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Genetic identification of units for conservation in tomato frogs, genus *Dyscophus*

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Abstract

Dyscophus antongilii and *D. guineti* are two morphologically very similar microhylid frogs from Madagascar of uncertain taxonomy. *D. antongilii* is currently included in Appendix I of the Convention on the International Trade in Endangered Species (CITES) and its exportation is banned completely. In contrast, *D. guineti* does not receive any legal protection and it is regularly exported. Field data on ecology and behaviour are to a large extent lacking. Here we report on a genetic survey of *D. antongilii* and *D. guineti* using nuclear and mitochondrial DNA markers. Sequences of a fragment of 501 bp of the mitochondrial cytochrome *b* gene from one population of *D. antongilii* and two populations of *D. guineti* resulted in a single haplotype network, without haplotype sharing among the populations. However, haplotypes of *D. guineti* were only 1–4 mutational steps from those of *D. antongilii*, and did not form a clade. The analysis of eight microsatellites newly developed and standardized for *D. antongilii* revealed an excess of homozygotes and the absence of Hardy–Weinberg equilibrium. The microsatellite data clearly distinguished between *D. antongilii* and *D. guineti*, and fixed differences were observed at one locus. Although confirmation of the status of *Dyscophus antongilii* and *D. guineti* as separate species requires further data, our study supports the definition of these two taxa as different evolutionary significant units under the adaptive evolutionary conservation concept.

Introduction

The resolution of taxonomic uncertainties is a necessary step to distinguish entities for conservation purposes (Frankham et al. 2002). An incorrect taxonomy may lead to ineffective or even erroneous management decisions. Direct consequences could result in populations of common species receiving legal or protective status while undiagnosed species can continue to be potentially exploited without restraint. Unrecognized or cryptic species could even become extinct before they are identified as such (Frankham et al. 2002). Additionally, any reintroduction program requires an accurately defined taxonomy and biology of the organism.

The tomato frog, *Dyscophus antongilii*, is an example of the need for resolving taxonomic uncertainties. It is the only amphibian species from Madagascar currently listed in the Appendix I of the Convention on the International Trade in Endangered Species, CITES (22/10/87), which implies a complete ban on trade. The placement of this frog in Appendix I was based on the fact that several decades ago large quantities of specimens were exported from Madagascar. This species has been described from the Antongil Bay, around the town of Maroantsetra, where it is also known from Foizana, Voloina and Rantabe. It has historically been recorded also from Andevoranto (Blommers-Schlösser and Blanc 1991). At present, this species is still commonly observed in Maroantsetra (Glaw and Vences 1994). However, so far, data on its ecology are lacking, except a few notes (e.g., Pintak 1987). The general level of knowledge about *Dyscophus antongillii* makes any conservation assessment difficult. The tomato frog was classified as Vulnerable in the IUCN classification (Raxworthy and Nussbaum 2000) and Near Threatened in a recent comprehensive update of the IUCN classification (Andreone et al. 2005). Considering the widespread global appearance of amphibian declines (Stuart et al. 2004), more precise data on the status and genetic variation of this emblematic species are crucial for the prioritization of amphibian conservation efforts in Madagascar.

A similar congeneric microhylid frog, also endemic to Madagascar, is *Dyscophus guineti*. This species is known from low- and mid-altitude sites along the Malagasy East coast. *D. antongilii* and *D. guineti* have not been recorded in sympatry. They are both large frogs with very similar bright red dorsal colouration that makes them appealing for the pet-trade. Their advertisement calls are also similar (Glaw and Vences 1994). *D. guineti* mainly differs from *D. antongilii* by the presence of an extended black lateral band on the flanks and some dark dorsal marbling. Whereas the specific status of the third species of *Dyscophus*, *D. insularis* from western Madagascar, is beyond question (Glaw and Vences 1994), it cannot be excluded that *D. antongilii* and *D. guineti* may be indeed conspecific.

Classification of these tomato frogs is of immediate relevance for taxonomy-based conservation programs. *D. antongilii* is currently included in CITES Appendix I with complete ban on trade, while *D. guineti* has no legal protective status yet. Since *D. guineti* (Grandidier 1875) was described 2 years before *D. antongilii* (Grandidier 1877), it has taxonomic priority. If the two names were to be considered synonyms, all *Dyscophus* populations were to be assigned to *D. guineti*. Subsequently, they would be legally unprotected unless *D. guineti* were proposed to be included in the CITES list.

