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Mitochondrial evidence for distinct phylogeographic units in the endangered Malagasy poison frog *Mantella bernhardi*

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Abstract

***Mantella bernhardi* is an endemic species of Malagasy poison frog threatened by loss and fragmentation of its natural habitat and collection for the pet trade. It is classified as threatened according to the International Union for Conservation of Nature and Natural Resources (IUCN) categories and included in Appendix II of the Convention on the International Trade of Endangered Species (CITES). A recent survey has increased the known distributional range of the species from one to eight populations across southeastern Madagascar, but little is known about its biology and genetic diversity. Here we estimate inter- and intrapopulation mitochondrial genetic variation of four populations. Populations from the northern and southern parts of the distributional range showed a high degree of divergence (maximum of 11.35% in cytochrome *b*) and were recovered as reciprocally monophyletic groups. Nine haplotypes were detected in the northern and 12 in the southern populations. The population from Ranomafana National Park showed the lowest number of haplotypes and nucleotide diversity, and shared its most common haplotype with the second northern population from Tolongoina. All the other detected haplotypes were unique to each of the four populations. This suggests the existence of important barriers to gene flow, pre-dating human colonization of Madagascar at about 2000 years ago, in distinct contrast to other *Mantella* species that show a high degree of haplotype sharing throughout their range. The continued habitat fragmentation within the distribution range of *M. bernhardi* prevents any connection between its populations. Our data indicate the existence of at least two different management units for conservation in this species, corresponding to the North and South of its distribution range, and highlight the existence of strong regional endemism in southeastern Madagascar.**

Keywords: amphibians, conservation, cytochrome *b*, Madagascar, *Mantella bernhardi*, phylogeography

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Introduction

The evolution of species diversity in rainforests has captured much attention of researchers due to the high biodiversity of tropical regions. There have been a number of hypotheses that explain diversification in rainforest faunas, based on neutral or adaptive processes (reviewed by Moritz *et al*. 2000). Among these, the riverine model, with major rivers acting as an isolating barrier driving allopatric differentiation, seems to be especially relevant for taxa of low dispersal capacity, and has initially been applied to explain differentiation patterns of primates (Wallace 1852; Ayres & Clutton-Brock 1992). In Sulawesi,

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areas of endemism were defined using phylogeographic patterns of monkeys. These areas show a remarkable congruence with those defined using similar methods for toads. This indicates that similar processes may drive differentiation in these taxa (Evans *et al*. 2003).

Amphibians are a group belonging to the lower end of the relative dispersal ability spectrum (e.g. Inger & Voris 2001; Brown & Guttman 2002), although transoceanic colonizations have been demonstrated for a number of taxa (Hedges *et al*. 1992; Kaiser *et al*. 1994; Vences *et al*. 2003). Molecular studies in amphibians have usually revealed strong phylogeographic structure and limited gene flow among populations (e.g. García-París *et al*. 2000; Wake & Jockusch 2000; Kraaijeveld-Smit *et al*. 2005), although several species from the Palearctic and Nearctic have colonized wide ranges after the last glaciation and show very limited mitochondrial variability in these areas (e.g. Alexandrino *et al*. 2000; Steinfartz *et al*. 2000; Babik *et al*. 2004; Kuchta & Tan 2005). Many other amphibian species, however, show high levels of intraspecific mitochondrial divergence (e.g. James & Moritz 2000; Vences *et al*. 2005).

Madagascar is an ideal model region to test for general patterns of phylogeographic differentiation in amphibians. This island harbours a highly diverse and endemic amphibian fauna, which largely evolved in isolation due to the limited dispersal to and from other landmasses (Glaw & Vences 2003). Moreover, the Madagascan geography is of a relatively simple pattern, with a western arid region separated from the eastern rainforests by a central mountain chain. The eastern rainforest is interrupted only by rivers that flow down from the central highlands in a west–east direction into the Indian Ocean. The western dry and spiny forests are separated by rivers flowing in the opposite direction into the Mozambique Channel. These rivers are known to form significant phylogeographic barriers for lemurs (Pastorini *et al*. 2003). A mid-domain effect largely influences amphibian diversity, being highest at mid-altitudes and in the central-eastern rainforests (Lees 1996; Lees *et al*. 1999). So far, phylogeographic studies in Madagascan amphibians have mainly focused on Malagasy poison frogs, genus *Mantella*, and have found a relatively low degree of phylogeographic structure and common haplotype sharing in species from mid-altitude rainforest (Chiari *et al*. 2004, in press; Vences *et al*. 2004).

