23"S, 48°29'03"E	9 m	*M. ebenaui*	2
Berara	14°18'33"S, 47°54'55"E	170 m	*M. ebenaui*	1
Marojejy	14°26.33’S, 49°46.57’E	411 m	*M laevigata*	9
Marojejy	---	---	*M. manery*	1
Nosy Mangabe	15°30'S, 49°46'E	<100 m	*M. laevigata*	1
Nosy Boraha	16°54.54'S, 49°52.01'E	20 m	*M. ebenaui*	7
Bemaraha	18°42'31"S, 44°43'08"E	146 m	*M.* aff. *expectata*	11
Kirindy	20°04’35’’S, 44°40’30’’E	55m	*M. betsileo*	13
Isalo Andrehitogna	22°32’19’’S, 45°24’40’’E	756 m	*M. betsileo*	4
Isalo (FA)	---	---	*M. betsileo*	2
Isalo Oasis	22°37’37’’ E, 45°21’12’’S	776 m	*M. expectata*	4
Ambatondradama	19°38.85’S; 46°02.99’E	922 m	*M. betsileo*	1
South of Tranomaro	24°27.36'S, 46°31.90'E	443 m	*M.* aff. *expectata*	6

Two main clades were recovered: (1) *M. laevigata* and *M. manery*, and (2) the *M. betsileo* group. The *M. betsileo* group is further partitioned, by our data, in two main lineages, a northern lineage containing *M. viridis* and several population of "brown" *Mantella*, and a southern lineage consisting of *M. expectata* and the remaining populations of "brown" *Mantella*.

"Brown" specimens, which all had previously (e.g., Blommers-Schlösser & Blanc 1991; Glaw & Vences 1994) been assigned to *Mantella betsileo,* are now suggested to include: (1) *M. betsileo sensu stricto,* corresponding to the species occurring at Kirindy, Ambatondradama near Antsirabe, and Isalo, a clade supported by 100% bootstrap and 100% posterior probability (see Discussion for the rationale of assigning the name *M. betsileo* to these populations); (2) populations from Bemaraha and from Tranomaro which are sister to *M. expectata* (90% bootstrap and 100% posterior probability) and are here named *M.* aff *expectata*; (3) the population from Ankarana which shows haplotype sharing with *M. viridis* and is here named *M.* aff. *viridis;* (4) the populations from Nosy Boraha, Manongarivo, Antsirasira, Berara and Nosy Be, which are paraphyletically arranged basal to a lineage containing *M. viridis* and *M.* aff. *viridis,* and are here named *M. ebenaui*.