In an effort to contribute to resolving this taxonomic uncertainty, and to assess the genetic population structure of these two taxa, we isolated new microsatellite markers and collected a 501 bp portion of the mitochondrial cytochrome *b* gene from a large subset of individuals of the studied populations. Our study revealed two genetically distinct units that correspond to the formally named species.

Methods

Sample collection and DNA extraction

Fieldwork was carried out in February of 2003 and 2004. Three populations (one of *D. antongilii* and two of *D. guineti*: Maroantsetra, Andekaleka, and Fierenana) were sampled for 19–63 individuals and the geographic coordinates and altitude above sea level were recorded with Global Positioning System (GPS) devices (Table 1). The Maroantsetra population was sampled in both years, and some analyses in the following were performed separately for these two batches. Additional tissues of single individuals of *D. antongilii* were obtained from localities near to the population of Maroantsetra (Figure 1, Table 1). Tissue samples were collected by toe-clipping all encountered individuals, most of which were subsequently released. Representative voucher specimens were deposited in the collections of the Zoological Museum Amsterdam and the Zoologische Staatssammlung München.

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

Microsatellite characterization

A plasmid library was constructed using genomic DNA isolated from a *D. antongilii* samples.

Table 1. Coordinates, altitude above sea level, species and sample size for each locality used for (a) the mitochondrial and (b) the microsatellites analyses

Locality	Locality number	Coordinates	Altitude (m)	Species	Sample size (a)	Sample size (b)
Andekaleka	1	18°42.61′ S, 48°34.75′ E	650	*D. guineti*	3	50
Fierenana	2	18°34.900′ S, 48°28.128′ E	935	*D. guineti*	9	19
Ambalamahogo*	3	15°21′18″ S, 49°33′26″ E	–	*D. antongilii*	1	1
Ambatofotsy*	4	15°19′39″ S, 49°32′42″ E	–	*D. antongilii*		1
Ambodivoangy*	5	15°17′50″ S, 49°36′47″ E	–	*D. antongilii*	1	1
Andaparaty*	6	15°13′57″ S, 49°36′42″ E	–	*D. antongilii*	1	1
Andranotsara*	7	15°8′53″ S, 49°41′30″ E	–	*D. antongilii*	1	1
Andasinantimaroa*	8	15°7′11″ S, 49°39′29″ E	–	*D. antongilii*		1
Mahafidina*	7	15°23′12″ S, 49°52′30″ E	–	*D. antongilii*	1	1
Maroantsetra	8	15°25′36″ S, 49°44′26″ E	24	*D. antongilii*	50	63
Marovovonana*	9	15°20′2″ S, 49°33′26″ E	–	*D. antongilii*		1
Masondrano*	10	15°25′27″ S, 49°54′32″ E	–	*D. antongilii*		1
Sahafota*	11	15°22′0″ S, 49°51′2″ E	–	*D. antongilii*	1	1
Soanafindra*	12	15°11′27″ S, 49°35′29″ E	–	*D. antongilii*	1	1
Sonisika*	13	15°15′57″ S, 49°49′13″ E	–	*D. antongilii*	1	1
Total					70	144

*Localities surrounding Maroantsetra.
Locality numbers refer to the locality order in Figure 2 where each locality is represented by a different symbol.

Procedures for construction of the genomic DNA library followed an enrichment method based on Kandpal et al. (1994) as modified by Moranga-Amador et al. (2001) and described in An et al. (2003). Of a set of positive clones screened and sequenced, 11 clones contained a microsatellite insert. Of those 11, 8 were standardized to generate the nuclear genotype data. Primers for the polymerase chain reaction (PCR) amplification were designed from the flanking regions of the eight simple sequence repeats (Table 2) using MACVECTOR 6.5.3 software package (Oxford Molecular Group).

