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Genetic variation of an endangered Malagasy frog, *Mantella cowani*, and its phylogeographic relationship to the widespread *M. baroni*

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Abstract

We investigated the degree and distribution of the genetic variation, and phylogeography, of two species of Malagasy poison frogs, *Mantella cowani* and *M. baroni*. The former is critically endangered due to its restricted distribution, habitat destruction and overcollection for the pet trade. Analysis of 526 bp of mtDNA (cytochrome *b*) resulted in separate haplotype networks for the two species, and discovered hybridization at a single locality. The two networks confirm the status of *M. baroni* and *M. cowani* as separate evolutionary species and units for conservation. Within both mitochondrial haplotype networks, specimens from different localities shared numerous identical haplotypes, even those from the most distant sample sites of *M. baroni*. Most populations were characterized by high haplotype diversity and no haplotype clades exclusive to geographical regions were observed. Protection of a few large populations of these species is therefore likely to conserve much of the mtDNA genetic diversity found in the entire species. While *M. baroni* is widespread and occurs in many nature reserves, we recommend efficient legal protection of some *M. cowani* habitats to protect this species against extinction.

Introduction

Amphibian populations are suffering worldwide declines, range contractions and extinctions (Stuart et al. 2004). This is due to different causes like habitat disturbance, diseases and pathogens, introduction of invasive species and exploitation for the pet-trade (reviewed e.g., in Semlitsch 2003). On Madagascar, the fourth largest islands of the world, over 99% of the amphibians are endemic. Amphibians are only represented on Madagascar by anurans with a number of species that surpasses 210 (Andreone and Luiselli 2003). Habitat destruction such as deforestation with the practice of slash-and-burn agriculture and heavy anthropogenic pressure negatively affects their survivorship and distribution (Raxworthy and Nussbaum 2000; Vallan 2000, 2002). In contrast, global declines caused by pathogens, especially chytrid fungi (Berger et al. 1998; Bosch et al. 2001; Carey et al. 2003) are so far apparently absent from Madagascar (Andreone et al. 2005). *Mantella cowani* is an endemic diurnal poison frog, which occurs mainly on the high plateau of east-central Madagascar. This species is confined to a small area of these highlands where primary forest has been almost completely destroyed or degraded (Vences et al. 1999; Andreone and Randrianirina 2003; Andreone et al. 2005). *M. cowani* is listed in the IUCN red list as critically endangered and in

CITES Appendix II for the decline of its habitat, drastic population reduction and the fragmented distribution (Andreone et al. 2005). This species is also over-exploited for the international pet-trade due to its conspicuous and attractive bright colouration. Little is known about its natural history, capacity for long-term survival in degraded habitats, and the status of most of its populations.

In the genus *Mantella*, five major groups can be genetically distinguished (Schaefer et al. 2002; Chiari et al. 2004; Vences et al. 2004). *M. cowani* belongs to the *M. cowani* group that also includes *M. baroni*, *M. haraldmeieri* and *M. nigricans*. The taxonomic history of these species has been complex (reviewed by Vences et al. 1999) and *M. cowani* and *M. baroni* may be sister species (Vences et al. 2004). *M. baroni* is known from a relatively wide distribution area in the central eastern mid-altitude rainforests, at elevations up to about 1000 m above sea level, whereas *M. cowani* is present only in the highland areas west of the rainforest belt and has been recorded up to 2000 m altitude at Ambatodradama (Vences et al. 1999). Probable hybrids between these two species have been identified in the pet trade, but the geographic origin of these individuals remained unknown (Glaw and Vences 2000).

Here we comparatively investigate the phylogeography and genetic variation of *M. cowani* and *M. baroni* by examining variation in 526 bp of the mitochondrial cytochrome *b* gene and discuss our results in regard to potential future conservation efforts.

