Molecular Ecology (2004) **13**, 3763–3774 doi: 10.1111/j.1365-294X.2004.02367.x

New evidence for parallel evolution of colour patterns in Malagasy poison frogs (*Mantella*)

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Abstract

Malagasy poison frogs of the genus *Mantella* are diurnal and toxic amphibians of highly variable and largely aposematic coloration. Previous studies provided evidence for several instances of homoplastic colour evolution in this genus but were unable to sufficiently resolve relationships among major species groups or to clarify the phylogenetic position of several crucial taxa. Here, we provide cytochrome *b* data for 143 individuals of three species in the *Mantella madagascariensis* group, including four newly discovered populations. Three of these new populations are characterized by highly variable coloration and patterns but showed no conspicuous increase of haplotype diversity which would be expected under a scenario of secondary hybridization or admixture of chromatically uniform populations. Several populations of these variable forms and of *M. crocea* were geographically interspersed between the distribution areas of *Mantella aurantiaca* and *Mantella milotympanum*. This provides further support for the hypothesis that the largely similar uniformly orange colour of the last two species evolved in parallel. Phylogenies based on over 2000 bp of two nuclear genes (*Rag-1* and *Rag-2*) identified reliably a clade of the *Mantella betsileo* and *Mantella laevigata* groups as sister lineage to the *M. madagascariensis* group, but did not support species within the latter group as monophyletic. The evolutionary history of these frogs might have been characterized by fast and recurrent evolution of colour patterns, possibly triggered by strong selection pressures and mimicry effects, being too complex to be represented by simple bifurcating models of phylogenetic reconstruction.

Keywords: Amphibia, cytochrome *b*, *Mantella madagascariensis* group, Mantellidae, *Rag-1*, *Rag-2*

Received 28 April 2004; revision received 26 July 2004; accepted 2 September 2004

Introduction

Coloration plays an important role in the life history and evolution of animals. Different colour patterns have been associated with mate choice, defence or mimicry, e.g. in fishes, birds and frogs (Seehausen *et al*. 1997, 1999; Summers *et al*. 1999; Uy & Borgia 2000). Brightness can be an indicator of health and absence of parasites in birds (Hamilton & Zuk 1982). Bright coloration is also often associated with toxicity of the organisms and acts as an aposematic warning signal for predators (Servedio 2000).

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Diurnal frogs are a good example of this latter association. Warning coloration usually involves red, orange and yellow; the 'bull's-eye' black and white ventral patterns of some frogs are also considered aposematic (Duellman & Trueb 1986).

In the South-American family of poison frogs, Dendrobatidae, a relationship between coloration and visual mate choice has been observed (Summers *et al*. 1999) and the possibility of a correlation between intensity of coloration and toxicity is debated (Summers & Clough 2001; Daly *et al*. 2002; Santos *et al*. 2003). In addition, Hagman & Forsman (2003) showed a positive association between conspicuous coloration and body size in this family.

Like dendrobatids, Malagasy poison frogs (genus *Mantella*, family Mantellidae) are a monophyletic group

of diurnal, terrestrial frogs with bright coloration (Schaefer *et al*. 2002). Their behaviour, feeding and mating mechanisms are considered to be a case of convergence with the Dendrobatidae. Similar to these, they feed primarily on ants and other small arthropods from which they derive their toxic skin alkaloids (Vences *et al*. 1998a). Daly *et al*. (1996) recognized the general alkaloid composition of the skin of *Mantella* species to be similar to that of Dendrobatidae. Dendrobatid and mantellid frogs bred in captivity do not have any detectable alkaloids (Daly *et al*. 1994, 1997). Considering the toxicity of wild individuals, an aposematic function is the most probable explanation for the conspicuous colour patterns of *Mantella*. However, mate choice based on coloration cannot be totally excluded. For example, Staniszewski (2001) noted that the flanks of excited males of *Mantella pulchra* may shift from dark to iridescent blue or green and in *Mantella expectata* the colour might be more vivid in breeding specimens than in aestivating individuals (M. Vences, personal observation in captive specimens).

Many species of *Mantella* are highly valued in the pet trade for their bright coloration. For the same reason they have been considered as flagship-species to promote conservation of specific areas in Madagascar (Zimmermann 1996). All *Mantella* species are placed on Appendix II of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), and some, such as *Mantella cowani* and *Mantella aurantiaca*, have been assigned high conservation priorities (Vences *et al*. 1999; Raxworthy & Nussbaum 2000; Andreone & Randrianirina 2003).

Schaefer *et al*. (2002) subdivided *Mantella* into five major monophyletic species groups based on an analysis of mitochondrial sequences of the 16S rRNA gene and found evidence for homoplastic evolution of colour pattern in these frogs. Studies of Vences *et al*. (2004) have shown a high mitochondrial diversity within and among populations of *Mantella*. Among species of the *Mantella madagascariensis* group they found evidence for a sharing of relatively divergent haplotypes as a result of secondary introgression or, less likely, incomplete lineage sorting. This group consists of five species that display a high diversity in colour phenotypes: *M. aurantiaca*, *M. crocea*, *M. madagascariensis*, *M. milotympanum* and *M. pulchra*. Of these, *M. madagascariensis* and *M. pulchra* have a relatively wide, complementary distribution in the eastern rainforest belt. They show a black dorsal colour with yellow to green flank blotches that is highly similar to that of the often sympatric *M. baroni* and *M. nigricans* which belong to another species group (Vences *et al*. 1999; Schaefer *et al*. 2002). The remaining three species are more specialized to the forests that border larger inland swamps and occupy a very restricted range in central eastern Madagascar (Vences *et al*. 2004). They have a patchy distribution with only a few precisely known localities. *M. aurantiaca* and *M. milotympanum* are bright uniform orange, differing by a black spot in the tympanic and nostril region in the latter species, whereas *M. crocea* has a more cryptic yellow-green and black pattern.

Previous studies using allozyme (Vences *et al*. 1998b) and mitochondrial markers (Schaefer *et al*. 2002; Vences *et al*. 2004) have produced conflicting hypotheses of phylogeny within the *M. madagascariensis* group. This especially concerns the position of *M. madagascariensis*, which was grouped either with the chromatically similar *M. pulchra* (allozymes) or with *M. aurantiaca* (mtDNA), but the individuals studied originated from different populations. The locality maps produced by Vences *et al*. (2004) furthermore provided indications that the derived uniform orange colour of *M. aurantiaca* and *M. milotympanum* might be homoplastic, since geographically interspersed populations (*M. crocea*) were genetically closer to *M. milotympanum* and showed a different pattern. The relationships of the *M. madagascariensis* group within *Mantella*, crucial to understand the evolution of their colour and pattern, are also unclarified since different mitochondrial genes provided contradictory hypotheses (Vences *et al*. 2004).