Screening of microsatellites

Eight microsatellites were isolated and standardized. They were called: TOM151, TOM356, TOM487, TOM125, TOM314, TOM505, TOM717 and TOM197. All of these were $(CA)_n$ dinucleotide tandem repeats of perfect sequences. The size of the amplified microsatellite loci (excluding primer sequences) was at least 100 bp. Twenty-five μl PCR reactions were prepared using: 2.5 μl 10× KCl buffer, 1.5 μl 25 mM $MgCl_2$, 0.2 μl 40 mM dNTPs, 0.2 μl of each of the 20 μM primers (one labelled either Hex or 6-Fam), 0.2 μl 5U/μl *Taq* and 1 μl template. Each PCR reaction was preheated for 5 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at a specific annealing temperature (Table 2), 45 s at 72 °C with a final extension of 30 min at 72 °C. TOM151, TOM356 and TOM487 were standardized and scored manually on LI-COR sequencer at the Universiteit van Amsterdam. Further genotyping and standardization of PCR reactions was performed with ABI 3100 automatic sequencers at the Bill & Berniece Grewcock Center for Conservation and Research at the Omaha's Henry Doorly Zoo. Randomly chosen samples previously run in Amsterdam were repeated in Omaha. The results were fully congruent among the different sequencing techniques used in the two laboratories.

Mitochondrial DNA analysis

Fragments of 501 bp of cytochrome *b* of 1–50 specimens from each population (see Table 1) were amplified via PCR using the primers Cyt*b*-*c* and CBJ10933 from Bossuyt and Milinkovitch (2000) and the following conditions (see Chiari et al. 2004 for further details of protocols used): 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. Sequence data

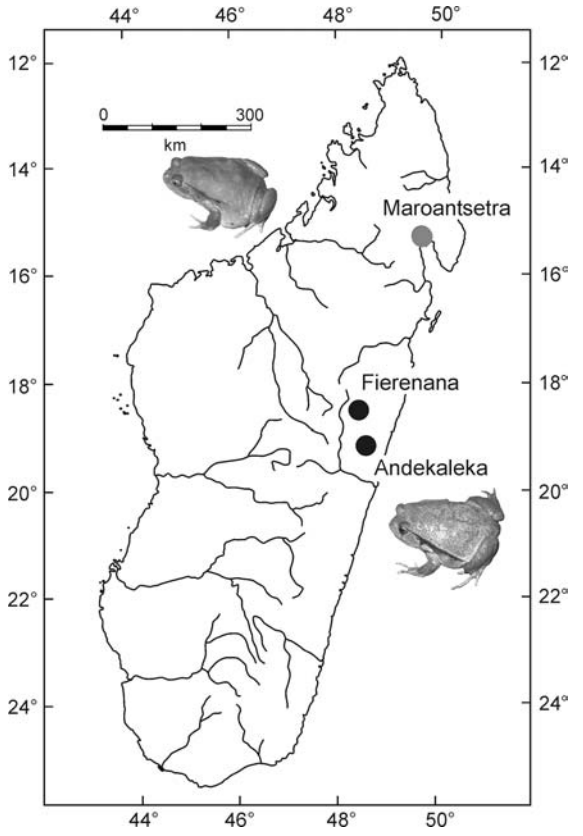

Figure 1. Map of Madagascar, showing the *Dyscophus* populations sampled. Only the three main populations Maroantsetra (*D. antongilii*, grey spot on the map, upper inset photograph), and Fierenana and Andekaleka (*D. guineti*, black spots on the map, lower inset photograph) are shown. The remaining populations from which single individuals of *D. antongilii* were obtained (Figure 2) are closely surrounding Maroantsetra (Table 1).

collection and visualization were performed on an ABI 3100 automated sequencer at the University of Konstanz.

Sequences were deposited in GenBank; accession numbers: DQ119669–DQ119738.

Data analysis

For all quantitative microsatellite analyses, only the Maroantsetra, Andekaleka, and Fierenana populations were used. Given the low sample sizes of the other studied populations (only one specimen each was included in our analyses, Table 1), we only tested these qualitatively for allele sharing with respect to the other three populations.

Average polymorphisms, unbiased heterozygosities, and linkage disequilibrium between loci were estimated from the microsatellite data with GENEPOP 3.3 (Raymond and Rousset 1995). Deviations from Hardy–Weinberg equilibrium were calculated by means of *U* tests and multiloci probability tests to detect an excess or deficiency of heterozygosity, as defined by GENEPOP 3.3. In all cases the Markov Chains followed 10,000 dememorizations, 1000 batches and 10,000 iterations per batch. Fst and Rst were used as measures of population differentiation (estimated with Fstat 2.9.1 (Goudet 1995) and RstCalc (Goodman 1997), respectively), and from these parameters, the average number of migrants per generation were estimated. For the migration estimation, Slatkin's (1995) private allele model was also implemented in GENEPOP 3.3. Effective population sizes were calculated based on the genetic diversity values under the Infinite Alleles Model (IAM) and the Stepwise Mutation Model (SMM).