Mantella bernhardi is a species of Malagasy poison frog endemic to southeastern Madagascar and classified as endangered in the International Union for Conservation of Nature and Natural Resources (IUCN) Red List (Andreone *et al*. 2005). It was until recently known from only a single locality that suffers from habitat destruction and represented the only source population for the pet trade (Raxworthy & Nussbaum 2000). Recent fieldwork has considerably extended the known range of this species by the discovery of seven new populations, all in low-altitude rainforests (Rabemananjara *et al*. 2005). Here we provide data on the mitochondrial genetic structure within and among four populations of *M. bernhardi* from the northern and southern parts of its distribution area. The data indicate a surprisingly strong phylogeographic structuring in this low-altitude species, which we discuss in terms of a possible differentiation due to riverine barriers. Our findings also have implication for conservation actions, indicating the need of at least two separate managing units within *M. bernhardi* for conservation purposes.

Materials and methods

Sample collection

Tissue samples were collected in January and February 2004 in four of the eight known populations, spanning over most of the distributional range of *Mantella bernhardi* (Rabemananjara *et al*. 2005): Ranomafana National Park (21.4 S, 47.5 E), Tolongoina (21.55 S, 47.52 E), Manombo Special Reserve (23.0 S, 47.7 E), and Vevembé (22.8 S, 47.0 E). We collected one toe-clip from each individual, which was preserved in absolute ethanol, releasing immediately the animals after treating wounds with antiseptic. Sample sizes are given for each locality in Table 1.

DNA extraction and sequencing

Total genomic DNA was extracted using proteinase K (final concentration 1 mg/mL), and isolated by a standard salt extraction protocol (Bruford *et al*. 1992). A fragment of the mitochondrial cytochrome *b* gene was amplified via polymerase chain reaction (PCR) using the primers Cytb-c and CBJ10933 (Bossuyt & Milinkovitch 2000). PCRs were performed in 25- L reactions using 50 ng genomic DNA, 10 pmol of each primer, 15 nmol of each dNTP, 50 nmol additional $MgCl_2$ and the RED*Taq* PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.1 mM $MgCl_2$ and 0.01% gelatine) and 1 U of RED*Taq* DNA polymerase (Sigma). PCR conditions were as follows: an initial denaturation step at 94 C for 90 s; 35 cycles at 94 C for 30 s, annealing temperature of 53 C for 45 s, extension at 72 C for 60 s; final extension of 10 min at 72 C. PCR products were purified using QIAquick spin columns (QIAGEN) prior to cycle sequencing. A 10- L sequencing reaction included 1–2 L of template, 1 L of sequencing buffer, 2 L of 2 pmol primer, 1.8 L of ABI sequence mix (BigDye Terminator version 3.1 Sequencing Standard, Applied Biosystems) and 3.2–4.2 L of water. The sequence reaction was 33 cycles of 10 s at 96 C, 10 s at 50 C and 4 min at 60 C. Sequence data collection and visualization were performed on an ABI 3100 automated sequencer (Applied Biosystems). Sequences were deposited in GenBank; accession numbers DQ278651–DQ278803.

Table 1 Summary of mitochondrial DNA diversity for samples of *Mantella bernhardi* used in this study and other species of *Mantella* for comparison