The goal of our study is to contribute to the clarification of these controversies by providing further mitochondrial and nuclear DNA data on Malagasy poison frogs, with particular focus on the *Mantella madagascariensis* group. We determined cytochrome *b* sequences of 143 specimens, including additional individuals from known populations and samples from four newly discovered populations of *M. aurantiaca*, *M. crocea* and *M. milotympanum*. In addition, we used sequences of two nuclear genes (*Rag-1* and *Rag-2*) in order to understand the phylogenetic relationships within the group and to identify their closest relatives.

Materials and methods

Sampling

Tissue samples from 13 species comprising five species groups used in this study were available from previous studies (Vences *et al*. 1998c; Schaefer *et al*. 2002; Vences *et al*. 2004) or collected during fieldwork in Madagascar. These five groups (Schaefer *et al*. 2002; Vences *et al*. 2004) include the *Mantella betsileo* group (*M. betsileo*, *M. expectata* and *M. viridis*), the *M. cowani* group (*M. baroni*, *M. cowani*, *M. haraldmeieri* and *M. nigricans*), the *M. madagascariensis* group (*M. aurantiaca*, *M. crocea*, *M. milotympanum*, *M. madagascariensis* and *M. pulchra*), the *M. bernhardi* group (*M. bernhardi*) and the *M. laevigata* group (*M. laevigata*).

Fieldwork was carried out in December 2001 and February 2003. Ten populations were sampled and geographical coordinates and altitude above sea level recorded by GPS instruments (Table 1). These localities extend along a north–south transect of *c*. 110 km in central eastern Madagascar (Fig. 1) that encompass the complete known ranges of all

Table 1 Coordinates, altitude and *Mantella* species for each locality

Locality	Locality number	Coordinates	Altitude (m)	Species
North of Fierenana	1	18°16′10″ S, 48°29′03″ E	1060	*M.* cf. *milotympanum*
Fierenana (Sahamarolambo)	2	48°26′56″ S, 18°32′36″ E	948	*M. milotympanum*
Andriabe	3	18°36′46″ S, 48°19′34″ E	1047	*M.* cf. *milotympanum*
Savakoanina	4	18°36′44″ S, 48°24′30″ E	959	*M.* cf. *milotympanum*
Ambohimanarivo	5	18°48′34″ S, 48°16′52″ E	1057	*M. crocea*
Ihofa	6	18°46′06″ S, 48°22′18″ E	1017	*M. crocea*
Torotorofotsy 1	7	18°52′29″ S, 48°22′21″ E	960	*M. aurantiaca*
Torotorofotsy 2	8	18°51′19″ S, 48°21′36″ E	950	*M. aurantiaca*
Andranomandry	9	19°02′22″ S, 48°10′34″ E	917	*M. aurantiaca*
Andranomena	10	19°01′30″ S, 48°10′0″ E	921	*M. aurantiaca*

Locality numbers are the same as those in Fig. 1.

