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Preliminary data on genetic differentiation within the Madagascar spider tortoise, *Pyxis arachnoides* (BELL, 1827)

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Abstract. The spider tortoise, *Pyxis arachnoides*, is one of the four extant endemic terrestrial chelonian species of Madagascar. Although this species is considered to be vulnerable, little is known about the status and genetic differentiation of its three subspecies. Here we report on field observations made in early 2004 during collection of blood samples of this species for genetic analysis. We investigated the genetic differentiation within the above mentioned subspecies using mtDNA sequences (cytochrome *b*). This marker showed a low differentiation among the three subspecies of *P. arachnoides* and an extremely low variation within and among populations of each of the subspecies. In contrast the differentiation to the second species of *Pyxis*, *P. planicauda*, was much higher. Our data therefore confirm current taxonomy and suggest that the three subspecies of *P. arachnoides* should be treated as separate entities for conservation.

Key words. Reptilia: Testudinidae: *Pyxis arachnoides*; subspecies; cytochrome *b*; Madagascar.

Introduction

The high biodiversity and high degree of endemism make Madagascar one of the top hotspots for biodiversity conservation worldwide (MYERS et al. 2000). At the species level, endemism in amphibians and reptiles is greater than 95%. Of the seven endemic chelonian species that historically inhabited the island (six terrestrial and one aquatic) only five are still present in Madagascar. The four land tortoises (*Geochelone radiata*, *Geochelone yniphora*, *Pyxis planicauda* and *Pyxis arachnoides*) share a common ancestor as shown by mitochondrial DNA data (CACCONE et al. 1999) that probably arrived by rafting from Africa (BOUR 1988, 1994). The other species of tortoise, *Kinixys belliana*, seems to have been introduced from Africa by humans (ANGEL 1941). All endemic species are considered to be endangered or vulnerable according to IUCN classification (RAXWORTHY & NUSSBAUM 2000, IUCN Red List), whereas the status of the non-endemic *Kinixys belliana* on Madagascar is undetermined. The decline of tortoise populations is the result of many centuries of commercial exploitation (PEDRONO et al. 2000) and it has been associated with habitat loss and population fragmentation (RAXWORTHY & NUSSBAUM 2000). Human exploitation is considered to be the cause of the extinction of Madagascar's giant tortoises, *Dipsochelys grandidieri* and *D. abrupta* (BOUR 1994).

The systematic history of the two *Pyxis* species is controversial. BELL (1827) defined the genus *Pyxis* for its movable anterior part of the plastron. Afterwards, GRANDIDIER (1867) described *Testudo planicauda*, a species close to *Pyxis* (BELL 1827) but without movable plastron. Based on osteological characters, SIEBENROCK (1902) created the genus *Acinixys* for the species described by GRANDIDIER (1867). Within the family Testudinidae, BOUR (1979) and OBST (1978, 1980) placed *Acinixys planicauda* within the genus *Pyxis*, designating *Acinixys* as a subgenus. This classification was supported from juvenile traits shared also with juveniles of other species. ERNST & BARBOUR (1989) used *Pyxis*

Fig. 1. Sampling localities of *Pyxis arachnoides*. The different level of grey correspond from North to South to the distribution area of each subspecies (lighter grey: *P. a. brygooi*; medium grey: *P. a. arachnoides*; darker grey: *P. a. oblonga*). Numbers refer to Tab. 1.

and *Acinixys* as different monotypic genera, whereas the results of CACCONE et al. (1999) agreed with the placement of the two species in a single genus, as previously proposed by BOUR (1979) and OBST (1978, 1980).

Pyxis arachnoides, the Madagascar spider tortoise, is a small chelonian with a carapace length of up to 15 cm. The carapace is domed and has a starlike pattern with a black rounded edge and yellow center from which several broad rays extend. The plastron varies from immaculate yellow to having some dark blotches at the bridge. The head is black with some yellow markings (PEDRONO & SMITH 2003).