Materials and methods

Sampling

Fieldwork was carried out in February 2002 and 2003. Eleven populations (eight of *M. baroni* and three of *M. cowani*) were sampled and geographic coordinates and altitude above sea level recorded by GPS (Table 1). The sampling localities extend along a North–South transect of ca. 400 km in central eastern Madagascar (Figure 1), covering a large part of the distribution area of the two species. Sympatry of the two species was recorded at one locality (Farimazava) (Figure 1). Frogs were classified morphologically either as *M. cowani* ($n = 33$), *M. baroni* ($n = 67$), or "putative" hybrids ($n = 6$), based on their morphology when their colouration was intermediate. "Putative" hybrids had an orange-yellowish colouration, more extended lateral spots (versus small and rounded red spots in *M. cowani*), residuals of cephalic lines (clearly delineated in *M. baroni* and lacking in *M. cowani*) and presence of yellowish shading on tibiae (versus red bands in *M. cowani* and black-orange patterned tibiae in *M. baroni*). Tissue samples were collected by toe-clipping all encountered individuals, most of which were subsequently released. Representative voucher specimens were preserved in the collections of the Museo di Scienze Naturali di Torino, the Zoological Museum Amsterdam and the Zoologische Staatssammlung München.

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

Locality	Locality number	Coordinates	Altitude (m)	Species	Sample size
Fierenana	1	18°32′36″ S–48°26′56″ E	948	*M. baroni*	1
Andriabe	2	18°36′46″ S–48°19′34″ E	1047	*M. baroni*	5
Vohidrazana	3	18°57′57″ S–48°30′37″ E	731	*M. baroni*	10
Mantady	4	18°49′48″ S–48°25′56″ E	966	*M. baroni*	1
Andranomena	5	19°01′30″ S–48°10′0″ E	921	*M. baroni*	2
Tsinjoarivo region	6	No precise coordinates	–	*M. baroni*	3
Ranomafana[a]	7	21°13′34″ S–47°22′10″ E	1152	*M. baroni*	13
Farimazava	8	20°50′06″ S–47°19′95″ E	1380–1420	*M. cowani*–*M. baroni*	8 *M. cowani*–33 *M. baroni*
Soamazaka	9	20°45′22″ S–47°17′38″ E	1600–1650	*M. cowani*	4
Vohisokina	10	20°42′18″ S–47°17′14″ E	1580–1620	*M. cowani*	20
Vatolampy	11	20°49′40″ S–47°19′08″ E	1540–1580	*M. cowani*	6

Locality numbers refer to those in Figure 1.
[a]The coordinates from Ranomafana refer to a bridge near Vohiparara where most samples were collected; some samples from other sites within Ranomafana National Park were pooled with these for analysis.

Laboratory techniques

Total genomic DNA was extracted from toeclips preserved in 99% ethanol using a proteinase K digestion (final concentration 1 mg/ml). DNA was isolated by a standard salt extraction protocol (Bruford et al. 1992).

Fragments of 526 bp of cytochrome *b* were amplified via the polymerase chain reaction (PCR) using the primers Cytb-c and CBJ10933 from Bossuyt and Milinkovitch (2000). To amplify the cytochrome *b* fragment, PCRs were performed in 25 μl reactions containing 1.0 unit of REDTaq DNA Polymerase (Sigma, Taufkirchen, Germany), 50 ng genomic DNA, 10 nmol of each primer, 15 nmol of each dNTP, 50 nmol additional $MgCl_2$ and the REDTaq PCR reaction buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.1 mM MgCl2 and 0.01% gelatine) using the following conditions: an initial denaturation at 94 °C for 1:30 min; 35 cycles at 94 °C for 30 s, annealing temperature of 53 °C for 45 s, extension at 72 °C for 1:30 min; final extension of 10:00 min at 72 °C.

PCR products were checked on 1% agarose gels and purified using QIAquick spin columns (Qiagen) prior to cycle sequencing. Sequence data collection and visualisation were performed on an ABI 3100 automated sequencer. Sequencing reactions were prepared according to the manufacturers instructions, using ABI sequence mix (BigDye® Terminator V3.1 Sequencing Standard, Applied Biosystems).

