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Species boundaries in Malagasy snakes of the genus *Madagascarophis* (Serpentes: Colubridae sensu lato) assessed by nuclear and mitochondrial markers

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Abstract

We studied mitochondrial divergence in 27 individuals of colubrid snakes of the genus *Madagascarophis* Mertens from most of its distribution area in Madagascar. Combined analyses of 16S rRNA and cytochrome *b* sequences identified six major clades which only partly agreed with previously proposed classifications. Analysis of nuclear DNA sequences of the c-mos gene as well as of ISSR fingerprints revealed consistent differences only among three clades which we consider as distinct species: a widespread *Madagascarophis colubrinus* (Schlegel), with *M. citrinus* (Boettger) as a junior synonym, a southern *M. meridionalis* Domergue, and a presumably undescribed species from the extreme north of Madagascar. The species *M. ocellatus* Domergue was not available for our study. Within *M. colubrinus* there are two populations from the north-west, each showing two divergent haplotypes with pairwise divergences of up to 5.2% in the cytochrome *b* gene. Maximum divergence in this gene within *M. colubrinus* was 7.1%. These high values emphasise that caution needs to be applied before genetic distance values are used for species delimitation. Phylogeographically, most of the genetic variation in *M. colubrinus* is found in northern Madagascar, indicating that the species might have originated in this region. Later one haplotype clade colonised western and eastern Madagascar, with a putative secondary introgression into north-western populations.

Keywords: Madagascar; *Madagascarophis*; Species boundaries; Phylogeography; mtDNA; Nuclear DNA

Introduction

Molecular divergence in mitochondrial DNA sequences is increasingly used as an indicator of the species status of populations (e.g. Bradley and Baker 2001). However, significant discordance between matrilineal haplotype distribution and nuclear genetic relationships

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have been encountered (Shaw 2002; Monsen and Blouin 2003). Sharing of highly divergent haplotypes among individuals of the same population is known for some animals (Thomaz et al. 1996), and species in mitochondrial gene trees are remarkably often mapped as paraphyletic or polyphyletic units (Avise 2000; Funk and Omland 2003; see also Joger et al. 1998; Nagy et al. 2003b).

Completeness of lineage sorting and degree of haplotype sharing vary widely among animal groups (Avise 2000). Although the number of apparently polyphyletic species in reptiles does not appear to be particularly high as compared to other vertebrates (Funk and Omland 2003), recent research has revealed a number of striking cases of complex mitochondrial variation among reptile populations that challenge prevailing taxonomic schemes (e.g. Burbrink et al. 2000; Nagy et al. 2002; Harris et al. 2003, 2004). So far, most of the comprehensive phylogeographic studies in reptiles have focused on species from temperate areas, whereas widespread tropical species are less regularly studied. Reptile populations in Madagascar are less disturbed by recent anthropogenic influence than in Europe or North America where much translocation has taken place. As a consequence, the risk of artefacts caused by non-indigenous species or populations is lower.

Among tropical biota, those of Madagascar are especially suitable for studies on speciation and adaptive radiation because they contain several endemic radiations that have evolved in isolation and are sufficiently species-rich to permit statistical comparisons of evolutionary patterns once sufficient information is available. Analyses of phylogeography and genetic differentiation of the Malagasy radiations in various groups of organisms will contribute to establish Madagascar as a model region for the understanding of mechanisms of adaptive radiation and speciation, and for the factors leading to different rates of molecular evolution among clades.

The colubrid snakes of Madagascar, with the exception of the genus *Mimophis* Günther, constitute one of those speciose radiations. According to recent molecular data, it originated most probably in the upper Oligocene (Nagy et al. 2003a). Several of the ca. 18 known Malagasy colubrid genera have been reviewed in the past decade (e.g. Raxworthy and Nussbaum 1994; Cadle 1996a, b, 1999; Ziegler et al. 1997; Andreone and Raxworthy 1998; Nussbaum and Raxworthy 2000). The genus *Madagascarophis* Mertens, 1952 has been revised by Domergue (1987), who added two new species (*M. ocellatus*, *M. meridionalis*) to the original two (*M. colubrinus*, *M. citrinus*), and divided *M. colubrinus* into five subspecies. The status of *M. citrinus*, described from Nosy Be in north-western Madagascar, remained uncertain. It was thought to occur syntopically with *M. colubrinus insularis* Domergue, and the two taxa to differ only by yellow (*M. citrinus*) versus brown-grey colour (Glaw and Vences 1994). While *M. ocellatus* has been diagnosed by differences in genital morphology and reportedly occurs sympatrically with *M. meridionalis*, all other taxa distinguished by Domergue (1987) have similar hemipenial structures and are distributed in allopatry.