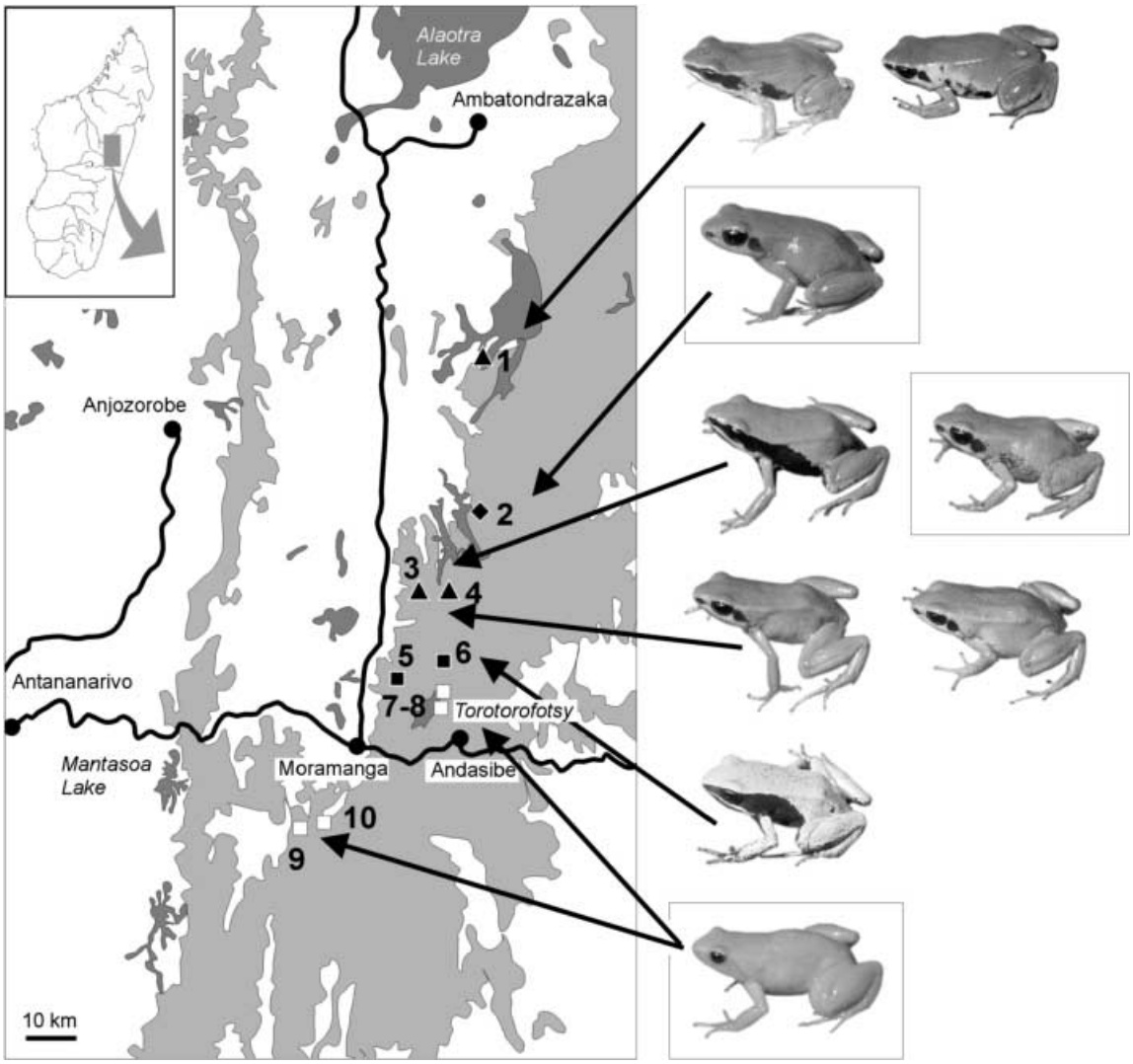

Fig. 1 Map of localities of *Mantella aurantiaca*, *Mantella crocea* and *Mantella milotympanum*: black squares, *Mantella crocea*; white squares, *Mantella aurantiaca*; Black diamond, *Mantella milotympanum*; black triangle, uncertain assignation, variable or intermediate colour and pattern. The photographs show individuals found in the populations indicated by arrows (or identical to the typical patterns found in the populations). Populations 2 and 7–10, and some individuals from population 3, are uniformly orange (uniform orange individuals included in boxes) whereas the others show a pattern of at least partly black flanks or a green-yellow colour. Localities are as follows: 1, North of Fierenana; 2, Fierenana; 3, Andriabe; 4, Savakoanina; 5, Ambohimanarivo; 6, Ihofa; 7–8, Torotorofotsy; 9, Andranomandry; 10, Andranomena.

three species. No other reliable locality records for these species have been published to date.

We here follow two approaches with different sampling. On one hand we study differentiation of three species in the *M. madagascariensis* group (*M. aurantiaca*, *M. crocea* and *M. milotympanum*) at the population level, using partial sequences of the mitochondrial cytochrome *b* gene. On the other hand we determined *Rag-1* and *Rag-2* sequences from a less extensive number of individuals in a broader taxonomic sampling. This included (1) representatives of each of the species groups recognized in the genus (Schaefer *et al*. 2002) and (2) individuals from crucial populations of all five species of the *M. madagascariensis* group. From this latter group we chose two specimens of *M. madagascariensis* that in previous studies were placed at different phylogenetic positions and three *M. aurantiaca* specimens that clustered in the two separate mitochondrial haplotype networks. A species of the mantellid genus *Mantidactylus*, *M. wittei*, was used as outgroup in phylogenetic analyses. Representative voucher specimens were preserved in the collection of the Zoologische Staats-sammlung Munich, Germany, and the Zoological Museum, Amsterdam, The Netherlands.

Laboratory techniques

Genomic DNA was extracted from toeclips or muscle tissues fixed in 99% ethanol using a Proteinase K digestion (final concentration 1 mg/mL). DNA was isolated by a standard salt extraction protocol (Brufford *et al*. 1992).

Fragments of cytochrome *b* and two nuclear genes (*Rag-1* and *Rag-2*) were amplified via polymerase chain reaction (PCR). For the population genetic part of our study, a fragment of 528 bp of the cytochrome *b* gene was amplified using the primers Cytb-c and CBJ10933 from Bossuyt & Milinkovitch (2000). For the phylogenetic part of our work a fragment of 1367 bp of *Rag-1* and 666 bp of *Rag-2* were amplified using a combination of degenerate primers (Hoegg *et al*. 2004) (Table 2).

To obtain the *Rag-1* and *Rag-2* fragments, PCRs were performed in 25 μL reactions containing 0.5–1.0 units of REDTaq DNA Polymerase (Sigma), 0.01 units of *Pwo* DNA polymerase (Roche), 50 ng genomic DNA, 10 pmol 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 $MgCl_2$ and 0.01% gelatine). To amplify the cytochrome *b* fragment, the same reaction was performed using 1.0 unit of REDTaq DNA Polymerase without *Pwo* DNA polymerase, using the following conditions: an initial denaturation 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 90 s, and final extension for 10 min at 72 °C. Cycle conditions for *Rag-1* were adapted from a long range PCR protocol (Barnes 1994), with an initial denaturation step at 94 °C for 5 min, followed by 10 cycles at 94 °C for 30 s, annealing temperatures increasing by 0.5 °C per cycle from 54 °C to 57 °C for 40 s and extension for 3 min at 68 °C. An additional 25 PCR cycles were performed at 94 °C for 30 s, 57 °C for 40 s and 68 °C for 3 min. Final extension was at 68 °C for 5 min. Cycle conditions for *Rag-2* were an initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, annealing temperature of 50 °C for 40 s and an extension step of 68 °C for 3 min; final extension was at 68 °C for 5 min.

Table 2 Names and sequences of primers used for amplification of cytochrome *b*, *Rag-1* and *Rag-2* gene fragments

Locus	Primer	Primer sequence
Cyt b	Cytb-c	5′-CTACTGGTTGTCCTCCGATTCATGT-3′
	CBJ 10933	5′-TATGTTCTACCATGAGGACAAATATC-3′
Rag-1	Amp F2	5′-ACNGGNMGICARATCTTYCARCC-3′
	Amp F1	5′-ACAGGATATGATGARAAGCTTGT-3′
	Amp R2	5′-GGTGYTTYAACACATCTTCCATYTCRTA-3′
	AmpRI	5′-AACTACGCTGCATTKCCAATRTCACA-3′
	Mart FL1	5′-AGCTGGAGYCARTAYCAYAARATG-3′
	Mart R6	5′-GTGTAGAGCCARTGRTGYTT-3′
Rag-2	Lung.35F	5′-GGCCAAAGAGRTCYTGTCCIACTGG-3′
	Lung.320R	5′-AYCACCCATATYRCTACCAAACC-3′
	Lung.460R	5′-GCATYGRGCATGGACCCARTGICC-3′
	31 FN. Venk	5′-TTYGGICARAARGGITGGCC-3′

The PCR products were loaded on 1.2% agarose gels, stained with ethidium bromide, and visualized on a Gel Doc system (Bio-Rad). If results were satisfactory, products were purified using QIAquick spin columns (Qiagen) prior to cycle sequencing. A 10 μL sequencing reaction included 1–2 μL of template, 1 μL of sequencing buffer, 2 μL of pmol/μL primer, 1.8 μL of ABI sequence mix (Applied Biosystems) and 3.2–4.2 μL of water. The sequence reaction was 30 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.

We obtained cytochrome *b* sequences of 5–26 specimens from each population of the *M. madagascariensis* group sampled (except for only two specimens from North of Fierenana, see Table 1). Sequences were deposited in GenBank; accession numbers AY723515–AY723696.