Species and population	Sample size	Haplotypes	Polymorphic sites	Haplotype diversity (Hd)	Nucleotide diversity (Pi) × 100
M. bernhardi					
Ranomafana — N (1)	25	2	2	0.08 ± 0.07	0.03 ± 0.03
Tolongoina — N (1)	37	8	9	0.47 ± 0.10	0.15 ± 0.04
Manombo — S (1)	66	7	21	0.33 ± 0.07	0.27 ± 0.09
Vevembe — S (1)	25	5	4	0.38 ± 0.12	0.10 ± 0.04
M. milotympanum					
Fierenana (2)	20	9	14	0.79 ± 0.09	0.65 ± 0.39
M. crocea					
Ihofa (2)	26	10	11	0.85 ± 0.05	0.50 ± 0.31
Ambohimanarivo (2)	7	2	4	0.57 ± 0.12	0.43 ± 0.31
Savakoanina (2)	15	8	7	0.87 ± 0.07	0.38 ± 0.25
Andriabe (2)	13	7	9	0.73 ± 0.13	0.35 ± 0.24
North of Fierenana (2)	2	2	2	1.00 ± 0.50	0.38 ± 0.46
M. aurantiaca					
Torotorofotsy 1 (2)	6	2	7	0.62 ± 0.11	0.33 ± 0.22
Torotorofotsy 2 (2)	5	2	2	0.40 ± 0.24	0.15 ± 0.15
Andranomena (2)	10	4	48	0.68 ± 0.12	2.47 ± 1.32
Andranomandry (2)	16	11	36	0.93 ± 0.05	2.00 ± 1.08
M. madagascariensis					
Marolambo (3)	7	4	6	0.81 ± 0.13	0.32 ± 0.22
M. baroni					
Farimazava (4)	33	26	42	0.97 ± 0.02	1.24 ± 0.29
Mantady (4)	1	1	—	—	—
Vohidrazana (4)	10	8	12	0.93 ± 0.08	0.49 ± 0.10
Andranomena (4)	2	2	3	1.00 ± 0.50	0.57 ± 0.29
Ranomafana (4)	13	12	20	0.99 ± 0.04	0.70 ± 0.10
Tsinjoarivo (4)	3	2	1	0.67 ± 0.31	0.13 ± 0.06
Andriave (4)	5	3	2	0.80 ± 0.16	0.19 ± 0.05
M. cowani					
Soamazaka (4)	4	3	4	0.83 ± 0.22	0.38 ± 0.15
Vohisokina (4)	20	8	15	0.77 ± 0.08	0.54 ± 0.10
Vatolampy (4)	6	3	2	0.73 ± 0.16	0.22 ± 0.16
Farimazava (4)	8	5	25	0.79 ± 0,15	1.88 ± 0.71

References are given in parentheses: 1, this study; 2, Chiari *et al*. (2004); 3, Vences *et al*. (2004); 4, Chiari *et al*. (in press). For *M. bernhardi*, N and S identify northern and southern populations, respectively. Values are given ± standard deviation.

Data analysis

Sequences (502 bp) were edited and aligned using SEQUENCE NAVIGATOR software (Applied Biosystems). We did not detect stop codons or indels in the alignment. Haplotypes were merged using the program COLLAPSE version 1.2 (Posada 1999). Phylogenetic analyses were performed using the programs PAUP*, version 4b10 (Swofford 2002), and MRBAYES, version 3.1 (Ronquist & Huelsenbeck 2003). We performed both a Bayesian and maximum-parsimony (MP) analysis in order to check for consistency in the results using different algorithms based on different assumptions of molecular evolution. MP analysis was performed in PAUP* 4b10 using heuristic searches with tree-bisection–reconnection (TBR) branch swapping, step addition starting tree, and random addition sequence with 1000 replicates, using distinct haplotypes. We used two species of mantellids as outgroups: *Mantella baroni* and *Boophis ankaratra*. For the Bayesian analysis, we partitioned our data by codon position, as this partitioning strategy performs better with protein-coding mtDNA (Brandley *et al*. 2005). MRMODELTEST version 2.2 [Nylander 2004; modified version of MODELTEST 3.6 (Posada & Crandall 1998)] was employed to choose the appropriate

model of sequence evolution for each partition. We standardized the initial tree used for calculation of every partition model, by using a neighbour-joining (NJ) tree with Jukes–Cantor substitution model of the whole data set as starting tree, instead of a random NJ tree as is defined in MRMODELTEST by default. The models selected were K80 + G, HKY and GTR for the partitions of the first, second and third codon positions, respectively. The analysis consisted of four Markov chains that ran for 10×10^6 generations, sampled every 1000 generations, with a random starting tree, default priors and equal branch lengths for each partition. The burn-in parameter was empirically estimated by plotting –ln L against the generation number, and the trees corresponding to the first million generations discarded.