Here, we present data on differentiation of mitochondrial and nuclear DNA sequences as well as ISSR genetic fingerprints of *Madagascarophis* specimens from most of the range of the genus. Our results indicate discordance between several morphological–chromatic characters and haplotype lineages at shallow phylogenetic levels. These results challenge current species definitions in the genus, and demonstrate the existence of divergent conspecific mitochondrial haplotypes.

Material and methods

Sampling of material

The pseudoxyrhophiine (Serpentes: Colubridae sensu lato) genus *Madagascarophis* is one of the most common representatives of the endemic Malagasy serpent fauna (Glaw and Vences 1994). It comprises medium-sized, mainly nocturnal or crepuscular snakes which are easily recognised by their vertical pupils and typical dorsal patterns. They are widespread in Madagascar (Fig. 1), and often observed close to human settlements. Specimens of *Madagascarophis* were collected and examined from wide ranges of its distribution (Figs. 1 and 2), with special regard to the northern parts of Madagascar. We included samples assignable to all species and subspecies, except for *M. ocellatus* and *M. colubrinus pastoriensis* Domergue. Data on the analysed samples are summarised in Table 1. The pseudoxyrhophiine snake species *Langaha madagascariensis* Bonnaterre was used as the outgroup for tree rooting (for its relation to *Madagascarophis*, see Nagy et al. 2003a).

Laboratory procedures

Tissue samples removed for genetic analyses were stored in 70–96% ethanol. A standard method for isolating total genomic DNA was used (Sambrook et al. 1989). Three marker genes showing different evolutionary and functional characteristics were chosen and amplified in polymerase chain reactions (PCR):

(1) the complete sequence of the mitochondrial cytochrome *b* (cyt *b*) gene (1114 bp including a terminal 'T'), amplified with primers L14910, L14919, and H16064 (Burbrink et al. 2000; modified by de Queiroz et al. 2002), and sequenced with L14903

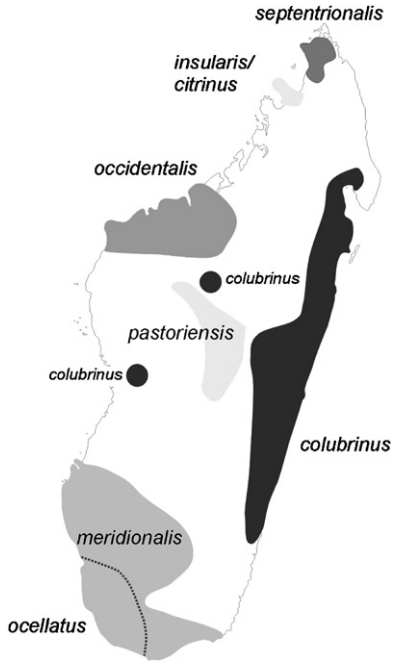

Fig. 1. Approximate distribution of *Madagascarophis* species and subspecies according to Domergue (1987). Dotted line delimits approximate area of *M. ocellatus*, in which the latter occurs sympatrically with *M. meridionalis*.

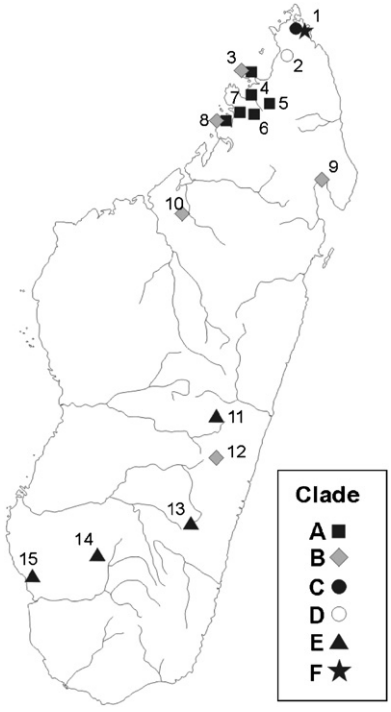

Fig. 2. Collecting localities of *Madagascarophis* specimens studied. Symbols mark different clades as identified by phylogenetic analysis of mitochondrial sequences (Fig. 3). Localities — 1: Montagne des Français; 2: Ankarana; 3: Nosy Be; 4: Benavony; 5: Tsaratanana; 6: Manongarivo; 7: Ambanja-Maromandia; 8: Berara; 9: Andranofotsy; 10: Ankarafantsika; 11: Antoetra; 12: Ranomafana; 13: Andringitra; 14: Isalo; 15: Ifaty.