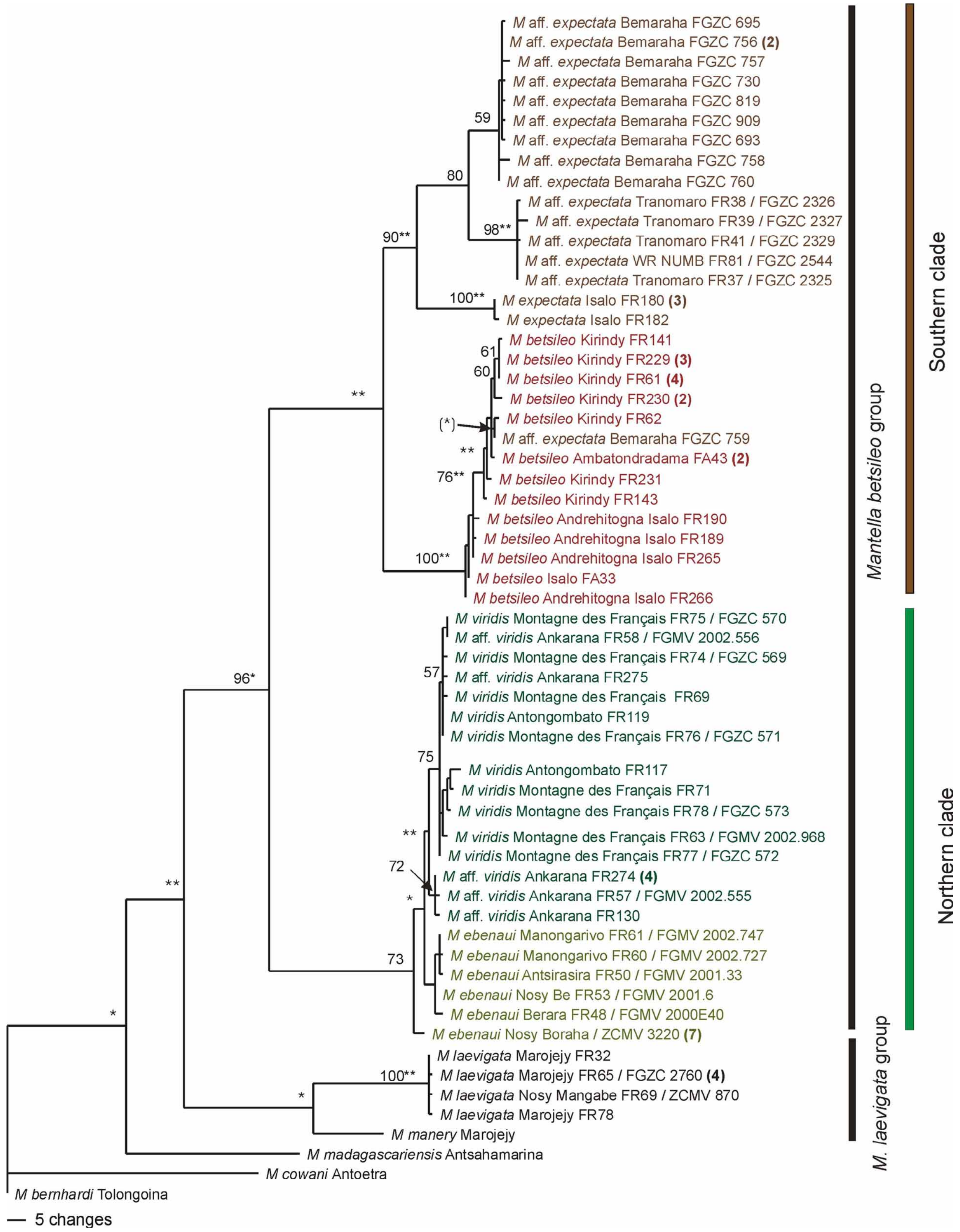

FIGURE 2. Phylogram from a Maximum Parsimony analysis, representing a 50% majority-rule consensus tree of 65700 equally most parsimonious trees. *Mantella bernhardi* was defined as outgroup. Specimens with identical haplotypes were merged; numbers in brackets after names of taxa give the number of specimens with the same haplotype. Numbers at nodes are bootstrap values in percent from a Maximum Parsimony bootstrap analysis with 250 replicates. Asterisks denote posterior probabilities from a partitioned Bayesian analysis: (*) >90%; * >95%; ** >99%.

Despite very obvious sequence similarities, not all clades were supported by both Bayesian and MP analysis. For example, the clade of *M. ebenaui*, *M.* aff. *viridis* and *M. viridis* was not supported as monophyletic group by the Bayesian analysis, although all haplotypes in this clade are extremely similar to each other. The same was true for the set of sequences of *M.* aff. *expectata* from Bemaraha. A possible explanation is that in data sets of short sequences, with many taxa that are highly similar to each other, likelihood methods in general may perform poorly. However, further speculations or analyses on this problem are far beyond the scope of the present paper. Independent from these analytical problems, most of our main conclusions remain unaffected since in no case did an analysis method provide any relevant support for an alternative topology contradicting the placements as summarized above. Additionally, unpublished data of K. Wollenberg and M. Vences indicate that sequences from other genes (16S rRNA and cox1) support all of the phylogenetic relationships in the *M. betsileo* group reported here.

Between the species of the *M. laevigata* and *M. betsileo* groups, the uncorrected genetic divergence (p-distance, transformed into percent) ranges between 12.9% (*M. manery*-*M. betsileo*) and 16.1% (*M. laevigata*-*M. betsileo*). *M. laevigata* as well is differentiated from *M. manery* by a high distance (10.4%). The two major lineages (northern - southern) of the *M. betsileo* group are separated by 6.5% to 7.5%. The southern lineage consisting of *M. betsileo*, *M*. aff. *expectata* and *M. expectata*, is constituted by well separated entities with distances of the order of 3.0% (*M. expectata*-*M*. aff. *expectata*) to 9.2% (*M. expectata*-*M. betsileo*). On the contrary, inside the northern lineage, constituted by *M. viridis*, *M*. aff *viridis* and *M. ebenaui* a low differentiation was found (1.5% to 2.3% between *M. ebenaui* and *M.* aff. *viridis*-*M. viridis*).