Alignment of the cytochrome *b* sequences was unambiguous and performed manually by visual inspection. No indels were present. 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 probability of parsimony according to Templeton et al. (1992).

Results

Microsatellite differentiation

Data were obtained from 75 individuals of *D. antongilii* and 69 individuals of *D. guineti* from the three main populations (Table 1). Allele diversity in *D. guineti* ranged from fixation (TOM197, TOM125, TOM505 and TOM707) to a maximum of nine alleles (TOM487), while in *D. antongilii* it varied from fixation (TOM197, TOM125 and TOM707) to 23 alleles (TOM487). The average number of alleles per locus was 3.38 for *D. guineti* to 5.88 for *D. antongilii*. Observed heterozygosity ranged per polymorphic locus from 0.014 to 0.717 with an overall average of 0.212 for *D. guineti*, while *D. antongilii* was more polymorphic, ranging from 0.281 to 0.91 with an overall average of 0.367 (Table 3). Average loci population observed

Table 2. Primers designed for microsatellite loci in *Dyscophus antongilii* and *D. guineti*

Locus	Annealing	Primer sequences (5′-3′)	No. of alleles	Size range (bp)
TOM125	60°C	CCA TCC ACA GAC AAT AAA CAA AAG CCT CAC ATA ATT GAG CAC TGT CAG	1	138
TOM151	52°C	ATG AGA GCC CAA GAT GGT TC AGT GAT TAG GTG GTT GTG TGA TG	5	186–196
TOM197	53°C	GGT GTT TCT TTG CCA GTG CC GCT TCT GTC ACC ACA TTT GTC AAG	2	127–135
TOM314	56°C	TCC CCC AAG CAA TCA GAC AAT GGA TGG ATA GAT GGA TGG	16	130–192
TOM356	54°C	CTA ATA GGG AAT GGG TGG GAC TCA GGG TTA CAC AGG AAG CC	7	210–228
TOM487	53°C	GGT TAC GGA TGC TAA AGC CC CAC TCC AGC GTC TTG ATT GC	24	147–203
TOM505	54°C	GAA GAG AAC CTT TGG AGA ACT TAT C CCC ATA CAC ACA ATC AAC CAT C	4	107–113
TOM717	56°C	TGG GTC AGC ACA CTT CTC C TAA GGG CAA AAC ACT CAG ATA AG	1	132

heterozygosity values were Fierenana = 0.220, Andekaleka = 0.153, Maroantsetra (samples from 2003) = 0.404 and Maroantsetra (samples from 2004) = 0.332.

None of the possible comparisons between polymorphic loci showed significant linkage disequilibrium, providing evidence of the independent segregation of the loci analysed. Hardy–Weinberg equilibrium was absent in a pooled analysis of all populations, as supported by the overall loci probability test (12 d.f., χ^2: infinity). The significant departure from Hardy–Weinberg equilibrium was determined by an excess of homozygotes (probabilities of significance ranged between 0.0001 and 0.0035) due to the division of the dataset into two main groups (*D. antongilii* and *D. guineti*). The multi-loci-multi-population analysis shows an absence of Hardy–Weinberg equilibrium due to an excess of homozygotes with a significant probability of 0.0001.

Both Fst and Rst values were high, suggesting a clear differentiation between the two species

Table 3. Sample sizes (N), numbers of alleles (A), observed heterozygosity (H_O) and gene diversity (H_E) detected at each of the eight microsatellites loci for *Dyscophus antongilii* and *D. guineti*

Species	Locus	N	A	H_O	H_E
D. guineti	TOM125	69	1	0.000	0.000
D. antongilii		75	1	0.000	0.000
D. guineti	TOM151	69	2	0.015	0.014
D. antongilii		72	5	0.625	0.619
D. guineti	TOM197	69	1	0.000	0.000
D. antongilii		75	1	0.000	0.000
D. guineti	TOM314	59	8	0.717	0.709
D. antongilii		69	11	0.837	0.829
D. guineti	TOM356	54	4	0.238	0.235
D. antongilii		70	6	0.281	0.279
D. guineti	TOM487	61	9	0.717	0.709
D. antongilii		75	23	0.910	0.901
D. guineti	TOM505	69	1	0.000	0.000
D. antongilii		60	3	0.279	0.275
D. guineti	TOM717	69	1	0.000	0.000
D. antongilii		75	1	0.000	0.000