(a 5′ end of L14910), L-410, H-391 (Nagy et al. 2003a);

(2) part of the mitochondrial 16S rRNA gene (513 bp aligned), using primers 16SA and 16SB (Palumbi et al. 1991);

(3) an exon fragment of the nuclear c-mos gene (570 bp), amplified and sequenced with the primers S77 and S78 (Lawson et al. 2005).

Clean-up of PCR products, cycle sequencing reaction and sequencing were performed following manufacturers' instructions. For detection of DNA sequences, the automatic capillary sequencers ABI Prism Genetic Analyzer 310 and 3100 (Applied Biosystems) were used.

In addition, a genetic fingerprinting method, ISSR-PCR (inter simple sequence repeats), was performed for comparison with results obtained by DNA sequencing. This method uses microsatellite sequences as single primers to amplify various fragments of DNA located between microsatellites. Based on previous attempts at finding appropriate candidates in colubrid snakes (Nagy et al. 2002), the PCR primers $(GACA)_4$ and $(GAA)_5$ were used to generate multiband fingerprints. Reactions were carried out according to the protocol of Wink et al. (2001). PCR products were separated on high-resolution polyacrylamide gel, and detected and visualised autoradiographically. Fingerprint patterns were evaluated manually.

Phylogenetic reconstruction

DNA sequences were validated and corrected for sequencing errors and for stop codons in protein-coding genes. The latter sequences were aligned manually, whereas 16S rRNA sequences were aligned using the multiple alignment algorithm implemented in ClustalX 1.81 (Thompson et al. 1997). The incongruence length difference (ILD) test – known as the partition homogeneity test in PAUP* 4.0b10 (Swofford 2002) – was performed for the combined set of cytochrome *b* and 16S rRNA sequences, after the exclusion of constant sites. This test used heuristic searches with 1000 replicates. The *P*-value of 0.837 failed to reject the null hypothesis of congruence between the genes, thus – in addition to the single gene analyses – combined analyses were performed. Therefore, under maximum parsimony and maximum likelihood criteria, heuristic searches were conducted with 16S (MP: 25 of 513 nucleotides parsimony informative) and cyt *b* (225 of 1114 nucleotides parsimony informative) sequences separately as well as combined.

To infer phylogenies, maximum parsimony (MP) and maximum likelihood (ML) analyses using heuristic searches were carried out with PAUP* 4.0b10, as well

Table 1. *Madagascarophis* samples analysed, including collection numbers and provenance of voucher specimens, main external morphological characters, and GenBank accession nos. of marker-gene sequences

Voucher	Locality	Sex	Clade	Scales		Colour	Tail tip	GenBank accession nos.		
				Dorsal	Ventral			16S	cyt b	c-mos
MV 2001.1249 (UADBA)	Nosy Be	?	A	–	–	–	–	AY586206	AY586243	–
ZSM 603/2003	Manongarivo	M	A	25	197	Uniform grey	White	AY586211	AY586248	–
ZSM 240/2002	Nosy Be (Andranobe)	?	A	25	185	Uniform grey	White	AY586205	AY586242	–
MV 2001.1237 (UADBA)	Nosy Be (Andranobe)	?	A	–	–	–	–	AY586204	AY586241	–
MV 2001.161 (UADBA)	Tsaratanana	?	A	–	–	–	–	AY586201	AY586238	–
ZSM 571/2001	Tsaratanana	?	A	25	185	Light brown	White with black tip	AY586200	AY586237	–
ZSM 476/2000	Nosy Be	M	A	25	191	Uniform grey	White with black tip	AY586197	AY586234	AY586221
MRSN R1966	Anbanja-Maromandia	?	A	25	204	Yellow	–	AY586195	AY586232	–
ZSM 403/2000	Benavony	?	A	25	188	Light brown	White with black tip	AY188067	AY188028	AY187989
MRSN R1838	Berara	?	A	27	196	Yellow	–	AY586193	AY586230	AY586218
ZSM 238/2002	Andranofotsy	M	B	27	197	Brown	–	AY586198	AY586235	–
ZSM 572/2001	Ankarafantsika	?	B	27	196	Dark brown	White with black tip	AY586202	AY586239	–
ZSM 602/2003	Ranomafana	?	B	27	196	Light brown	White	AY586209	AY586246	AY586225
MV 2001.378 (UADBA)	Ankarafantsika	?	B	–	–	–	–	AY586199	AY586236	–
ZSM 239/2002	Nosy Be (Andranobe)	?	B	25	186	Yellow	White	AY586203	AY586240	AY586222
MRSN R1967	Berara	?	B	25	197	Brown	–	AY586196	AY586233	AY586220
ZSM 549/2000	M. des Français	M	C	27	201	Brown with markings	–	AY586194	AY586231	AY586219
ZSM 600/2003	Ankarana	M	D	27	204	Brown	Normal	AY586208	AY586245	AY586224
ZSM 601/2003	Ankarana	M	D	27	198	Light brown	Black	AY586210	AY586247	AY586226
FGMV 2002.585 (UADBA)	Ankarana	?	D	–	–	–	–	AY586214	AY586251	–
FGMV 2002.584 (UADBA)	Ankarana	?	D	–	–	–	–	AY586215	AY586252	–
FGMV 2002.3094 (UADBA)	Ankarana	?	D	–	–	–	–	AY586216	AY586253	–
ZSM 629/2000	Ifaty	?	E	29	224	Light brown	Normal	AY188066	AY188027	AY187988
ZSM 604/2003	Isalo	?	E	29	201	Grey with markings	Normal	AY586207	AY586244	AY586223
ZMA 19528	Antoetra	?	E	27	187	Melanic	Normal	AY586212	AY586249	AY586227
ZSM 606/2003	Andringitra	?	E	27	190	Grey with markings	Normal	AY586213	AY586250	AY586228
ZMA 19622	M. des Français	?	F	29	187	Brown	Normal	AY586217	AY586254	AY586229