Cases of haplotype sharing were noted between *Mantella viridis* and *M.* aff. *viridis*, which are geographically neighbouring forms, genetically very close to each other. A further possible case was noted among *M.* aff. *expectata* specimens from Bemaraha, one of which had a deviant haplotype clustering in the *M. betsileo* lineage.

Discussion

Taxonomy of the *Mantella betsileo* group

Our data corroborate previous assumptions (Vences *et al*. 1999) that the *Mantella betsileo* group is composed of several species, although the encountered pattern of distribution of these species distinctly differs from any previous hypothesis. The "brown" *Mantella*, previously all subsumed under the name *M. betsileo*, can in fact be divided into three major genetic lineages: (1) a sublineage of the southern clade (sister to *M. expectata* and *M.* aff. *expectata*), here considered as *M. betsileo*; (2) a lineage distributed mainly in the north and closely related to *M. viridis*, here considered as *M. ebenaui* (and including *Mantella attemsi* Werner, 1901 as junior synonym, as argued by Glaw & Vences 2006); (3) a lineage sister to *M. expectata* (here named *M.* aff. *expectata*) which further contains relevant genetic differences between the two populations included here. Additional "brown" *Mantella* population from the north (here represented by Ankarana and named *M.* aff. *viridis*) resulted to share haplotypes with *Mantella viridis* and may be considered to be conspecific with this species. The sympatric occurrence of two representatives of the southern lineage (*M. betsileo* and *M. expectata*) in Isalo contradicts an extreme view that would interpret all of these lineages to be colour morphs or geographical variants of the same species. Consequently, our data support the partition of *Mantella betsileo sensu lato* as anticipated by Glaw & Vences (2006), and in addition indicate the existence of at least one further undescribed species (*Mantella* aff. *expectata*).

The identity of *Mantella betsileo* has long remained a mystery. Its original description by Grandidier (1872), as *Dendrobates betsileo*, was based on specimens collected in the Betsileo region, which refers to an ethnic group in the province of Fianarantsoa. Ten years later, Boettger (1880) described a brown-coloured *Mantella* from the island of Nosy Be in the north-west as *Dendrobates ebenaui*. Interestingly, the type locality of *Mantella betsileo* has never been confirmed since Vences *et al*. (1999) and Glaw & Vences (2006) hypothesized that the collecting locality of the types of *M. betsileo* may have been located along the travel route of Grandidier *towards* the Betsileo region, but not exactly *in* the Betsileo region. The discovery by one of us (FA) of a population of "brown" *Mantella* at Ambatondradama near Antsirabe, which is close to the Betsileo territory, has been anticipated by Glaw & Vences (2006) but is first reported in detail in the present paper (specimens captured on 20 October 2004 by J. E. Randrianirina, in a sort of gallery forest along streams, rich in *Ravenala* traveller palms). This is the first such record and confirms that it is the species mostly distributed in central-western Madagascar that is to be named *Mantella betsileo*, whereas the northern populations should be named *Mantella ebenaui*.

The taxonomic status of the populations here named *M.* aff. *expectata* requires further study. These frogs occur in western and south-western Madagascar where suitable habitat is scarce and populations are likely to be patchily distributed. Nevertheless, there are further locality records of "brown" *Mantella* from the south-western part of Madagascar (Vences *et al.* 1999), and of *M. expectata* from the Mikea forest (Thomas & Kidney 2005) and possibly from near Morondava (see Vences *et al.* 1999) which need to be evaluated, before a new species can possibly be described. Most important is to elucidate the relationships between *M.* aff. *expectata* and *M. expectata*. The latter species displays a blue-yellow colour pattern that is unique among *Mantella* and almost certainly a derived state. *M.* aff. *expectata* from Tranomaro, where we collected several adult male and female specimens, does not share with *M. expectata* this blue-yellow pattern, but there is at least one chromatic character that is in agreement with its placement close to *M. expectata:* both share a light stripe along the upper lip (frenal stripe) that does not reach the tip of the snout, while it does reach the snout tip in all other taxa in the *M. betsileo* group. Unfortunately, all specimens of *M.* aff. *expectata* that we collected in the Bemaraha massif were juveniles, and therefore their adult colouration remains unknown. However, from available photos taken by other researchers it is clear that at this locality, specimens with a orange-brown dorsal colouration and diamond-shaped markings on the back occur, different from *M. expectata* which has a yellowish dorsum and no diamond-shaped marking. Furthermore, the juvenile specimens collected by us, in preservative, all have a typical pattern of "brown" *Mantella* populations, including a frenal stripe that reaches to the tip of the snout (e.g., ZSM 15/2006, 32/2006, 44/2006, 58/2006, 73/2006 and 120/2006).