(Rst = 0.546 and Fst = 0.606). Numbers of migrants per generation between the two species as estimated from the two fixation indexes were 0.206 and 0.163, respectively. One locus alone was able to assign all specimens to their respective species: at the microsatellite TOM197, *D. guineti* was fixed for an allele with a length of 127 bp while *D. antongilii* was fixed for an alternate allele size (135 bp). Slatkin's Private Allele Model indicated that between the two species an average of 0.1624 individuals might migrate per generation.

Population differentiation between populations of each species was minimal (Table 4), and the two *D. guineti* populations resembled each other more than each resembled the *D. antongilii* population. Analyses of migrants per generation, estimated from the Fst and Rst, established a close correlation between those populations displaying lower differentiation values, i.e., between *D. guineti* populations Fst (Nm) = 1.568, Rst (Nm) = 6.669. Migration numbers estimated from private allele frequencies showed that the *D. guineti* populations have an average frequency of private alleles of 0.104 and an Nm of 0.499 individuals per generation.

The populations represented by single individuals, although all of them considered *a priori* to be *D. antongilii*, surprisingly showed, in some cases, alleles that are specific of *D. guineti*. The Andasinantimaroa *D. antongilii* specimen had for TOM487 a private allele, with its complementary copy indicative of a *D. guineti* allele (157 bp), and it was homozygous for a *D. guineti* allele in TOM314 (156 bp). Three other individuals as well showed alleles that were characteristic for *D. guineti* but not for the Maroantsetra population of *D. antongilii*. These individuals were: the Andranotsara specimen (allele 161 bp for TOM487), the Sonisika specimen (allele 156 bp for TOM314), and the Masondrano specimen (allele 157 bp for TOM487). The remaining heterozygote alleles in these individuals were typical of *D. antongilii* alleles or were alleles that were present in both species.

Table 4. Fst (above the diagonal) and Rst (below the diagonal) values between populations

Rst/Fst	Fierenana	Andekaleka	Maroantsetra	
			2004	2003
Fierenana		0.1375	0.6463	0.5887
Andekaleka	0.03613		0.6620	0.5897
Maroantsetra				
2004	0.56814	0.57485		0.0166
2003	0.52560	0.53034	0.02238	

Data for Maroantsetra are considered separately for the two sampling periods.

Effective population sizes varied between the species. *D. guineti* had a lower effective population size in both mutation models implemented, ranging from 673 individuals (IAM) to 764 individuals (SMM). *D. antongilii*, due to its higher allelic variability, had larger estimates of its effective population sizes. These ranged from 1450 individuals (IAM) to 1870 individuals (SMM), a rather high value for an amphibian (Beebee 2005).

Mitochondrial structure

We generated 501 bp of cytochrome *b* sequences for 70 individuals from three populations of *D. antongilii* and *D. guineti*. Only nine different haplotypes with a maximum divergence of four steps were detected. The TCS analysis produced a single haplotype network, without haplotype sharing between the two recognized species (Figure 2). The network contains 58 *D. antongilii* individuals from the Maroantsetra population and surrounding sites, and 12 individuals of *D. guineti* from two populations. The most abundant haplotype is in a central position and consists of a group of 30 individuals of *D. antongilii*. Only one step is necessary to connect individuals of *D. guineti* from Fierenana with this most common haplotype of *D. antongilii*, and only two steps for individuals of the same species from Andekaleka. Individuals from the two different localities of *D. guineti* do not form a separate haplotype clade.