Abbreviations: ? = sex unknown; FGMV = F. Glaw & M. Vences field number; M = male; MRSN = Museo Regionale di Scienze Naturali, Torino; MV = M. Vences field number; UADBA = Université d'Antananarivo, Département de Biologie Animale (not yet catalogued); ZMA = Zoölogisch Museum Amsterdam; ZSM = Zoologische Staatssammlung München. For most specimens, reliable morphological diagnosis and assignment to one of Domergue's subspecies was impossible; for geographic attribution of sample sites to subspecies, see Figs. 1 and 2.

as Bayesian inference of phylogeny (BI) using MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001). In MP analyses, gaps were treated as "fifth bases", and no weighting scheme was used for the characters. For likelihood methods, an appropriate model of nucleotide substitution was inferred with Modeltest 3.06 (Posada and Crandall 1998) which uses hierarchical likelihood ratio tests (hLRTs) and the Akaike information criterion (AIC). With MP analyses, bootstrapping (Felsenstein 1985) with 1000 replicates using heuristic

searches was carried out to evaluate statistical support for clades. In BI analysis, we ran four Markov chains (MCMCMC) for one million generations simultaneously, and sampled every 100th generations. The first 500 of the 10,000 trees were ignored, and a consensus was calculated based on the remaining 9500 trees.

Because of the very low genetic variation in the c-mos nuclear marker (only 7 variable characters in the present dataset), we used a distance-based reconstruction method to build a dendrogram: the Kimura two-parameter model (Kimura 1980), which differentiates between transitions and transversions, in combination with the neighbour-joining (NJ) reconstruction algorithm.

Morphological analyses

The available voucher specimens were studied morphologically by assessing those scale counts and configurations that are known to be relevant in colubrid snake taxonomy and that had been found to differ among *Madagascarophis* species and subspecies by Domergue (1987): (1) number of dorsal scale rows at midbody; (2) number of ventral scales; (3) number of subcaudal scales; (4) divided or undivided state of anal scale; (5) number of supralabial scales; (6) number of infralabial scales; (7) number of periocular scales. In addition, we assessed (8) the general dorsal colouration, and (9) the colour of the tail tip, which varies among some *Madagascarophis* (Domergue 1987).

Fig. 3. Maximum likelihood phylogram based on mitochondrial 16S rRNA and cytochrome *b* gene sequences in specimens of *Madagascarophis* (TrN + I + G model of nucleotide substitution). Capital letters at right mark major clades identified in phylogenetic analysis. Node labels: number pairs = bootstrap support values under maximum likelihood (100 replicates)/maximum parsimony (1000 replicates) optimality criteria; asterisks indicate Bayesian posterior probability 99–100%. Tree rooted with *Langaha madagascariensis* as outgroup (not shown). For data on numbered vouchers see Table 1.

Results

Phylogeny of *Madagascarophis*

For all ML analyses, the nucleotide substitution model TrN+I+G was recommended by Modeltest. All combined analyses of MP, ML, and BI were in very good agreement and yielded practically the same relationships. These results are summarised in Fig. 3. Separate analyses (not shown) based on cyt *b* revealed the same topology as found in the combined analyses; separate 16S analyses supported the clades A+B+C, D, E, and F, as represented in Fig. 3.