Rediscovery and relationships of *Mantella manery*

Mantella manery is an enigmatic species known only from two specimens collected in the Marojejy Massif in north-eastern Madagascar, of which only one - the holotype - has been preserved. Despite the lack of data, Vences *et al*. (1999) felt forced to describe this species because, based on published photos, it had already been given invalid scientific names by various hobbyists, a situation that was leading to nomenclatural confusion. The rediscovery of the holotype later permitted to provide an adequate morphological description of the species (Vences *et al*. 2004b) but its relationships remained obscure. The species has several similarities with *Mantella laevigata*, which occurs in north-eastern Madagascar as well, such as the black belly with relatively small bluish dots, and the green-yellowish dorsal color which does not fully cover the posterior part of the dorsum. However, based on several morphological and especially chromatic characters (presence of a horseshoe marking on the throat and of a frenal stripe along the upper lip) *M. manery* had been placed in the *M. betsileo* group, while *M. laevigata* was kept in an own group based on its many derived characters (e.g., tree-hole breeding and enlarged discs of digits) (Glaw & Vences 1994; Vences *et al*. 1999).

Mantella manery remained the only species of *Mantella* for which no molecular data were available thus far. One of us (RD) could recently (November 2005) find one specimen of the species at the type locality, near a site in Marojejy National Park locally known as "Camp Mantella" (which is at the geographical coordinates 14°26'S, 49°47'E, 481 m above sea level). The precise locality was a small stream flowing into the main river, where after a search of 15 minutes a specimen was found around 10 am on a fallen bamboo trunk, following heavy rains in the previous night. At the same site, FG and MV had unsuccessfully searched for the species in

February of 2005, and FR had been with various colleagues collecting *Mantella laevigata* in 2004, both in the wet and dry seasons, without finding *M. manery.* This indicates that the species is probably rare at the type locality, but it may be more common at other, yet unknown sites. The newly collected specimen was a large female, and a molecular sample of it could be taken and analyzed herein. The results corroborate that this species is a genetically very distinct unit, surprisingly not belonging to the *M. betsileo* group but the sister species of *Mantella laevigata*. This relationship was not strongly supported in our analysis, but is backed up by analysis of sequences of other genes (K. Wollenberg and M. Vences, unpublished data).

Mantella laevigata is a largely arboreal species that can occur at sites with partly open canopy cover, whereas *M. manery* appears to be living on the floor of dense forest next to streams and rivers (RD, personal observation). Considering these different ecologies and the very high genetic differentiation among these species (more than 10%), it is unlikely that any hybridization takes place despite the close syntopy of both forms. Most adaptations of *M. laevigata* in its habits (arboreality), morphology (broad tips of fingers and toes) and reproduction (single-egg laying, parental care) are clearly derived and unique among *Mantella*. If these two species are indeed sister to each other, then it is likely that *M. laevigata* evolved from a less specialized ancestor which may not have been too different from *M. manery.* Since both species live in sympatry, and indeed can occur in close syntopy, evolutionary hypotheses that can be drawn at this stage are that (a) speciation may have occurred in sympatry or parapatry by ecological divergence of the ancestor of *M. laevigata*, and that (b) the very similar colour patterns of the two species may be an ancestral character, although selection through Müllerian mimicry may contribute to maintain this pattern stable over evolutionary time in the two species (Schaefer *et al.* 2002).

Molecular systematics and biogeography

In a recent study, Wilmé *et al*. (2006) hypothesized that the high faunal richness and local endemism in Madagascar may be explained by the river catchments with sources at relatively low elevations being zones of isolation leading to speciation of locally endemic taxa, whereas those with sources at higher elevations were zones of retreat and dispersion and hence contain proportionately lower levels of microendemism. The existence of geographically restricted lineages in the *Mantella betsileo* group, especially in western Madagascar, could offer a good opportunity to test this hypothesis but at present the ranges of these lineages are insufficiently known for any such explicit analyses.