We obtained cytochrome *b* sequences of 1–41 specimens from each population of the two species (see Table 1). Sequences were deposited in GenBank; accession numbers: AY862201–AY862306.

Phylogeography and population genetics

Unambiguous alignment of the sequences was possible by eye, as they contained no indels. Sequences were verified and aligned with Sequence Navigator (Applied Biosystems) software. Different haplotypes were identified by Collapse v 1.1 (Posada 1999).

A minimum-spanning network was constructed using the TCS software package (Clement et al. 2000), which employs the method of Templeton et al. (1992). It calculates the number of mutational steps by which pairwise haplotypes differ and computes the probability of parsimony (Templeton et al. 1992) for pairwise differences until the probability exceeds 0.95. In addition, we performed a maximum likelihood phylogenetic analysis of all identified haplotypes. The topology inferred using this method was then used to choose among unresolved connections in the haplotype network (Figure 2). Maximum likelihood (ML) analyses were carried out using PAUP* (Swofford 2002), using the heuristic search option with tree-bisection-reconnection (TBR) branch swapping and 10 random addition sequence replicates, following substitution model parameter estimation with hierarchical likelihood ratio tests as implemented in Modeltest version 3.06 (Posada and Crandall 1998).

Results

We obtained 526 bp of cytochrome *b* sequences of 106 individuals from 11 populations of *M. baroni* and *M. cowani*. These contained 57 different haplotypes, of which 47 were unique. The TCS analysis produced two main haplotype networks (Figure 2). Haplotype sharing was restricted to putative and *"a posteriori"* hybrids at the locality Farimazava (Figure 1). We defined *"a posteriori"* hybrids as those individuals that were morphologically identified as *M. baroni* but contained a haplotype of *M. cowani*. The first network contains sequences of 36 *M. cowani* individuals from four populations (Soamazaka, Vohisokina, Farimazava and Vatolampy) (Figure 1) and four *"a posteriori"* hybrids from Farimazava, grouped in 14 haplotypes. The most abundant haplotype is in the central position and consists of a group of 16 individuals of *M. cowani* in which all the sampling localities are represented and two of the four hybrids. The second haplotype network contains sequences of 64 *M. baroni* individuals from eight populations (Mantady, Vohidrazana, Andranomena, Ranomafana, Tsinjoarivo region, Fierenana, Andriabe, Farimazava) and two "putative" hybrids grouped in 43 haplotypes. The most abundant haplotype is in the central position and was found in 14 individuals from five localities (Andriabe, Tsinjoarivo region, Ranomafana, Farimazava, Vohidrazana). From 18 to 25 pairwise substitutions are necessary to connect the two separate networks. However, we inferred high levels of gene flow within the two species, in particular for *M. baroni*, which contained 36 different haplotypes in our entire sample. In this species, the