With *M. ocellatus* unavailable for molecular analysis, the most basal representative of *Madagascarophis* is a peculiar juvenile specimen (ZMA 19622) collected in northernmost Madagascar. It stands at a considerable distance from all other clades and could not be assigned to any known species. When the sequence of this specimen was added to a larger data set of pseudoxyrophiines, including two other *Madagascarophis* (from Nagy et al. 2003a), these three sequences were always grouped together in phylogenetic analyses (not shown). Hence, based on mitochondrial as well as nuclear markers, *Madagascarophis* appears to be monophyletic.

Within the genus, remarkable genetic variation was found, in the form of several distinct genetic clades. Some representatives of different clades occur sympatrically (see phylogeographic results). Both the analysis with cyt *b* and 16S sequences separate (not shown) and the combined analysis revealed clades A–F as represented in Fig. 3. Bootstrap analyses under maximum parsimony criteria, and clade credibility values of Bayesian inference supported this topology. Furthermore, these clades show internal genetic divergence where distinct haplotype lineages can be observed.

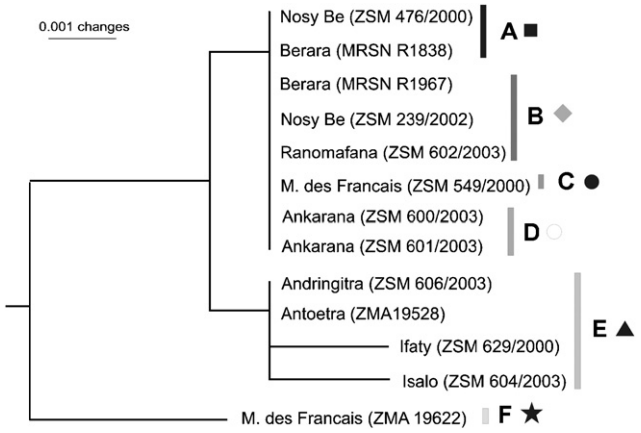

Fig. 4. Distance tree (NJ; Kimura two-parameter distances) based on nuclear c-mos gene sequences in representative samples of *Madagascarophis*. Capital letters at right mark major mitochondrial clades (see Fig. 2). Tree rooted with *Langaha madagascariensis* as outgroup (not shown).

In contrast to the mitochondrial phylogeny, the less variable nuclear c-mos gene did not distinguish between clades A–D; they all show uniform sequence characteristics (Fig. 4). Interestingly, two subgroups with different c-mos sequences in clade E were observed. This agrees with the mitochondrial gene phylogeny.

ISSR-PCR fingerprints were made with the primers $(GAA)_5$ and $(GACA)_4$, respectively. In the first case, some samples did not work well, and rather little banding variation was observed, therefore these results were not considered further. The fingerprint pattern produced by the $(GACA)_4$ primer, separated on a denaturing Sequagel matrix, is shown in Fig. 5. In general, a number of clade-specific patterns can be observed; these are marked with white frames in the figure. However, exclusively characteristic patterns are found only for clades E and F. All other clades (A–D) share their clade-specific bands in various combinations: e.g. A+B, C+D, or A+B+C+D.

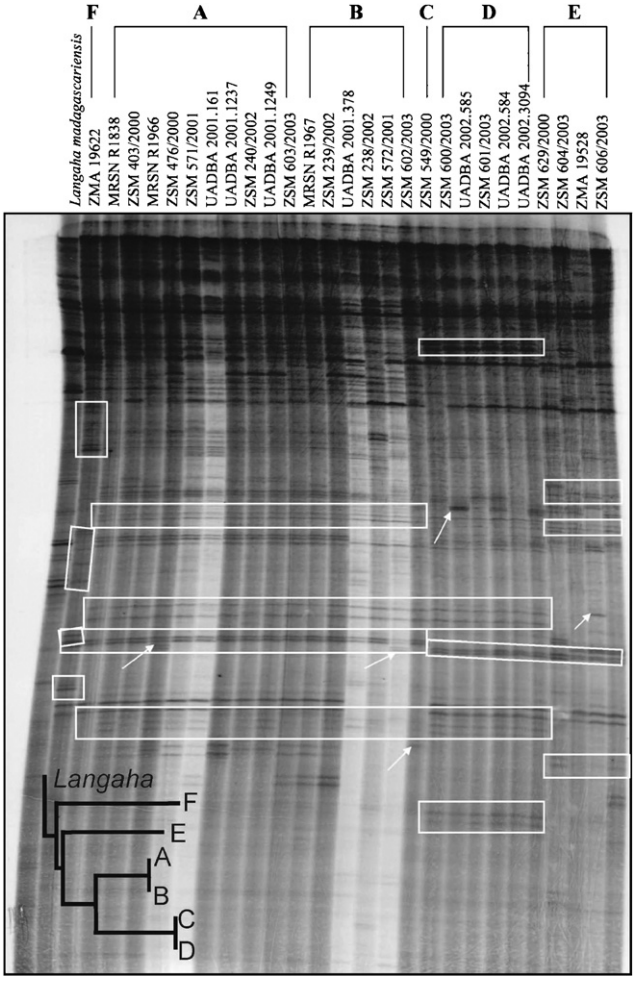

Fig. 5. ISSR-PCR genetic fingerprint of *Madagascarophis* specimens using $(GACA)_4$ microsatellite primer. White frames indicate group-specific banding patterns; arrows indicate peculiar individual variations. Inset shows a neighbour joining tree based on a 0/1 data matrix of clade-specific patterns.