The distribution of *P. arachnoides* is limited to the south-western region of the island, in a semi-arid area with thornbush vegetation from south of Morombe to Fort-Dauphin (see Fig. 1) (BOUR 1978, 1981; KUCHLING 1989). This species is sympatric with *Geochelone radiata* but to date it is not known to be heavily exploited or consumed for food by local people as *G. radiata*, except in places where radiated tortoises have disappeared (PEDRONO et al. 2000). Except a few field observations by KUCHLING (1989), little is known about the ecology, behaviour and status of *P. arachnoides*. This is probably related to the secretive life style of this species and the difficulties of access to large parts of its range. These tortoises are active early in the morning, particularly during the rainy season. For the rest of the day they hide mostly underground (JESU & SCHIMMENTI 1995).

Morphological examination of specimens of *P. arachnoides* from different localities has revealed the existence of three separated geographic entities that are presently considered as subspecies (BOUR 1978): *Pyxis arachnoides brygooi* (VUILLEMIN & DOMERGUE 1972), (Fig. 2a), *P. a. arachnoides*, BELL 1827, (Fig. 2b) and *P. a. oblonga,* GRAY 1869, (Fig. 2c). *P. a. brygooi* occupies the northernmost part of the distribution area shown in Fig. 1, in the south of Morombe; *P. a. arachnoides* occurs around Toliary; *P. a. oblonga* occurs in the extreme southern coast of Madagascar to Fort-Dauphin (BOUR 1987). The main character used to identify the different subspecies is the movable hinge in the anterior part of the plastron and its coloration (BOUR 1987). *P. a. brygooi* presents a rigid and uniform yellow colored plastron; *P. a. arachnoides* has a uniform yellow plastron in which the anterior lobe is more or less mobile; *P. a. oblonga* shows a yellow plastron with black spots on the sides in which the anterior lobe is completely mobile (BOUR 1987) (Fig. 2).

Three of the four taxa within the genus *Pyxis* (*P. arachnoides arachnoides*, *P. a. brygooi*, *P. a. oblonga*) are currently considered subspecies, but exceptional sympatric occurrence has been quoted (BOUR 1987). The taxonomic history of the taxa within the genus *Pyxis* has been quite chaotic. Together with the above mentioned taxonomic confusion between *Pyxis arachnoides* and *P. planicauda*, also *P. a. oblonga* has been previously considered as *P. a. matzi* by BOUR (1979) but the same author later recognised *P. a. oblonga* as a valid senior synonym of *P. a. matzi* (BOUR 1982). Moreover, VUILLEMIN & DOMERGUE (1972) introduced the genus *Pyxoides*, characterised by the absence of the movable hinge in the plastron and described *Pyxoides brygooi*. OBST (1978, 1980) and

BOUR (1978) agreed with the identification of *Pyxoides brygooi* as a geographic subspecies of *P. arachnoides*.

We here provide preliminary insights into genetic differentiation within the species *P. arachnoides*. Our goal is to explore whether the geographical variants identified as subspecies are also genetically isolated units, which is of particular importance for conservation actions. We also want to evaluate whether the three subspecies of *P. arachnoides* are best considered as subspecies rather than species by comparing the genetic distance between subspecies with the genetic distance between the distinct species *P. arachnoides* and *P. planicauda*. To address these questions we used DNA sequences of the mitochondrial gene cytochrome *b* of individuals of each subspecies from different localities. In addition, we report on field observations made during our sampling trip in south-western Madagascar.

Materials and Methods

Sampling

Blood samples of 36 individuals used in this study were collected during fieldwork in Madagascar in February 2003 and January/February 2004. These samples belong to the three subspecies (*Pyxis arachnoides arachnoides, P. a. brygooi, P. a. oblonga*) and to *Pyxis planicauda* (Fig. 3). Blood was taken from the forelimbs of the tortoises with insulin syringes. Each tortoise was photographed and subsequently released at the place of capture.

Ten populations were sampled and geographical coordinates were recorded by GPS (Tab. 1). The sampling locations extend along the southern part of Madagascar (Fig. 1) and encompass the known ranges of the three subspecies.

We studied differentiation of three subspecies at the population level, using partial sequences of the mitochondrial cytochrome *b* gene. As outgroup we used a cytochrome *b* sequence of *Geochelone radiata* (Genbank accession number AF020897).

Laboratory techniques

DNA was extracted from 170 µl of blood tissues preserved in modified Queen's lysis buffer (SEUTIN et al. 1991) using Proteinase K (final concentration 1 mg/ml). DNA was isolated by a standard salt extraction protocol (BRUFORD et al. 1992).