For each population we assessed nucleotide diversity and haplotype diversity using the program DNASP version 4.0 (Rozas *et al*. 2003). The method of statistical parsimony (Templeton *et al*. 1992) implemented in the TCS software package (Clement *et al*. 2000) was employed to depict phylogenetic and geographical relationships among the identified haplotypes. The program first defines the uncorrected distance between haplotypes above which the parsimony criterion is violated with more than 5% of probability, and then establishes connections between the haplotypes until the 'parsimony' limit is reached. Ambiguities were solved following the frequency, topological and geographical criteria (Crandall & Templeton 1993; Templeton & Sing 1993; Crandall *et al*. 1994; Posada & Crandall 2001).

Results

We obtained sequences for 153 individuals of *Mantella bernhardi*, ranging from 25 to 66 individuals per population (Table 1). Of the 502 bp of the cytochrome *b* gene analysed, 420 were constant and 64 were parsimony-informative. The *M. bernhardi* sequences contained 77 variable sites, which defined 23 haplotypes; haplotype and nucleotide diversity values are summarized in Table 1. Haplotype diversity was highest in Tolongoina, and lowest in Ranomafana. Nucleotide diversity was highest at Manombo, second highest at Tolongoina, and lowest at Ranomafana.

The trees resulting from Bayesian (Fig. 1) and MP analyses consistently recovered two reciprocally monophyletic haploclades. MP searches recovered a single most parsimonious tree (consistency index 0.90, retention index 0.97; not shown). The two clades, corresponding to the two northern and the two southern populations, respectively, were supported with high (100%) bootstrap values. The northern populations constituted a single haploclade, while in the southern clade the Vevembé population was supported by high bootstrap values as a different entity. The Bayesian tree agreed in the general topology with the MP tree, with high support for the two above-mentioned clades.

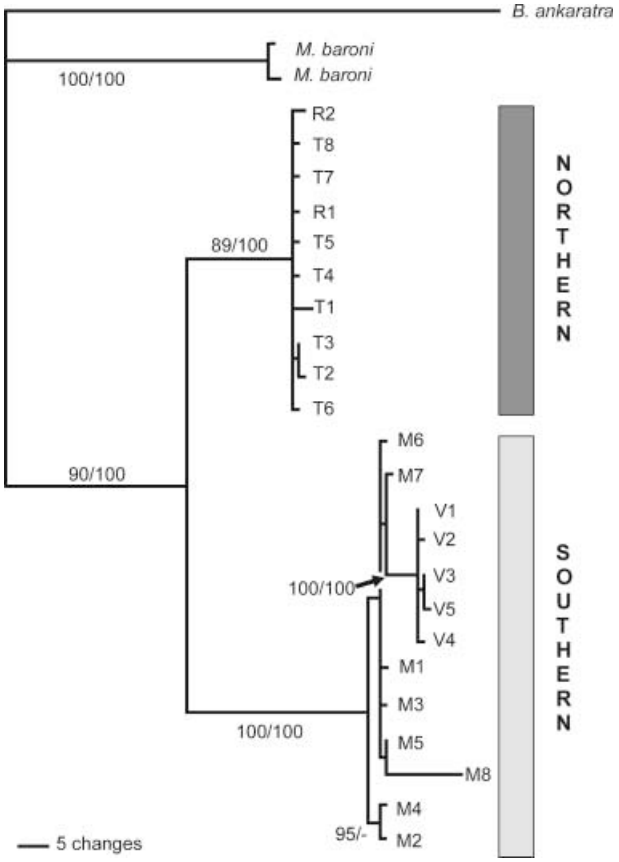

Fig. 1 Bayesian phylogram of the observed haplotypes showing the two clades of *Mantella bernhardi*. Haplotype codes refer to the first letter of each population and the number of that haplotype (R, Ranomafana; T, Tolongoina; V, Vevembé; M, Manombo). Bayesian posterior probabilities and bootstrap values higher than 85% in the Bayesian and MP analyses, respectively, are shown (BY/MP).