Genetic differentiation among *Madagascarophis* clades

Plotting the uncorrected divergences of the two mitochondrial genes (Fig. 6) resulted in three distinct clusters: (a) divergences within the major clades as defined above; (b) divergences among clades A, B, C, and D; and (c) divergences among the cluster of clades A–D and clades E and F. The uncorrected pairwise differences within clades were 0–1.2% in the 16S rRNA gene and 0–3.6% in the cytochrome *b* gene. Haplotypes of different clades shared by the populations of Nosy Be and Berara differed by 1.2–1.4% (16S) and 5.0–5.2% (cyt *b*). Between-clade differences were 0.8–2.0% (16S) and 4.8–7.1% (cyt *b*) among clades A, B, C and D, and 2.4–5.0% (16S) and 11.0–13.6% (cyt *b*) among clades (A–D), E, and F.

Phylogeography

Correlating the haplotype clades with the geographic provenance of the corresponding *Madagascarophis* individuals reveals that representatives of clade A and B, and of clade C and F, occur in sympatry. Both at Berara and at Nosy Be, individuals of clades A and B occurred. In the two cases, they were captured in immediate syntopy within a range of a few hundred metres in the same period.

The second instance of sympatry occurs in the Montagne des Français, an isolated calcareous massif in extreme northern Madagascar. The two individuals from this area formed clades C and F.

While some of the haplotype clades seem to occupy restricted ranges, especially in northern Madagascar (clades C, D, and F seem to be restricted to the Montagne des Français and Ankarana massifs), others were found across large areas. This is especially true for clade B which comprised specimens from eastern and north-western Madagascar, and for clade E which is widespread in south-western and central-southern Madagascar. However, while the haplotypes in clade B were very similar to each other, even between the localities Ankarafantsika and Ranomafana which are about 560 km apart, the four individuals grouped in clade E had relatively divergent haplotypes.

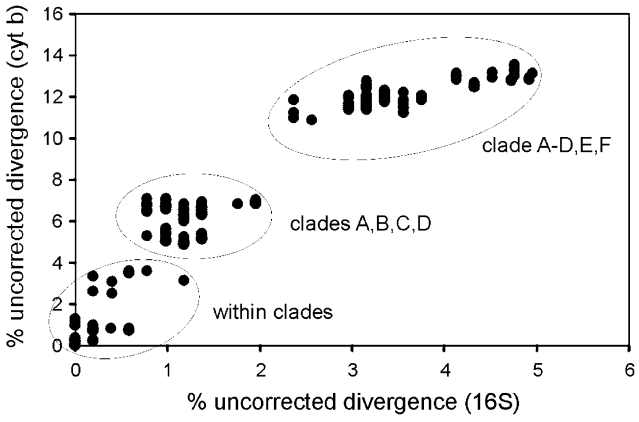

Fig. 6. Scatterplot of uncorrected pairwise divergences among *Madagascarophis* samples studied in the 16S rRNA and cytochrome *b* genes. Encircled areas group divergences, from bottom left: within each of the clades defined in Fig. 3, among putatively conspecific individuals belonging to clades A, B, C, or D, and among individuals belonging to clades A–D, E, or F that are considered to represent three separate species.

Morphology

Of the nine morphological and chromatic characters assessed, we discarded the number of subcaudals because many specimens had a mutilated tail, either naturally or as a result of tissue sampling for genetic analysis. One character was invariable: the anal scale was divided in all specimens. Three further characters, the numbers of supralabials, infralabials, and perioculars, showed no relevant variation and are therefore not analysed in detail here: all specimens had 8 supralabials, except for one individual from Berara (MRSN R1967) which had 7 supralabials on the left side of the head and 6 on the right side, and for the specimen from Antoetra which had 9 supralabials. Infralabials also showed no relevant variation, with values of 10–11 present in representatives of all clades, and 12–13 occurring in all but clade B. The number of perioculars was 8–9 in all specimens except one individual from Ankarana (ZSM 600/2003) that had 10.