Phylogeography and population genetics

This part of our study was based on cytochrome *b* sequences from 143 individuals from the *M. madagascariensis* group. A 528 bp segment of this gene was available from all specimens and contained no indels. 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. The number of mutational differences associated with the probability just before the 0.95 cut-off is then the maximum number of mutational connections between pairs of sequences justified by the 'parsimony' criterion, and these justified connections are applied in a haplotype network (Clement *et al*. 2000). In addition we performed a maximum likelihood phylogenetic analysis of all identified haplotypes using methods described below. The topology inferred using this method was then used to choose among various unresolved connections in the haplotype network. Values of nucleotide diversity and gene diversity were obtained with the software ARLEQUIN version 2.0 (Schneider *et al*. 2000).

Phylogenetic analyses

Sequences were checked and aligned with the SEQUENCE NAVIGATOR (Applied Biosystems) software. Sequence alignment was done by eye since there was no length variation. We calculated phylogenetic trees using each marker separately. Maximum parsimony (MP) and 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 MODELTEST version 3.06 (Posada & Crandall 1998).

Two-thousand bootstrap replicates were calculated under the MP optimality criterion, and 500 replicates under the ML criterion. All bootstrapping was carried out using heuristic searches with 10 random addition sequence replicates and TBR branch swapping. Bayesian posterior probabilities were calculated using MRBAYES version 2.01 (Huelsenbeck & Ronquist 2001) under a GTR substitution model with parameters estimated from the data. A total of 300 000 generations were run, every 10th tree collected, and the number of initial generations needed before convergence on stable likelihood values was empirically estimated at 15 000; the 'burn in' parameter was consequently set at 5%.

Competing phylogenetic hypotheses were tested using SH-tests (Shimodaira & Hasegawa 1999) as implemented in PAUP*. We performed maximum likelihood searches under various constraints and compared the obtained trees simultaneously with the best tree from the unconstrained search.

Results

Population genetic analysis

The TCS analysis of cytochrome *b* of 143 specimens from 10 populations produced two main haplotype networks (Fig. 2). We had to force the TCS program to employ a minimum of 17 steps to connect them (this minimum distance is the one of the haplotype aur2 from the *M. aurantiaca* network to aur21, cro2 or mil8 of the *M. milotympanum*/*M. crocea* network in pairwise comparisons). Of these two main groups one includes only *M. aurantiaca* individuals, while the other contains mainly *M. crocea* and *M. milotympanum*. A newly sampled population (Andranomandry) confirms the data obtained from Vences *et al*. (2004), showing haplotype sharing between the *M. aurantiaca* and the *M. crocea*/*M.milotympanum* networks. The first network contains eight *M. aurantiaca* individuals from two populations (Andranomandry and Andranomena) and all *M. crocea* and *M. milotympanum*. The second haplotype network contains 53 *M. aurantiaca* individuals from four populations divided in 17 haplotypes, with a maximum divergence of eight steps. The number of pairwise substitutions is 17–34 between the two networks. Within populations we found 2–11 haplotypes, with nucleotide diversities of 0.15–2.4% and haplotype diversities of 40–100% (Table 3).

Phylogenetic analyses

In an effort to construct a robust phylogeny of *Mantella* we amplified fragments of 528 bp of cytochrome *b*, 1367 bp of *Rag-1* and 666 bp of *Rag-2* from 17 individuals of 13 *Mantella* species. A total of 333 positions of the cytochrome *b* were invariant, 45 were parsimony-uninformative but variable, and 150 were parsimony-informative characters. Of those 150 parsimony-informative characters 85% were third position substitutions and 13% and 2%, respectively, referred to substitutions at first and second codon positions. Of the 1367 total characters of *Rag-1*, 1279 were constant and 57 variable characters were parsimony-uninformative. Of the 31 parsimony-informative characters, 74% were at third codon positions and 16% and 10% at first and second positions, respectively. In *Rag-2*, 605 of the total of 666 characters were constant, and of the variable characters, 41 were parsimony-uninformative and 20 were parsimony-informative; 65% were at third codon positions and 20% and 15% at first and second positions, respectively.

In *Rag-1*, one to four amino acid substitutions among *Mantella* species groups were identified. No amino acid substitutions were found within groups, except for the *M. cowani* group, which has up to three amino acid substitutions between species. *Rag-2* sequences differ for one amino acid substitution of the *M. betsileo* and *M. bernhardi* groups relative to other *Mantella*. Within-group variation is limited to one amino acid substitution each in the *M. cowani* and in the *M. madagascariensis* groups. Cytochrome *b* is more variable, with two to six amino acid substitutions between groups, and some within each of the groups (up to six within the *M. madagascariensis* group).

MODELTEST suggested a TrN + I + G substitution model (Tamura & Nei 1993) with a gamma distribution shape parameter of 1.78 as best fitting the cytochrome *b* data