These results were congruent also with TCS analysis, which recovered two haplotype networks corresponding to the northern and southern populations, respectively (Fig. 2). Manombo (with eight haplotypes) and Vevembé (five haplotypes) had no haplotypes in common, nor did either share any haplotype with northern populations. In the two northern populations (Ranomafana and Tolongoina), one haplotype predominated (R1, Fig. 2), and was shared between both. The Ranomafana population showed very low genetic diversity, with only two haplotypes detected vs. eight unique haplotypes in Tolongoina. Most of the sampled specimens in the northern populations (96%) shared the same haplotype. We had to force the program to a minimum of 48 steps to connect the two networks between the haplotype R2 and M1 (Fig. 2). In the southern haplotype network, Vevembé haplotypes constituted again a separate entity within the southern clade.

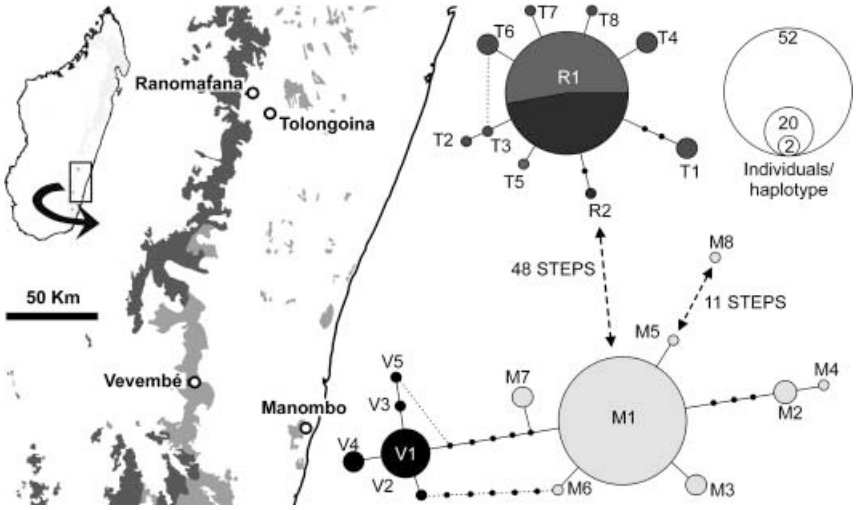

Fig. 2 Haplotype network of the four studied populations under the 95% cladogram estimation in TCS. Haplotype codes are the same as in Fig. 1, and alternative nodes are indicated by dashed lines. Only Ranomafana and Tolongoina shared a haplotype; all other haplotypes were unique to single populations. The program had to be forced to connect the two haploclades (see text). Geographic relationships of the sampled populations is shown on the left; light grey and dark grey represent, respectively, low-altitude and mid-altitude natural rainforest.

Discussion

Riverine barriers and phylogeography

Our data suggest the presence of two reciprocally monophyletic mitochondrial lineages within *Mantella bernhardi*, corresponding to the northern and southern populations, respectively. The high mtDNA divergence between these haploclades (with a maximum of 57 substitutions and 11.35% divergence between haplotypes T1 and V4), indicates long-term differentiation. This pattern stands in remarkable contrast to other species of *Mantella* from mid-altitude rainforest areas in Madagascar (Table 1). In one of these species, *Mantella baroni*, based on the analysis of a cytochrome *b* fragment homologous to the one used here, populations from the northernmost and southernmost regions largely shared similar or even identical haplotypes (Chiari *et al*. in press). Of the 17 species of *Mantella* recognized by Vences *et al*. (1999), six have so far been studied from a phylogeographic perspective: *M. baroni*, *Mantella cowani* (Chiari *et al*. in press), *Mantella aurantiaca*, *Mantella crocea*, and *Mantella milotympanum* (Chiari *et al*. 2004), and *Mantella madagascariensis* (Vences *et al*. 2004). Values of haplotype diversity (= gene diversity) and nucleotide diversity for these species are summarized in Table 1. Haplotype diversity ranged from 0.6 to 0.99 (counting populations where more than five individuals were sequenced), with most values higher than 0.6. Haplotypes within the *M. crocea*/*milotympanum* complex, and within *M. aurantiaca*, had maximum divergences of eight steps. Compared with these data, *M. bernhardi*, by contrast, is characterized by relatively lower haplotype diversity within populations (Table 1), but a higher among-population haplotype differentiation.