While specimens from most southern localities (11, 13–15) had high numbers of dorsals and were lacking a white tail tip, characteristic of *Madagascarophis meridionalis*, most of the other specimens could not be morphologically diagnosed as clearly belonging to one of the subspecies of *M. colubrinus* described by Domergue (1987). Important variation was found in the dorsal and ventral scale counts, and in colouration (Table 1). The differences were not consistent among genetic clades. Clade A is characterised by 25 dorsal scales, with one individual from Berara (MRSN R1838) having 27. In most other clades, there are 25–27 dorsals, but clades E and F comprise individuals with 29 dorsals. Ventral scales range from 185 to 224, but without any clear association to the genetic clades (Table 1).

Clades A, B, and D contained yellowish and brown-greyish coloured specimens. At Nosy Be, Ankarana, and Berara, these colour morphs co-exist. This leads to the superficial impression that two syntopic species are involved. The genetically most divergent specimen (constituting haplotype clade F) did not show any conspicuous morphological or chromatic feature except for its 29 rows of dorsal scales.

Discussion

Nuclear versus mitochondrial markers

Comparison of the mitochondrial (cytochrome *b*, 16S rRNA) and nuclear (c-mos, ISSR fingerprinting) markers provides novel insight into patterns of geographic variation and species boundaries in *Madagascarophis*. The mitochondrial data defined six major haplotype clades. Also among some of the less differentiated clades, chromatic differences were found, e.g., the lack of a white tail tip in clade D as compared to clades A and B. It could be hypothesised that these six clades represent six distinct species. The lack of distinct genital (Domergue 1987) and meristic differences (Table 1), and the complex pattern including syntopic occurrence of different colour morphs and of different haplotypes cast doubt on such an interpretation, but do not allow for its rejection.

Combination with the results from the nuclear data permits more definitive conclusions. Both c-mos analysis and ISSR fingerprinting suggest the presence of only three distinct clusters within our samples, one corresponding to clades A–D, one to clade E, and one to clade F. Because of the few substitutions identified in the c-mos sequences, the results from this gene could also be interpreted as due to insufficient resolution among the younger clades or species. The ISSR fingerprinting results, however, revealed extensive sharing of bands among clades A–D which suggests relatively substantial gene flow, in agreement with the hypothesis that these four clades may be conspecific.

Based on the differences in c-mos and ISSR fingerprinting, clades E and F are best considered as distinct species, which is in agreement with the morphological differences of clade E (higher number of dorsals) and its specific status as *M. meridionalis* advocated by Domergue (1987). We consider it as most likely that clades A–D represent a single species, and refer to the protocol for species delimitation proposed by Wiens and Penkrot (2002). Defining clades A–D as the focal species, we find (1) exclusive haplotypes relative to the non-focal species (clade E) and (2) gene flow between basal lineages of the focal species, which corroborates the definition of the focal species as one single species (Wiens and Penkrot 2002). We are aware, however, that sample size and geographic coverage of our sampling is limited, and that future studies may challenge the above conclusions.

Classification and biogeography

Leaving aside *Madagascarophis ocellatus* which was not included in our sampling, we interpret our samples to correspond to two described and one undescribed species of *Madagascarophis*. The most inclusive taxon is *M. colubrinus*, corresponding to clade A + B + C + D. This species was considered by Domergue (1987) as widespread and polytypic, but the genetic results presented here only partially correspond to that author's subspecific distinctions. In the context of Domergue's (1987) classification, the individuals belonging to the east-coast subspecies *M.c. colubrinus* (Andronofotsy near Maroantsetra and Ranomafana) have haplotypes similar to the individual of *M.c. occidentalis* Domergue, 1987 (Ankarafantsika), indicating that *M.c. occidentalis* might be a junior synonym of *M.c. colubrinus*. Furthermore, the identification key of Domergue (1987) does not allow for any distinction between the two subspecies based on scale counts (the only difference being melanic colouration in *M.c. occidentalis* versus 'normal' colouration in *M.c. colubrinus*).

Clades C and D from Ankarana and Montagne des Français correspond to the subspecies *M.c. septentrionalis* Domergue, 1987. Clade A (including the two clade B specimens from Nosy Be and Berara) is assignable to the subspecies *M.c. insularis*. However, the fact that both clades A and B contain greyish specimens assignable to *M.c. insularis* as well as yellowish specimens assignable to *M. citrinus*, which was considered as full species by Domergue (1987), provides compelling evidence that these two taxa are synonyms. Therefore, we propose the following new synonymy: *M. citrinus* (Boettger, 1877) is a junior synonym of *M. colubrinus* (Schlegel, 1837), and a senior synonym of *M.c. insularis* Domergue, 1987. If a subspecific name is given to these northwestern populations, the proper one is *Madagascarophis colubrinus citrinus* (Boettger, 1877). A recognition of subspecific units may be justified, considering that some chromatic and meristic characters are typical for particular clades. A white tail tip, sometimes with black at the extreme apex, characterises clades A and B but not C and D. Dorsal and ventral colour is often polymorphic (yellow or dark) at north-western localities (clades A, B, and D). *Madagascarophis c. pastoriensis* (type locality: lac Mandroseza, near Antananarivo) was not included in our study; its status therefore cannot be evaluated with current data.