Locality	Locality number	GPS coordinates	Subspecies	N° of individuals
Manombo Atsimo	0	22°56'44"S; 43°28'04"E	*P. a. brygooi*	1
Manombo Atsimo nr	1	22°57'05"S; 43°28'29"E	*P. a. brygooi*	2
Manombo Atsimo sr	2	22°58'10"S; 43°29'02"E	*P. a. brygooi*	1
Manombo Atsimo-Antsira	3	23°00'26"S; 43°30'41"E	*P. a. brygooi*	2
Ifaty	4	23°07'50"S; 43°36'55"E	*P. a. brygooi*	19
Miary (Toliary)	5	23°18'58"S; 43°44'11"E	*P. a. brygooi*	1
Beheloka	6	23°56'36"S; 43°41'17"E	*P. a. arachnoides*	2
Mangoro-Andranotohoka	7	23°78'00"S; 43°66'00"E	*P. a. arachnoides*	3
Faux Cap	8	25°34'07"S; 45°31'24"E	*P. a. oblonga*	3

Tab. 1. Geographical coordinates of the sampling sites, subspecies of *Pyxis arachnoides* collected at each locality, and number of samples for each site. Locality numbers correspond to the locations shown on the map.

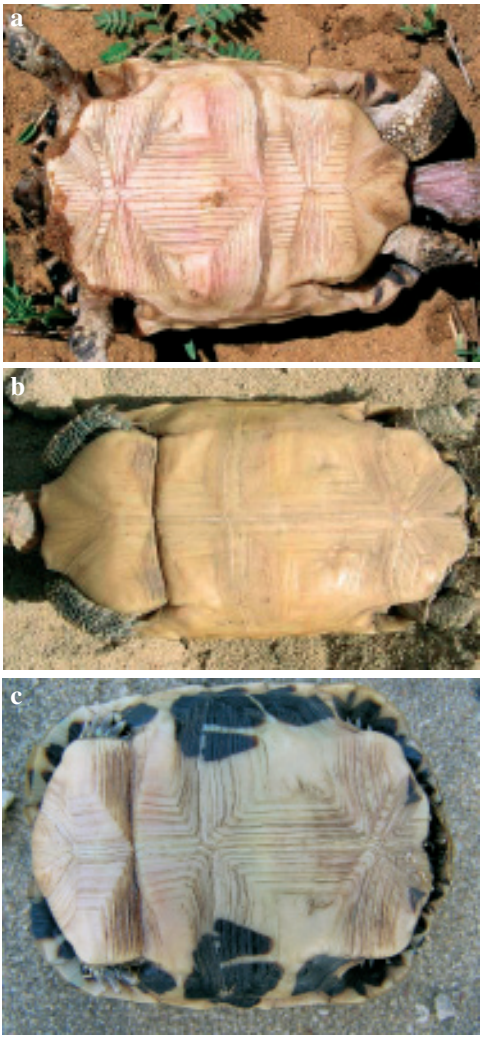

Fig. 2. Morphological differences of the three subspecies of *Pyxis arachnoides*: 2a *Pyxis a. brygooi* (ventral view); 2b *Pyxis a. arachnoides (*ventral view); 2c *Pyxis a. oblonga* (ventral view).

A fragment of 427 bp of the cytochrome *b* gene was amplified via the polymerase chain reaction (PCR) using the primers cyt *b* GLU and cyt *b* B2 as in CACCONE et al. (1999).

PCRs were performed in 25 µl reactions containing 1.0 unit of REDTaq DNA Polymerase (Sigma, Taufkirchen, Germany), 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). We used the following PCR conditions: an initial denaturation at 94 °C for 1:00 min; 34 cycles at 94 °C for 45 seconds, annealing temperature of 50 °C for 30 seconds, extension at 72 °C for 45 seconds; final extension of 5:00 min at 72 °C.