To reliably ascertain whether this interpopulational differentiation in *M. bernhardi* is due to fully disrupted gene flow or to a possible lower dispersal capacity of females, nuclear markers such as microsatellites are necessary. Such a male-biased dispersal pattern has been inferred by Lampert *et al*. (2003) in túngara frogs, and observed by Joly & Grolet (1996) for juvenile Alpine newts, *Triturus alpestris*, although evidence for female-biased dispersal was found in bullfrogs, *Rana catesbeiana*, by Austin *et al*. (2000), and in common frogs, *Rana temporaria*, by Palo *et al*. (2004). A further hypothesis that needs to be taken into account is that phylogeographic discontinuities in nonrecombining units such as mitochondrial genes may arise in continuously distributed species in the absence of gene flow if individual dispersal distances and population sizes are low (Irwin 2002). For *M. bernhardi*, our own unpublished data indicate high local population densities of 170–820 individuals per hectare, but nothing is known about individual dispersal distances. However, the fact that very few populations of this species are known and that it had eluded scientific collection before the 1990s (Rabemananjara *et al*. 2005) indicate that a historically continuous distribution is very unlikely. A phylogeographic discontinuity is not only present between northern and southern populations of this species but also (although much less pronounced) between Manombo and Vevembé. The high interpopulational divergences and the complete absence of haplotype sharing between north and south, and between Manombo and Vevembé, are therefore best explained by a long genetical isolation of these populations. Although the habitat of *M. bernhardi* is currently heavily fragmented, the available information suggests that most of the forest disappeared recently (Green & Sussman 1990) due to human activities. The amount of genetic divergence between the northern and southern haplotype groups are of a level that indicates the presence of barriers to gene flow pre-dating human colonization of Madagascar which is likely to have occurred less than 2000 years ago (Burney *et al*. 2003).

Comparisons of phylogeographic patterns for multiple codistributed species is a powerful tool to detect potential long-term spatial barriers to gene flow (Bermingham & Moritz 1998). Based on the Riverine Barrier Model (Wallace 1852; Ayres & Clutton-Brock 1992), large rivers facilitate genetic diversification in terrestrial organisms reducing the gene flow. However, the influence of rivers in this respect is controversial. Gascon *et al*. (2000) found no influence of a river in the Amazon basin on the present-day pattern of community similarity and species richness of frogs and marsupials. Lougheed *et al*. (1999) found a limited influence of a riverine barrier on the phylogeographic pattern of a frog species, *Epipedobates femoralis*, and Lugon-Moulin *et al*. (1999) found no significance influence of riverine barriers on the gene flow of the common shrew, *Sorex araneus*, in France. On the other hand, rivers are known to provide barriers to gene flow in primates (Peres *et al*. 1996; Eriksson *et al*. 2004), reptiles (Pellegrino *et al*. 2005), and even in understorey forest birds (Capparella 1991).

In Madagascar, Pastorini *et al*. (2003) provided evidence for a significant influence of several large rivers in western Madagascar on lemur phylogeography, but their data were insufficient to analyse the situation in the east. However, several more detailed studies indicated the existence of a genetic barrier between Ranomafana and Manombo/Vevembé. The black and white ruffed lemurs (*Varecia variegata*) are present both in Ranomafana and in Manombo, showing a high degree of genetic differentiation between the two sites (Louis *et al*. 2005). The same pattern of genetic differentiation has been found in brown lemurs (*Eulemur fulvus/albocollaris*), although in this case a stable hybrid zone was recorded in Andringitra National Park (between Vevembé and Ranomafana) (Sterling & Ramarason 1996). In this species, a putative barrier was located in the Manampatrana River (= Iantara River), which divides northern and southern lemur populations, appearing to serve as an important boundary in this hybrid zone (Wyner *et al*. 2002). Unfortunately, these studies on lemurs do not use a homologous genetic marker, and a direct comparison of the depth of the genetic divergences encountered in lemurs and frogs is therefore not possible. However, although there is little phylogeographic information concerning Malagasy amphibians, and existing works concern species from other parts of Madagascar (i.e. Chiari *et al*. 2004, in press; Vences *et al*. 2004), our data reinforce recent evidence that they might be diverged in response to similar barriers to gene flow as primates (Evans *et al*. 2003).