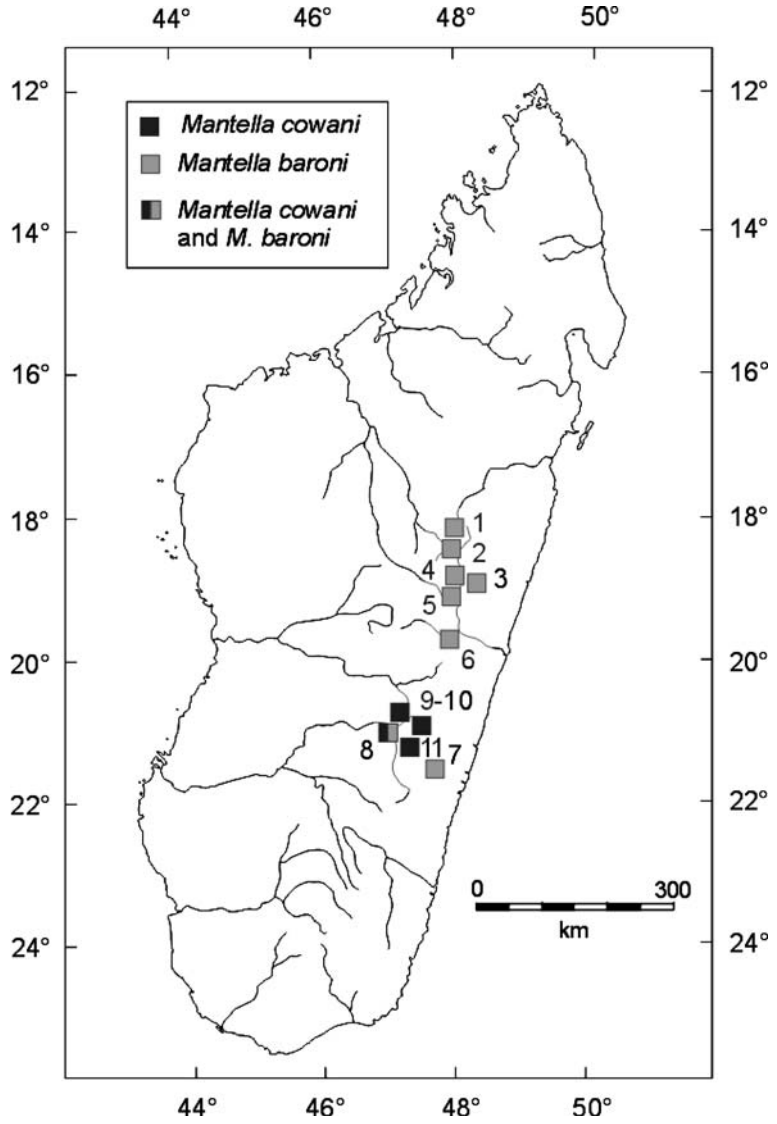

Figure 1. Map of sampled localities of *Mantella cowani* and *M. baroni*. Localities are coded as in Table 1.

most common haplotype was found in individuals from many populations, including the relatively most distant northern and southern ones.

Discussion

Defining a species has become a particularly important issue in conservation biology efforts (Moritz 1994). Hybridization phenomena make species definition more complex but do not invalidate them. Hybridization is widely known in amphibians (e.g., Szymura 1993; Vines et al. 2003). In the genus *Mantella* hybridization among different species has been recorded in captivity (Glaw et al. 2000). Haplotype sharing among taxa has also been observed recently (Chiari et al. 2004; Vences et al. 2004).

Mantella cowani differs by a number of morphological features from *M. baroni*. It is larger, has shorter hind limbs, less expanded toe-tips, smaller relative tympanum and eye diameters. *M. cowani* is also coloured differently, being characterized by a black body colouration with small red-orange flank blotches and red-orange bands on the hind limbs vs. large yellow-green flank blotches and