PCR products were loaded on 1 % agarose gels, stained with ethidium bromide, and visualised on a "Gel Doc" system (BioRad). If results were satisfactory, products were purified using QIAquick spin columns (Qiagen) prior to cycle sequencing. A 10 µl sequencing reaction included 1 µl of template, 1 µl of sequencing buffer, 2 µl of 2 pmol/µl primer, 1.8 µl of ABI sequence mix and 4.2 µl of water. The sequence reaction was 33 cycles of 10 seconds at 96 °C, 10 seconds at 50 °C and 4:00 min at 60 °C. Sequence data collection and visualisation were performed on an ABI 3100 automated sequencer. Se-

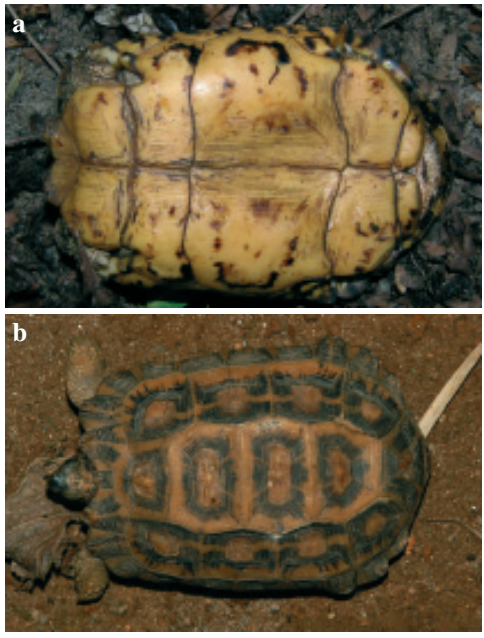

Fig. 3. *Pyxis planicauda,* ventral (a) and dorsal (b) view.

Fig. 4. Maximum Parsimony tree based on 427 bp of cytochrome *b* gene (1000 replicates) showing the relationships between the four *Pyxis* taxa. *Geochelone radiata* has been used as outgroup. Numbers correspond to specimen number.

quences were deposited in Genbank (accession numbers: AY834895- AY834930).

Sequence comparisons

Cytochrome *b* sequences were obtained from 36 individuals of *Pyxis*. A 427 bp segment of this gene was available from all specimens. Sequences were checked and aligned with the Sequence Navigator (Applied Biosystems) software. Sequence alignment was done visually since there was no length variation.

Maximum parsimony (MP) 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. One thousand replicates were calculated under the MP optimality criterion.

Fig. 5. These remains of a dead adult of *Geochelone radiata* were found near the village of Bevoalavo. We observed numerous carapaces of dead *G. radiata* along roads to Bavoalavo. Their numbers increased approaching the village, where people normally consume these tortoises for food.

Results

Field observations

We collected *Pyxis arachnoides* samples along its distribution area in southern Madagascar (Fig. 1). We observed many active individuals (and also many tracks in the sand) just after rain or during cloudy weather south of the village Beheloka and at Ifaty. However, it was relatively difficult to find individuals during sunny weather when they stay hidden by burying themselves in the sandy substrate. In such conditions, traces left by individuals on the surface sometimes allowed detection. However, during our field work it was sometimes difficult for us to find and collect specimens without the assistance of local people.

In three cases, we observed locals keeping tortoises as pets. At Manombo Atsimo for example, a sample of *P. a. brygooi* was ob-

tained from a child who was keeping it as pet. A rope was tightened to the tortoise's hind limb, where it caused a deep wound. *Pyxis arachnoides* is also intensively collected for consumption by locals in the Manombo Atsimo region. We observed several *P. arachnoides* in the south of Manombo Atsimo, at a place called Antsira, but despite of intensive searching by four people and for several hours only two individuals were observed in the remaining area around Manombo Atsimo. The villagers said that this animal was more common at this place in the past, but we were not able to verify this statement. At least four times, we saw several *P. arachnoides* individuals kept in houses as pets .

Sample collection was also difficult due to local circumstances. In the village of Bevoalavo tortoises were considered "fady" (taboo). Here, locals did not touch or collect these animals whereas in the neighbouring village people were unwilling to help collecting tortoises because here they were considered to be a food resource.

During our field work we collected samples of *Geochelone radiata*, that turned out to be rare near and north of Toliary. However, we found this species occurring in sympatry with *P. a. arachnoides* in the area south of Toliary, particularly in Mangoro, Mahatsandry, Vohombe and Beheloka (sampling localities of *G. radiata* are not indicated in Fig. 1). We found carapaces of dead *G. radiata* along roads in the south of the island at least two times (Fig. 5). This number increased as we approached Bevoalavo; there we found four broken carapaces near the village alone, with holes clearly made by humans. We also discovered a veritable "graveyard" of this species in a desert-like area near Bevoalavo. The place is a huge sandy flat area with no vegetation, between the sandy dunes close to the sea and the village. In 6.7 kilometers by car we counted 138 carapaces or pieces there of dead *G. radiata* spread everywhere in the area. Local people told us that this area is completely flooded by the sea at yearly intervals, suggesting that tortoises drown when the sea level rises.