Discussion

A wide array of studies have used mitochondrial DNA sequence data to clarify taxonomic uncertainties and phylogeography in amphibians, but their application does not always provide sufficient resolution (McKnight et al. 1991; Moritz 1994a; Palumbi and Backer 1994). Data from recent DNA barcoding efforts have shown that many species of amphibians show deep mitochondrial divergences among conspecific populations whereas instances of closely related species of low

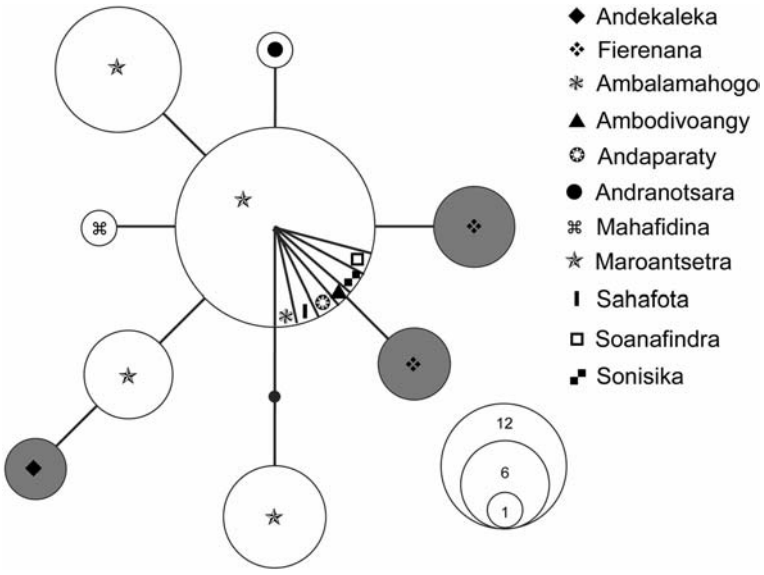

Figure 2. Haplotype network of specimens from populations assigned to *Dyscophus antongilii* (white) and *D. guineti* (grey).

mitochondrial differentiation are also found (Vences et al. 2005). In Malagasy frogs, mitochondrial cytochrome *b* sequences have been successfully used to identify species and their relationships, and to detect hybridization (Chiari et al. in press). Species like *Mantella crocea* and *M. aurantiaca*, and *M. baroni* and *M. cowani* (Vences et al. 2004; Chiari et al. 2004, in press) exhibited a large number of diagnostic differences in their haplotypes, correlated to differences in colouration, morphology and ecology, although in some populations haplotype sharing was observed and morphological evidence pointed to local instances of hybridization.

For the tomato frog dataset reported herein, the haplotypic data are less conclusive, and taxonomic and conservational conclusions depend more strongly on the theoretical framework applied. Advocates of a phylogenetic species concept have suggested to view species as elements forming the groundwork of life which do not necessarily correspond to 'real' entities for which different character data could constitute critical tests (Goldstein et al. 2000, 2005). In such a framework, diagnosis of entities by fixed differences becomes the major criterion, and by naming phylogenetically distinct populations of organisms as species, these become the major unit for conservation purposes. We consider it as more appropriate to see amphibian species, instead, as real entities representing the largest lineages, or monophyletic groups of allopatric populations, that are not likely to be on independent phylogenetic trajectories under an evolutionary species concept (Wiley 1978), as advocated for amphibians and reptiles by Frost and Hillis (1990). In this theoretical framework, one critical step in delimiting allopatric cryptic species for which pre- or post-mating reproductive isolation is not obvious, is to distinguish geographically restricted hybridization and introgression along usually narrow contact zones from broad genetic admixture (Wake and Jockusch 2000). However, different signatures of mitochondrial and nuclear genetic markers in contact zones of species and populations are a common theme in amphibians (e.g., Wake and Jockusch 2000; García-Paris et al. 2003; Monsen and Blouin 2003; Kuchta and Tan 2005; Sequeira et al. 2005), probably as a result of a lower effective population size of the maternally inherited mtDNA (Moore 1995; but see Ballard and Whitlock 2004). Using diagnostic nucleotide sites of mitochondrial haplotypes in a character-based approach to species delineation (Goldstein et al. 2005) would, in these cases, lead to geographical entities that are in strong conflict with nuclear genetic variation, or even morphology (e.g., Babik et al. 2005). We therefore concur with Moritz (1994a, b) that the

combination of different types of markers is a crucial step to understand species borders in amphibians.

In tomato frogs, cytochrome *b* haplotypes of *D. antongili* and *D. guineti* are not reciprocally monophyletic (Figure 2). Using the tree-based species delimitation suggested by Wiens and Penkrot (2002), this would result in considering both taxa as a single species, with gene flow among its basal haplotype lineages (see also Sites and Marshall 2004). However, nuclear genetic differences between the two taxa are well evident by the excess of homozygosity observed in the Hardy–Weinberg equilibrium analysis of the microsatellite data. Most importantly, in one of the loci analysed (TOM197) a fixed difference was found, with diagnostic alleles for *D. antongilii* and *D. guineti*, respectively. This difference suffices the criterion of diagnosability, and under a phylogenetic species concept would be one argument for the distinction of both taxa as different species.