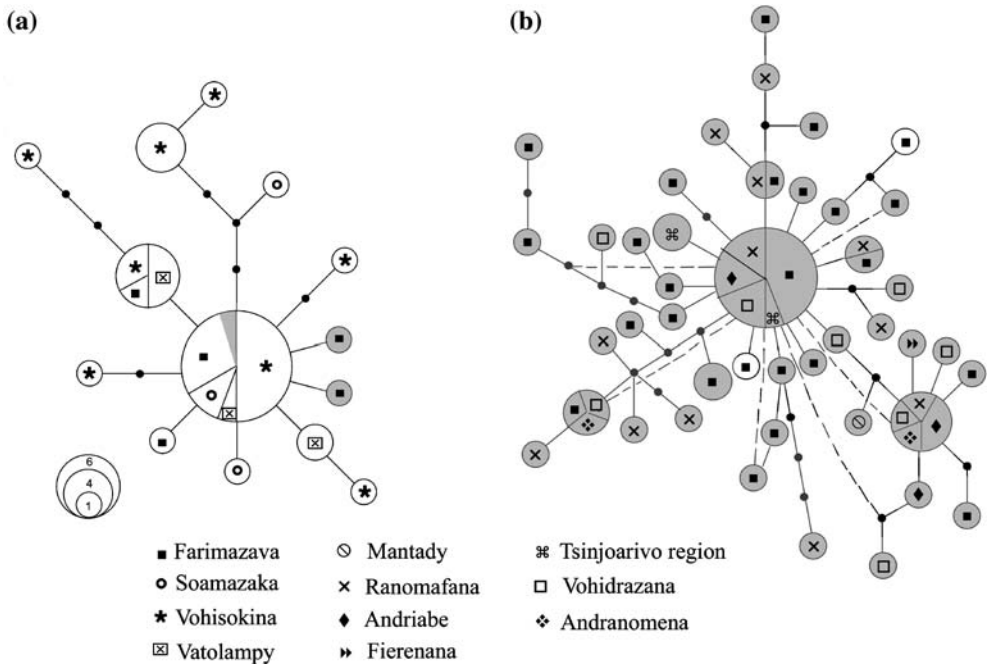

Figure 2. Haplotype networks of populations assigned to *Mantella cowani* (white; mainly in network A) and *Mantella baroni* (grey; mainly in network B). Network A contains four specimens (symbolized by grey colour) that were identified as *M. baroni* and not morphologically recognized as hybrids (''*a posteriori*'' hybrids). Network B contains two specimens (white circles) that had been morphologically identified as possible hybrids (''putative'' hybrids). Three other such putative hybrids are clustering within network A (not marked). All hybrids are from the single locality in which both species were found in syntopy (Farimazava). The dashed lines indicate links in the haplotype network with a lower level of ML support than the solid lines. Different sized circles indicate different haplotype counts, as indicated in the lower left corner.

orange-black patterning of hind limbs of *M. baroni* (Glaw and Vences 2000). *M. baroni* also has supraocular stripes, which are absent in *M. cowani*. Because the two species have similar advertisement calls consisting of series of click notes (Glaw and Vences 1994) they probably lack an efficient mechanism of prezygotic isolation. At Farimazava, we found hybrids in both of the haplotype networks. This result is referred only from mtDNA data (cytochrome *b*). The number of ''putative'' hybrids was actually larger than that detected with our analysis (*''a posteriori''* hybrids): of the total of eight individuals of *M. cowani* collected in Farimazava, five were recognized as ''putative'' hybrids. The reduced number of detected hybrids in our analysis compared to these morphological observations might be due to the use of a maternally inherited marker, mtDNA. The use of nuclear markers such as microsatellites would enable us to confirm the introgression since our data cannot exclude that the system represents a phylogeographic pattern of category II as defined by Avise (2000). In this scenario, some anciently separated lineages within a species might by chance have been retained whereas many intermediate genotypes were lost over time by gradual lineage sorting.

However, based on the relatively large number of steps (at least 18) separating the two haplotype networks in Figure 2 we here confirm the genetic differentiation between *M. cowani* and *M. baroni*. Together with the apparent ecological differences as reflected by different altitudinal occurrence, the available data support a status of *M. cowani* and *M. baroni* as separate species under the evolutionary species concept (Frost and Hillis 1990) and as separate entities for conservation. Within these, most populations are genetically diverse, containing many different haplotypes. This indicates that, for *M. cowani* and *M. baroni*, an intensive effort to protect a few populations would be sufficient to conserve much of their mitochondrial DNA genetic diversity. While *M. baroni* occurs in many nature reserves (Vences et al. 1999), no single occurrence

of *M. cowani* in a protected area is known to date. We therefore propose the inclusion into Madagascar's network of reserves of some parcels of the remaining high plateau habitat of *M. cowani*.

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