Populations from the southern clade (E) are characterised by a high number of dorsals and absence of a white tail tip, and correspond to *M. meridionalis*. This taxon was described as a distinct species by Domergue (1987), a conclusion supported by our data. The data presented here expand considerably the distribution range of *M. meridionalis* by its discovery at Andringitra and Antoetra, where it comes close to the distribution area of *M. colubrinus*, which is present at Ranomafana. However, these highland areas are also characterised by the presence of other reptiles otherwise typical for the arid south-west: *Acrantophis dumerili* at Antoetra (Vences and Glaw 2003), *Trachylepis vato* at Andringitra,

and *Oplurus quadrimaculatus* at Andringitra and Antoetra (M. Vences and F. Andreone, personal observation 2004). *M. meridionalis* shows no potentially species-specific differences in genital morphology from *M. colubrinus* (Domergue, 1987). Nevertheless, the apparent absence of gene flow between these two taxa, despite their neighbouring distribution areas in the Ranomafana–Andringitra–Antoetra region, corroborates the status of *M. meridionalis* as a separate species.

One specimen from Montagne des Français exhibits strong genetic divergence in all mitochondrial and nuclear markers. This juvenile individual has no distinct morphological features except for its 29 rows of dorsals; its hemipenial characters cannot be assessed because of immaturity. Nevertheless, we are convinced that this individual represents a new species possibly endemic to northern Madagascar. The Montagne des Français area has recently yielded a number of remarkable discoveries of apparently endemic reptiles, among them a new species of gecko (*Paroedura*) and two new species of colubrid snakes (*Heteroliodon* and *Liopholidophis*) (Glaw et al. 2001, 2005a, b).

Haplotype sharing and genetic assessment of species boundaries

Our study provides compelling evidence for the sharing of relatively highly differentiated mitochondrial haplotypes (divergence up to 1.4% for 16S rRNA, 5.2% for cytochrome *b*) within some populations of *Madagascarophis colubrinus* (Nosy Be and Berara). This may be explained by secondary introgression and genetic admixture among previously isolated populations and subspecies, but alternative hypotheses cannot be excluded without more dense sampling. Two instances of such deep haplotype sharing were detected despite a relatively low sample size analysed (a total of 27 *Madagascarophis* individuals only). In an analysis of several hundreds of sequences of mantellid frogs from Madagascar, only one instance of unambiguous interspecific haplotype sharing was detected (between *Mantella aurantiaca* and *M. crocea*, in two populations of the former species; up to 6.1% pairwise cytochrome *b* divergence; Chiari et al. 2004; Vences et al. 2004). A more comprehensive data set for various Malagasy reptile and amphibian species is necessary to ascertain whether there are real fundamental differences between the phylogeographic patterns in these two groups, for instance related to a putatively higher mobility of generalist reptile species.

According to our data, pairwise divergences among the three *Madagascarophis* species recognised are 2.4–5.0% in the 16S rRNA gene and 11.0–13.6% in the cytochrome *b* gene, whereas the various clades within one species, *M. colubrinus*, differ by pairwise divergences of up to 2.0% in the 16S rRNA gene and up to 7.1% in the cytochrome *b* gene. Particularly the latter value is rather high and cautions against the uncritical use of mitochondrial sequence divergences for species delimitations.

Especially for quick assessments of cryptic diversity in tropical environments, it would be most useful to define standard molecular markers which can be obtained from non-frozen tissue samples using standardised high-throughput protocols, a process that has gained attention recently under the terms DNA barcoding (Hebert et al. 2003) or DNA taxonomy (Tautz et al. 2003). Floyd et al. (2002) and Blaxter (2004) proposed the term Molecular Operational Taxonomic Unit (MOTU) for clusters of individuals with similar DNA barcodes. However, it needs to be emphasised that such MOTUs do not necessarily represent species, even if highly divergent as in our study. Additional characters such as nuclear markers, morphology, or the study of hybrid zones need to be taken into account (Wiens and Penkrot 2002), especially if conclusions are to be formalised by description of new taxa.

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