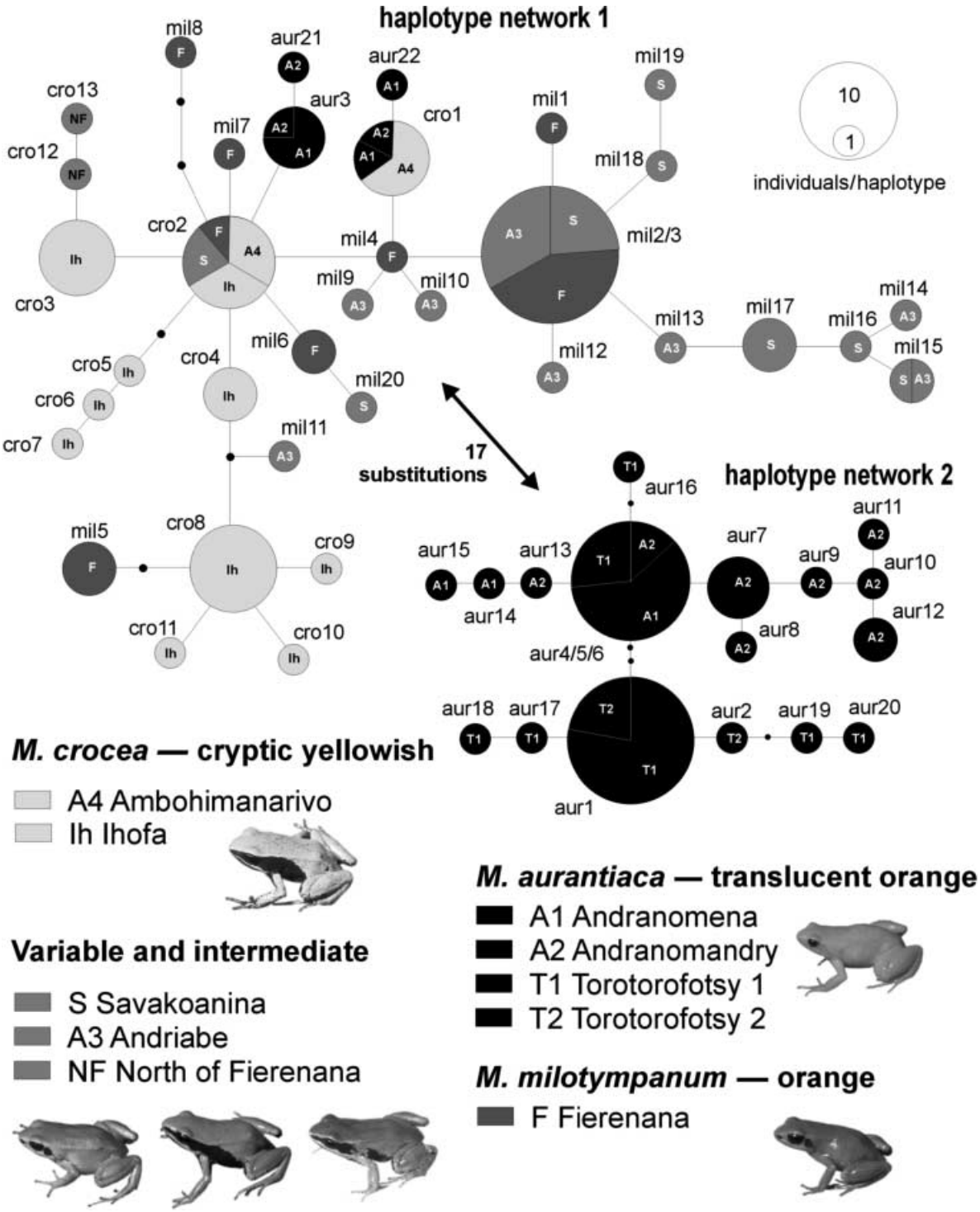

Fig. 2 Haplotype network of populations assigned to *Mantella aurantiaca*, *Mantella crocea* and *Mantella milotympanum*. The inset figures indicate which species and populations are uniformly orange and which show a pattern of at least partly black flanks.

set. The best model for *Rag-1* was the HKY + I + G model (Hasegawa *et al*. 1985) with gamma distribution shape parameters of 0.9873. The optimal model for *Rag-2* was HKY + G model (Hasegawa *et al*. 1985) with gamma distribution shape parameters of 0.1301.

The cytochrome *b*, *Rag-1* and *Rag-2* ML trees (Fig. 3) supported five main groups as indicated in Schaefer *et al*. (2002). The two nuclear markers are incongruent for the position of *M. laevigata*. This species is basal to the *M. madagascariensis* group based on *Rag-1*, but it clustered with

Table 3 Summary of colour and gene/nucleotide diversity in the *Mantella madagascariensis* group populations examined

Population	Locality number	Colour variability description	Colour variability score (0–4)	Gene diversity	Nucleotide diversity
Fierenana (Sahamarolambo)	2	Totally uniform pattern, slight variation in colour	0	0.7895 ± 0.0859	0.6465 ± 0.003851
Torotorofotsy 1	7	Totally uniform pattern, slight variation in colour	0	0.6166 ± 0.1068	0.3252 ± 0.002193
Torotorofotsy 2	8	Totally uniform pattern, slight variation in colour	0	0.4 ± 0.2373	0.1509 ± 0.001503
Andranomandry	9	Totally uniform pattern, slight variation in colour	0	0.9333 ± 0.0477	2.0047 ± 0.010799
Andranomena	10	Totally uniform pattern, slight variation in colour	0	0.6750 ± 0.1174	2.4701 ± 0.013152
Ambohimanarivo	5	Slightly variable dorsal pattern, moderately variable ventral pattern, uniform colour	2	0.5714 ± 0.1195	0.4313 ± 0.003070
Ihofa	6	Slightly variable dorsal pattern, moderately variable ventral pattern, uniform colour	2	0.8492 ± 0.0460	0.005039 ± 0.003089
Andriabe	3	Moderately variable dorsal pattern, strongly variable ventral pattern, uniform colour	3	0.7308 ± 0.1332	0.003532 ± 0.002425
North Fierenana	1	Strongly variable dorsal pattern, variable ventral pattern, uniform colour	3	1 ± 0.5	0.003774 ± 0.004622
Savakoanina	4	Moderately variable dorsal and ventral pattern, strongly variable colour	4	0.8667 ± 0.0673	0.003810 ± 0.002544

Populations are sorted according to the colour variability score, a subjective measure extending from no variability (0) to a maximum variability (4) as explained in the descriptions. Gene diversity is defined as the probability that two randomly chosen haplotypes are different in the sample. Nucleotide diversity is the equivalent to gene diversity at the nucleotide level (Nei 1987). Locality numbers refer to those in Fig. 1.

M. viridis in the *Rag-2* analysis (Fig. 3b,c). Nuclear and mitochondrial markers differed mainly for the basal position in the trees. Cytochrome *b* indicated *M. laevigata* as most basal, where the nuclear markers placed *M. bernhardi*.

The phylogenetic position of *M. madagascariensis* remained uncertain. In the cytochrome *b* and *Rag-2* ML analyses, the sample of *M. madagascariensis* from Ranomafana was basal to the rest of *M. madagascariensis* group. The other *M. madagascariensis* sample had a haplotype almost identical to *M. pulchra* in the cytochrome *b* analysis and an unresolved position within the *M. madagascariensis* group for *Rag-2* (Fig. 3a,c). In the *Rag-1* ML analysis the sample from Ranomafana clustered with *M. pulchra* and the other sample clustered with one of the *M. aurantiaca* samples (Fig. 3b).