A further influence of large rivers is that they may play a role as riverine barriers to gene flow (Wallace 1852; Ayres & Clutton-Brock 1992; Bermingham & Moritz 1998). The importance of rivers in western Madagascar for species formation and genetic isolation of lemurs has already been emphasized by Pastorini *et al.* (2003). Especially the Betsiboka river, the longest river of Madagascar which flows in a SE-NW direction, exerts a very important influence in this respect. Our results suggest, at a large geographical scale, the presence of the major phylogenetic subdivision in the *Mantella betsileo* group separating a clade occurring north of the Betsiboka from a clade occurring south of this river. However, again, without a more comprehensive geographic sampling, especially of populations closer to the river basin itself, we cannot confirm that it is really this river separating the major clades in the *M. betsileo* group.

Mantella betsileo as defined here is, of all species in the clade (and indeed of the whole *M. betsileo* group), the one occurring over the widest altitudinal range (55–1200 m) and also over a large geographical area encompassing both sides of the Mangoky river which therefore apparently has not played the role of a barrier for this species.

The two populations here referred to as *M*. aff. *expectata* are sister to each other in one clade, supported by a bootstrap value of 80%. Surprisingly, these two populations come from rather different lowland areas in Madagascar: one from very dry areas in the extreme south of Madagascar, near Tranomaro, the second one from the limestone massif of Bemaraha in the central west. These two areas are separated by a wide geographical gap, in which both *Mantella betsileo* and *Mantella expectata* occur. This pattern can at present not be explained by any simple biogeographic scenario, but further data are necessary to assess whether some other, geographically intermediate populations of "brown" *Mantella* belong into this clade as well. It needs to be remarked that a similar pattern was found in another vertebrate group: snakes of the genus *Acrantophis* from the extreme south of Madagascar, usually considered as *Acrantophis dumerilii,* have haplotypes that form a clade with those of the western/north-western species *A. madagascariensis* rather than with other *A. dumerilii* specimens from the south-west (Vences & Glaw 2003).

In contrast to the southern clade, the main lineages in the northern clade of the *Mantella betsileo* group are far less clearly differentiated genetically, although their morphological variability is similar to that of the southern clade. Most populations of *M. ebenaui* consist of small-sized, brown-coloured frogs, whereas *M.* aff. *viridis* is distinctly larger and equally brown-coloured and *M. viridis* is large and usually uniformly bright green-yellow, but none of these forms turned out to be a clearly monophyletic and genetically divergent lineage. If the poorly supported phylogenetic pattern resulting from our tree is correct, it suggests a relatively recent separation of *M. viridis* from *M.* aff. *viridis* which at its turn probably evolved out of a paraphyletic *M. ebenaui*. However, since our dataset includes only few specimens from many populations, and no information on differentiation in nuclear genes, we cannot distinguish between possible phenomena of recent and ongoing introgression or incomplete lineage sorting that may apply to some of these populations.

Different from the *Mantella cowani* group, as studied by Rabemananjara *et al*. (2007), the species in the *M. betsileo* group show a relatively distinct geographical signature in their haplotypes. In contrast, in the widespread *M. baroni* and *M. nigricans* in the *M. cowani* group, one cytochrome *b* haplotype was present and most common in all populations, separated by up to 1000 km of geographical distance (Rabemananjara *et al*. 2007).

In the *M. betsileo* group, haplotype sharing occurs between the closely related *M. viridis* and *M.* aff. *viridis*, but it seems to be less common among genetically more divergent lineages. Despite the sympatric occurrence of *Mantella expectata* and *M. betsileo* at Isalo, the specimens studied here did not show haplotype introgression, and although these species may occasionally and in some direct contact zones hybridize, there seems to be no broad genetic admixture, confirming their status as biological species. Among the Bemaraha samples, our data point out one possible case of haplotype introgression, indicated by the presence of a *M. betsileo* haplotype in what otherwise appears to be a population of *M.* aff. *expectata*. However, as said before, all available samples from this site came from juvenile specimens that lacked many of the characteristic colour patterns of adults, and therefore we cannot further interpret this deviant Bemaraha haplotype which might be indicative of (1) haplotype sharing among *M. betsileo* and *M.* aff. *expectata* through introgression or incomplete lineage sorting, or (2) sympatric occurrence of these two species in Bemaraha.

Mantella laevigata populations from Marojejy seem to be genetically similar to populations from Nosy Mangabe, indicating that this species is relatively uniform over much of its distribution area.