As pointed out by Peres *et al*. (1996), the specific characters of the riverine barriers need to be taken into account when studying their effect on gene flow. These authors found gene flow among adjacent subspecies of saddleback tamarins across Rio Jurua in Amazonia, but restricted to the headwater section of the river. In eastern Madagascar, rivers originate in the central highlands and flow eastwards into the Indian Ocean. These rivers obviously become larger towards lower altitudes, and therefore may constitute important barriers to species restricted to low-altitude habitat. This scenario could still explain why there is distinct interpopulational differentiation in the low-altitude specialist *M. bernhardi* while the mid-altitude *M. baroni* shows no relevant population subdivision (Chiari *et al*. in press). More intensive sampling of low-altitude specialists among Madagascan frog species is necessary to assess the impact of rivers, especially the Manampatrana River, on their genetic differentiation.

Management units for conservation in Mantella bernhardi

Madagascar is one region that deserves highest priority for biodiversity conservation (Myers *et al*. 2000). Amphibians are a group that is globally affected by important declines (Stuart *et al*. 2004), and in Madagascar all amphibian species but one (recently introduced) are endemic (Glaw & Vences 2003). For many of them fundamental data are lacking to reliably assess conservation priorities (Andreone & Luiselli 2003; Andreone *et al*. 2005). Rainforest destruction has been identified as one of the major causes of the loss of the Malagasy biodiversity (Green & Sussman 1990; Achard *et al*. 2002). Over-exploitation for the pet trade has also been identified as threat for a few species of Malagasy amphibians, especially of the genera *Dyscophus* and *Mantella* (Behra 1993; Andreone & Luiselli 2003). *Mantella* are included on Appendix II of the Convention on the International Trade of Endangered Species (CITES), and the numbers exported from Madagascar amount to several thousand individuals per year.

One of the goals of modern conservation biology is not only to preserve species and habitats, but also their evolutionary potential in terms of maintaining the genetic diversity of the extant species. In this context, conservation or management units should be clearly defined. There are very different criteria for defining these units in practice (e.g. Ryder 1986; US Fish and Wildlife Service & National Marine Fisheries Service 1996), and some can be controversial (e.g. Ryder 1986; Avise 1994; Moritz 1994; see review of Fraser & Bernatchez 2001). We here follow the rather flexible concept of adaptive evolutionary conservation (AEC) as proposed by Fraser & Bernatchez 2001). In this theoretical framework, an evolutionary significant unit (ESU) is a lineage demonstrating highly restricted gene flow from other such lineages within the higher organizational level of the species, and the best available biological information is used to exercise ESU definitions on a case-by-case basis. In *M. bernhardi*, the lack of habitat connection between southern and northern populations as assessed by Rabemananjara *et al*. (2005) (see also Fig. 2), and their

strong genetic differentiation suggests considering them as ESUs under this concept. The comparatively low haplotype and nucleotide diversity found within populations of this species (Table 1) indicates that a rather limited genetic diversity may add to the vulnerability of single populations.

Amphibians seem to be less sensitive to reduction of habitat size than birds, small mammals or reptiles (Goodman & Raherilalao 2003; Vallan 2003). The current isolation of *M. bernhardi* populations therefore probably does not represent an immediate threat, as long as some suitable habitat remains. Manombo and Ranomafana are protected as Special Reserve and National Park, respectively, but Vevembé is not. We suspect that the phylogeographic pattern observed in *M. bernhardi* is paralleled by other species. There are several other amphibian species that have been found by us at Vevembé but not at the other sites, and at least one of them (an undescribed species close to *Boophis albilabris*) may be a regional endemism. Hence, our data strongly suggest that this site merits inclusion in Madagascar's network of protected areas.

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