SH-tests were carried out separately for the *Rag-1*, *Rag-2* and Cytochrome *b* data sets. The two nuclear genes yielded similar results. Trees calculated under the constraints of (1) monophyly of individuals of *Mantella aurantiaca*, (2) monophyly of uniformly orange individuals belonging to *M. aurantiaca* and *M. milotympanum*, and (3) monophyly of individuals of *M. madagascariensis* were not significantly different from the most likely trees obtained from the unconstrained searches and aredepicted in Fig. 3. In contrast, a monophyletic group containing (4) individuals of *M. madagascariensis* and *M. baroni* was significantly rejected in both cases ($P < 0.001$). In the cytochrome *b* dataset, all four comparisons yielded significant results, i.e. monophyly of the respective haplotypes of *M. aurantiaca* and *M. madagascariensis* was rejected as well as the monophyly of haplotypes of *M. baroni* + *M. madagascariensis* and of all orange individuals ($P < 0.05$).

Discussion

The M. madagascariensis *group is sister to the* M. betsileo *and* M. laevigata *groups*

Phylogenetic analyses based on morphology (Vences *et al*. 1998c), allozymes (Vences *et al*. 1998b) and mtDNA (Schaefer *et al*. 2002) placed the *M. betsileo* group (*M. betsileo*, *M. expectata* and *M. viridis*) and the *M. laevigata* group (*M. laevigata*) as most basal representatives of the genus *Mantella*, although the molecular analyses did not provide any significant bootstrap support for this placement. Vences *et al*. (1998c) identified two osteological character states in which these two species groups had plesiomorphic states, thereby defining a monophylenic group containing the *M. bernhardi*, *M. cowani* and *M. madagascariensis* groups. This basal position of the *M. betsileo* and *M. laevigata* groups was in apparent agreement with their partly less derived colour pattern (for example that of *M. betsileo* which is rather cryptic with a brown dorsum and black flanks).

A combined analysis of 2840 bp of three mitochondrial and one nuclear gene (Vences *et al*. 2004) differed from the previous hypotheses and identified a lineage containing *M. laevigata* and the *M. betsileo* group as the sister clade of the *M. madagascariensis* group, and *M. bernhardi* as the most

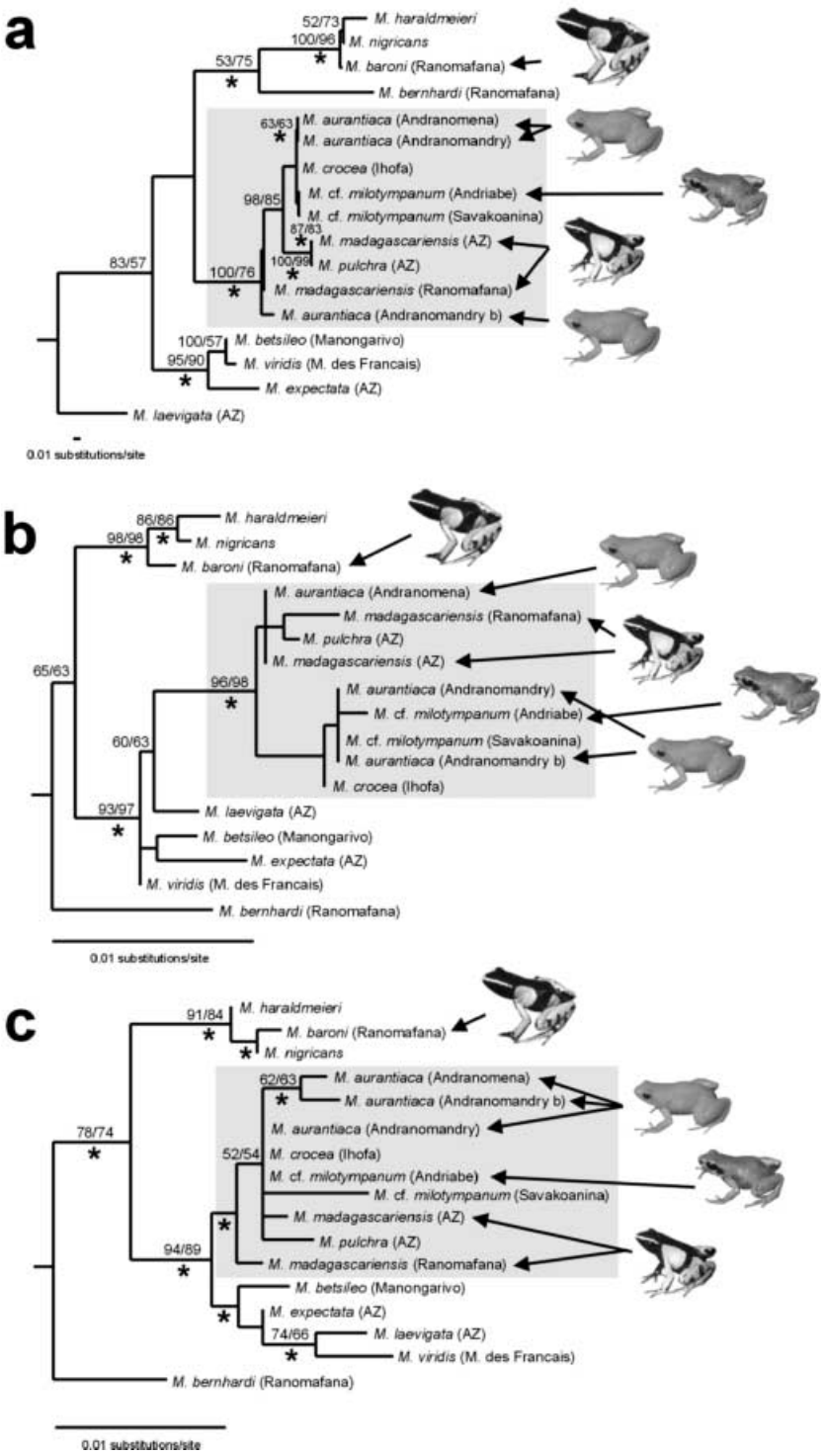

Fig. 3 Maximum Likelihood cladograms of 13 species of *Mantella*, based on 528 bp of cytochrome *b* (a), 1367 bp of *Rag-1* (b) and 666 bp of *Rag-2* (c). The tree was obtained by heuristic searches in PAUP*. Numbers indicate maximum parsimony (MP) and maximum likelihood (ML) bootstrap values in per cent (2000 and 500 bootstrap replicates, respectively). Asterisks indicate Bayesian posterior probabilities of 98% or higher. AZ, specimens used in the allozyme study of Vences *et al*. (1999); grey boxes, the *Mantella madagascariensis* group. The inset figures show individuals of similar colour and pattern: specimens of *M. madagascariensis* and *M. baroni*, which have a similar complex black-yellow-orange pattern, and *M. aurantiaca* that is invariably and uniformly orange. *Mantidactylus wittei* was used as the outgroup and is not shown in the figure.

basal *Mantella*. However, this hypothesis also received only low bootstrap support (59–67%).