To infer from the encountered differences whether the two taxa are on independent phylogenetic trajectories under an evolutionary species concept is more difficult, however, and is hampered by the lack of samples from direct contact zones. Our sampling includes most of the currently known populations of *D. antongilii* and *D. guineti* (Figure 1). Several other historical localities (Andevoranto; Sambava; Soavala; see Glaw and Vences 1994) have not been confirmed in the past 20 years, and other sites (Iharaka, Foizana, Voloina, Antsihanaka, Ancaye forest; Glaw and Vences 1994) are geographically very close to the populations sampled here. Our searches at Vevembe forest, in south-eastern Madagascar where a single specimen has been recorded recently by J. E. Cadle and E. Rajeriarison (personal communication in 2004) were unsuccessful to date. Although locally abundant, *Dyscophus* seem to be patchily distributed.

The cursorial data available so far indicate the possibility of an ecological difference between the two taxa: while *D. antongilii* lives in strongly degraded habitats in coastal lowlands (Glaw and Vences 1994), we found *D. guineti* around broad and near-stagnant sections of streams in mid-altitude rainforest. This may constitute an evolutionary significant divergence. Furthermore, the existence of one fixed microsatellite difference and the observed, albeit slight, diagnostic differences in colouration (Figure 1) also suffice the criterion of character concordance that has been proposed to avoid the risk of delineating nonevolutionary units as species (Grady and Quattro 1999).

Independent from species concepts, taxonomic changes in tomato frogs have to be applied with extreme care due to their immediate consequences for CITES listing and control of the pet trade. Under any species concept, the available information is insufficient to unequivocally refute the hypothesis of *D. antongilii* and *D. guineti* representing separate species. We therefore recommend to retain current taxonomy in a preliminary way, awaiting further fieldwork and more conclusive genetic data, that should mainly (1) provide information of the genetic structure of geographically intermediate populations, (2) investigate the existence of possible adaptive differences, and (3) use longer mitochondrial DNA sequences to critically test the apparent absence of reciprocal monophyly between the two taxa.

Delimiting species and their genetic variability is of obvious relevance to properly address conservation measures, but there is general agreement on the need to also introduce units of protection below the species level (e.g., Vogler and DeSalle 1994; Dimmick et al. 1998; Paetkau 1999; Crandall et al. 2000; Fraser and Bernatchez 2001; Frankham et al. 2002; DeSalle and Amato 2004). A major disagreement is whether these units should rather represent major historical lineages within recognized species (Avise 1994; Moritz 1994a, 2002) or reflect adaptive variation within species (e.g., Crandall et al. 2000). The concept of Evolutionary Significant Units (ESU), originally introduced by Ryder (1986), was defined by Moritz (1994a) for infraspecific lineages of reciprocal mitochondrial monophyly and a significant divergence of allele frequencies at nuclear genes. These criteria were selected because nuclear loci are expected to take substantially longer to show phylogenetic sorting due to their typically larger effective population size (Moritz 1994a). Our example from tomato frogs indicates the dilemma in applying such strict criteria to define ESUs, because we failed to find reciprocal monophyly in mtDNA but instead found a fixed difference at one nuclear (microsatellite) locus, contrary to theoretical expectations.

Fraser and Bernatchez (2001) integrate the ESU in their larger and more flexible concept of Adaptive Evolutionary Conservation (ACE). In

this theoretical framework, an ESU is a lineage demonstrating highly restricted gene flow from other such lineages within the higher organizational level of the species, and the authors suggest to use the best available biological information in exercising ESU definitions on a case-by-case basis. Following this recommendation, we see (1) the diagnostic difference at microsatellite locus TOM197, (2) the strong differences in allele frequencies at the other microsatellite loci, (3) the absence of mitochondrial haplotype sharing, (4) the differences in colouration and (5) the possible ecological (adaptive) differences between *D. antongilii* and *D. guineti* as clear evidence for considering them as separate ESUs for conservation under the ACE concept, even in the case that future analyses would indicate that from a taxonomic point of view they should best be considered as a single evolutionary species.

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