Conservation units

The genus *Mantella* is one of the most famous groups of Malagasy amphibians and is also prominent in the international commerce (Andreone *et al*. 2005; Rabemananjara *et al*. *in press*). One of the international strategies of *Mantella* species conservation has been the inclusion of all species of the genus in the Appendix II of the Convention on the International Trade of Endangered Species (CITES), but the lack of knowledge about the precise distribution and status of several *Mantella* species creates, every year, a very complex discussion about the opportunities and possible strategies for their sustainable exploitation. Actually, habitat loss constitutes the major threat of Madagascan fauna and the inexistence of a clear policy coupling conservation and development represents the major risk of extinction of a lot of species. The genetic data provided herein significantly contribute to a better knowledge of various species of *Mantella* and, therefore, could help to define more clearly some conservation priorities.

One first step for defining such priorities is to delimit the units to be considered separately for conservation efforts. In the *Mantella betsileo* and *M. laevigata* groups, according to our mitochondrial DNA data, there are a number of genetically well defined species which clearly qualify as such units: (1) *M. laevigata*, (2) *M. manery,* (3) *M. betsileo,* and (4) *M. expectata.* In addition, one undescribed form is genetically distinct and qualifies as unit for conservation, namely (5) *M.* aff. *expectata*. This latter form exhibits a clear differentiation between specimens from the two populations studied, but we here refrain from defining these two as independent conservation units until data from the whole range of this form become available and nuclear data will be gathered. Furthermore, there are two other species which are less easy to define based on the mitochondrial sequence data available, in particular *M. viridis* and the non-monophyletic *M. ebenaui.* However, in this case, there are distinct morphological and ecological differences such as *M. viridis* being larger, of a green-yellowish colour and adapted to much drier habitats than *M. ebenaui.* We suggest that in this case, the Adaptive Evolutionary Conservation concept proposed by Fraser & Bernatchez (2001) and evoked also in the studies of *Mantella bernhardi* (Vieites *et al*. 2006) and the *Mantella cowani* group (Chiari *et al*. 2005; Rabemananjara *et al*. 2007), is an adequate strategy to define also (6) *M. ebenaui* and (7) *M. viridis* as units for conservation. Because *M.* aff. *viridis* is morphologically intermediate between *M. ebenaui* and *M. viridis* (large body size and predominantly brown colouration) and shares haplotypes with *M. viridis,* we do not consider it as separate conservation unit but rather as a hybrid zone population.

In terms of red listing, *M. laevigata* is considered as Near Threatened, and its range encompasses several protected areas (Mananara Biosphere Reserve, Marojejy National Park, Masoala National Park, and Nosy Mangabe Special Reserve). Although the species is specialized to low-altitude forest which is under strong pressure, the Near Threatened category for this species appears to be appropriate.

The data deficient *M. manery* (also occurring in Marojejy National Park) deserves more thorough studies to ascertain its status before taking any decision about its conservation strategy, but as with other rare and localized species in northern Madagascar, it is likely that this species deserves being listed in one of the threatened categories.

For the *M. betsileo* group, our surveys confirmed localized occurrences of *M. expectata* and *M. viridis* which both are listed as Critically Endangered, and we support these categories.

Mantella betsileo is currently listed as Least Concern (Andreone *et al.* 2005), but this status is based on a wider taxonomic definition of this species. *M. betsileo* as redefined herein occurs in one protected area (Isalo National Park) and is known from only two additional sites (Kirindy and Ambatondradama). Despite a large extent of occurrence the known area of occupancy for the species is low, and there is no doubt that the distribution will continue to be patchy, even if new populations will be found in future surveys. Furthermore, forests and associated streams in western Madagascar are known to be in decline under heavy anthropogenic pressure which is almost certainly adversely affecting this species for which we therefore suggest a Red List category of Vulnerable.

Mantella ebenaui is a widespread and locally common species that tolerates high degrees of habitat disturbance. Populations now assigned to *M. ebenaui* were previously included in *M. betsileo* and their commonness and generalized habitat requirements were the reason for assigning the Least Concern status to that species. We consequently propose to consider *Mantella ebenaui* as Least Concern.

The last species for which we suggest special conservation attention is *M.* aff. *expectata*. Although not yet described as distinct species, this lineage occurs in the driest part of Madagascar and in a forested relict habitat in the central west (Tsingy de Bemaraha). There is no doubt that this species qualifies for inclusion in one of the threatened categories of IUCN, and we emphasize that more work is needed to understand the distribution, ecology, taxonomic status and genetic differentiation of this form.

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