The nuclear gene data presented here (Fig. 3b,c) are in agreement with the topology presented in Vences *et al*. (2004). So far, the only relevant support for any intergroup relationship within *Mantella* was the placement of *M. laevigata* with the *M. betsileo* group (Schaefer *et al*. 2002; Vences *et al*. 2004) that was also recovered here. In addition, both nuclear genes provide moderate bootstrap support (63–78%) for the placement of *M. bernhardi* as most basal species, sister to all other *Mantella* species groups, and good support (89–97%) for the placement of the *M. betsileo*/*M. laevigata* groups sister to the *M. madagascariensis* group. These results strongly suggest that the evolution of colour patterns in this

genus is homoplastic and, more interestingly, in parallel. This fact is most extreme in the two species *M. baroni* and *M. madagascariensis* that have very precisely the same pattern and often occur in syntopy (Schaefer *et al*. 2002). It also implies that the cryptic coloration of *M. betsileo*, and the lack of any flank blotches in the *M. betsileo* and *M. laevigata* groups (present in all other *Mantella*) are most probably cases of reversal.

The uniform colour of M. aurantiaca *and* M. milotympanum *is likely homoplastic*

Mantella aurantiaca and *M. milotympanum* show a very similar orange-red coloration, and *M. milotympanum* has a black spot in the nostril region and on the eardrum. *M. milotympanum* was considered as a variant of *M. aurantiaca* (Glaw & Vences 1994) until genetic data suggested its closer relationships to *M. crocea* (Vences *et al*. 1998b).

The cytochrome *b* data presented here (Fig. 3a) corroborate that the haplotype lineages of *M. crocea* and *M. milotympanum* are distinct from most *M. aurantiaca*, but they also confirm the existence of *M. aurantiaca* with haplotypes clustering in the *milotympanum*/*crocea* clade. In the DNA fragment analysed here, there was even one haplotype shared by *M. aurantiaca* and *M. crocea* (haplotype cro1). However, this applies to only a few *M. aurantiaca* individuals from Andranomena and Andranomandry (localities 9 and 10; Fig. 1). Geographically, these two populations are most distant from the *M. milotympanum* distribution area (Fierenana; locality 2 in Fig. 1), and the known sites of *M. crocea* are known to be geographically intermediate (Vences *et al*. 2004).

Two newly discovered populations, Savakoanina and Andriabe (localities 3 and 4 in Fig. 1), further fill the gap between the areas of *M. milotympanum* and *M. aurantiaca.* Specimens from these sites were intermediate in colour and pattern between *M. crocea* and *M. milotympanum* (Table 3), confirming that these two taxa are probably conspecific. To the north of Fierenana (locality 1 in Fig. 1) we further discovered a population of *Mantella* with a pattern similar to *M. crocea.* Individuals with *M. crocea*-like pattern are also known from the Zahamena reserve that is further to the north (F. Rabemananjara, personal observation).

These observations and the low incidence of haplotype sharing between *M. aurantiaca* and *M. milotympanum* suggest that one of three alternative explanations need to be invoked to explain their highly derived uniform orange colour. The first of these possible scenarios is retention of ancestral colour polymorphism in various populations, followed by local elimination of this polymorphism by genetic drift or selection, with only the orange phenotype remaining in populations today considered as *M. milotympanum* and *M. aurantiaca*. The second explanation is introgressive hybridization, with alleles for a uniform orange colour spreading into other populations. The third hypothesis is parallel or convergent evolution triggered by directional selection towards uniform orange colour.

As set out by Schaefer *et al*. (2002), in the case of *Mantella baroni* and *M. madagascariensis*, the similarity in colour pattern is most likely to have evolved through parallel evolution. These two taxa are consistently grouped into different species groups by all available data sets, and this distant placement was significantly confirmed by the SH-tests. A reconstruction of putative ancestral character states indicated different colour patterns in the most recent common ancestor of these two species. Even under the different phylogenetic scenario as proposed by Vences *et al*. (2004) and corroborated herein (Fig. 3b,c), it is obvious that assuming a retention of ancestral colour pattern implies a higher number of character state transformations than the assumption of convergent or parallel evolution, and therefore is less parsimonious.

The situation is different in the case of *M. aurantiaca* and *M. milotympanum* as described herein. These two taxa are closely related, as demonstrated by all available characters. They show mitochondrial haplotype sharing indicative of possible introgressive hybridization. However, the putatively introgressive haplotypes found in *M. aurantiaca* are not identical to those observed in *M. milotympanum* (Fig. 2). The fact that the uniformly orange *M. milotympanum* is geographically fully encircled by differently coloured populations makes an introgressive hybridization scenario further unlikely. In contrast, the hypothesis of ancestral polymorphism is more difficult to rule out. The populations in geographical proximity to *M. milotympanum* are characterized by colour polymorphism. One argument against such a hypothesis is that *M. aurantiaca* have a translucent shade which is lacking in *M. milotympanum*. This could indicate that the orange colour in these two taxa originated through different mechanisms of colour formation. We favour the hypothesis that the similar colour of *M. milotympanum* and *M. aurantiaca* evolved convergently, but a more thorough testing of this assumption is required.

The presence of the same coloration as a warning pattern in unpalatable species can be interpreted as a Müllerian mimicry. In this case, a toxic species will obtain a selective advantage by sharing the similar warning coloration (Müller 1879). The Müllerian mimicry theory would require the species presenting the same warning coloration to live in sympatry, in a way that each species can benefit from the learning capacity of the predator. Müllerian mimicry evidence is well supported when different populations living in sympatry with different model species mimic their distinct colour pattern. In frogs, Müllerian mimicry has been postulated for a Peruvian poison frog, *Dendrobates imitator*, that may mimic sympatric species in different geographical regions (Symula *et al*. 2001), and for the sympatric *M. madagascariensis* and *M. baroni* (Schaefer *et al*. 2002). Because

Table 4 Summary of sample sizes, number haplotypes and number of polymorphic sites in the cytochrome *b* fragments studied in populations of *Mantella aurantiaca*, *Mantella crocea* and *Mantella milotympanum*

Population	Locality number	Sample size	Haplotypes	Polymorphic sites
North of Fierenana	1	2	2	2
Fierenana (Sahamarolambo)	2	20	9	14
Andriabe	3	13	7	9
Savakoanina	4	15	8	7
Ambohimanarivo	5	7	2	4
Torotorofotsy 1	7	23	7	9
Torotorofotsy 2	8	5	2	2
Ihofa	6	26	10	11
Andranomandry	9	16	11	36
Andranomena	10	16	6	33

Locality numbers are the same as those in Fig. 1.

of their strictly allopatric distribution (Fig. 1) it is unlikely that such effects also explain the pattern similarity of *M. aurantiaca* and *M. milotympanum*, although the identification of predators and their home range would be needed to exclude this hypothesis.

Mantella madagascariensis *may not be a genetically homogeneous species*

Because of its relatively fast mutation rate (Brown *et al*. 1979), mitochondrial DNA is often used to reconstruct relationships among closely related species. Even if the taxa under study are well differentiated species, a limited amount of gene flow between their populations may occur in some cases. Because mtDNA is maternally inherited and no recombination takes place, incomplete lineage sorting or introgression of divergent haplotypes after speciation can lead to equivocal phylogenetic reconstructions. A surprisingly large number of species have been found to be paraphyletic using mitochondrial gene trees (Funk & Omland 2003). In such cases, nuclear-encoded markers could provide better means to estimate the true phylogenetic relationships among taxa and populations (Albertson *et al*. 1999). Conversely, the fourfold lower effective population size of mitochondrial DNA (Moore 1995) causes mtDNA haplotypes to coalesce and become monophyletic more quickly compared with nuclear markers, making these less reliable to estimate relationships among closely related and incipient species (Wiens & Penkrot 2002). However, independent of these considerations, simple bifurcating phylogenies may poorly represent the evolutionary history of species that have been exchanging genes (Machado *et al*. 2002; Machado & Hey 2003).

In *Mantella*, haplotype sharing with *M. crocea/milotympanum* was found in the Andranomena population of *M. aurantiaca* and confirmed herein a second nearby population of this species, Andranomandry (populations 9 and 10 in Fig. 1). In *M. madagascariensis*, the cytochrome *b* tree (Fig. 3a) also indicates that *M. madagascariensis* is not a genetically homogeneous species: one individual clustered with high support in a clade with *M. pulchra*, coinciding with allozyme results (Vences *et al*. 1998b) and karyological data (Odierna *et al*. 2001), whereas the individual from Ranomafana clustered as the most basal representative of the *M. madagascariensis* group. This separate clustering was confirmed by the SH tests that significantly rejected the alternative hypothesis of monophyly of the two *M. madagascariensis* haplotypes. This phylogeny would suggest that the colour pattern of *M. madagascariensis* evolved twice, possibly as a cause of independent events of Müllerian mimicry with the chromatically similar *M. baroni* (Schaefer *et al*. 2002). However, in the nuclear gene phylogenies, no consistent pattern was apparent. Neither the two *M. madagascariensis* nor the three *M. aurantiaca* individuals were placed in a monophyletic group, but their monophyly was not significantly excluded by the SH tests.

Analysis of more variable nuclear markers such as amplified fragment length polymorphisms (AFLPs) might be necessary to understand whether this is caused by a too low number of informative substitutions among these closely related taxa, an incomplete lineage sorting, or by a complex evolutionary history of repeated hybridization and admixture of populations.

Haplotype diversity is not strongly correlated with colour diversity

It is striking that some of the *Mantella* populations sampled in this study show a pronounced intrapopulational variation in colour pattern (Table 3). For example, at Savakoanina, some individuals were orange-red and others yellow-greenish, whereas at Andriabe different extension of black lateral pattern was observed. This contradicts classical theory of aposematism, which would predict stability of colour and pattern within a population (Guilford & Dawkins 1993). Our observations could be explained by

secondary admixture of chromatically uniform populations, e.g. having the colour and pattern typical for either *M. crocea* and *M. milotympanum*; it is known that *Mantella* hybrids show intermediate as well as new patterns (Glaw *et al*. 2000). If this hypothesis were true, populations with high colour variability would be expected to have a high heterozygosity and increased haplotype diversity. Cytochrome *b* diversity patterns (Table 4) do not seem to support this hypothesis (Spearman correlation of genetic diversity values with subjective scores of colour pattern diversity, $P > 0.2$). Alternatively, the variation in pattern and colour could also be an indication of disruptive or fluctuating selection, e.g. through heterogeneous or unstable populations of predators. To choose among these hypotheses, it will be necessary to screen more populations and to employ codominant and highly variable nuclear data (i.e. microsatellites) that also will provide data on levels of heterozygosity in chromatically uniform vs. variable populations. It will be crucial to perform field studies to identify which animals prey on *Mantella*, to assess the intensity of predation, and to study experimentally the selective prey choice and learning capacity of these predators.

Acknowledgements

We are grateful to Arie van der Meijden for comments on this paper and to Simone Hoegg and Marta Barluenga for, respectively, assistance in the phylogenetic and population genetic analyses. Euan Edwards, Marta Puente, Meike Thomas and Bertrand Razafimahatratra helped during field work. We are indebted to the University of Antananarivo and ICTE/MICET for logistic assistance. The Ministere des Eaux et Forets kindly issued research authorizations and export permits (036N-EA02/MG03, 038N-EA02/MG03, 074C-EA02/MG04). Y.C. was supported by a grant of the Landesgraduiertenförderung Baden-Württemberg, D. R. V. by a grant of the University of Vigo for research in foreign countries, and F.R. by a WOTRO/NWO DC-fellowship. Laboratory and field work received support by grants of the Deutsche Forschungsgemeinschaft to M.V. and A.M. (VE247/1–1 and VE247/1–2).

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This study is part of Ylenia Chiari's PhD research project. Her research interests focus on the conservation genetics and phylogeography of endangered Malagasy herpetofauna. The research was carried out in the laboratory of Axel Meyer at the University of Konstanz, Germany. Miguel Vences, Assistant Professor at the University of Amsterdam, is a specialist of the biogeography and evolution of the vertebrates of Madagascar, especially amphibians and reptiles, and his research interests currently focus on understanding the speciation mechanisms that generated Madagascar's extraordinary biodiversity. David R. Vieites is a postdoctoral researcher with a special interest in biogeographical patterns and population genetics of threatened species. Falitiana Rabemananjara and Parfait Bora are carrying out their PhD and MSc theses, respectively, on the biology of Malagasy poison frogs, this being supervised by Olga Ramilijaona Ravoahangimalala, Professor at the Zoology Department of the University of Antananarivo.