Genetic differentiation

Of the 427 bp of the cytochrome *b* amplified in 34 individuals of *Pyxis arachnoides* and two individuals of *P. planicauda*, 364 characters were invariant, 23 were parsimony-uninformative but variable, and 40 were parsimony-informative. Of those 40 parsimony-informative characters 75% were third position substitutions and 18% and 7% respectively referred to substitutions at first and second codon positions.

Absolute pairwise distances (absolute number of substitutions) among individuals from the same subspecies were 0-2 for *P. a. brygooi* and *P. a. arachnoides* and 0 within *P. a. oblonga*. Pairwise distance between *P. a. brygooi* and *P. a. arachnoides* was 6-7; between *P. a. brygooi* and *P. a. oblonga* 3-4 and between *P. a. arachnoides* and *P. a. oblonga* 3. There was no variation within populations. Pairwise genetic distance of *P. arachnoides* to *P. planicauda* was 34-35 pairwise substitutions. Tamura and Nei (TnR) genetic distances between *P. a. brygooi* and *P. a. arachnoides* were 0.014-0.017; between *P. a. brygooi* and *P. a. oblonga* 0.007- 0.0095; between *P. a. arachnoides* and *P. a. oblonga* 0.007. From our data set the TnR genetic distance to *P. planicauda* was 0.09 for *P. a. brygooi* and *P. a. arachnoides* and 0.086 for *P. a. oblonga*. The cytochrome *b* sequence of *Geochelone radiata* gave a TnR genetic distance of 0.1 for each of the three subspecies of *P. arachnoides*.

The cytochrome *b* tree (Fig. 4) supported three main groups corresponding to the three geographic subspecies accepted by BOUR (1978). The low variability within each subspecies did not permit further genetic analyses at the population level to be carried out.

Discussion

Three geographic subspecies are currently defined within the species *Pyxis arachnoides* based on morphology (BOUR 1978). Our genetic data, based on cytochrome *b* analysis, confirmed the identity of each subspecies

as a monophyletic entity (Fig. 4). For Indian Ocean tortoises the Tamura and Nei genetic distance based on a combined data set of mtDNA genes range between 0.08 and 0.24 at the species level (PALKOVACS et al. 2002). Compared to that the genetic distances observed from our dataset at the subspecies level are lower than between species, ranging between 0.007 and 0.017. However, the genetic distance between recognized species, as *P. arachnoides* and *P. planicauda*, is about 0.09. This fact is in agreement with current classification, *P. planicauda* being a valid species, strongly differentiated from *P. arachnoides*. *P. a. arachnoides*, *P. a. brygooi* and *P. a. oblonga* are well identified as subspecies, in agreement with the current taxonomy and morphological data. The genetic identification of *P. planicauda* and *P. arachnoides* at a divergence level typical for species has also been shown by AUSTIN et al. (2003). Surprisingly the pairwise distance between *P. a. brygooi* (west) and the neighboring *P. a. arachnoides* (southwest) is distinctly lower than the pairwise distance between *P. a. brygooi* (west) and *P. a. oblonga* (south). This fact is unexpected considering the biogeographic distribution of the three subspecies.

The situation we observed in the *Pyxis* subspecies of low mitochondrial diversity related to highly differentiated morphology has also been shown in the giant tortoises of the small islands in the Indian Ocean (AUSTIN et al. 2003; PALKOVACS et al. 2003). Mitochondrial DNA analysis supported the origin of *Pyxis* species from *Dipsochelys* (PALKOVACS ET AL. 2002). It has been proposed that giant tortoises of Mascarene islands and of northern Madagascar radiated into few species with similar variation in shell shape as those present in the Galapagos (AUSTIN et al. 2003). In this latter case, different shell shape is probably an adaptation to different habitats and is the most distinctive character among populations of tortoises (CACCONE et al. 1999a; BEHEREGARAY et al. 2003). Shell shape is a distinctive character also between the three *Pyxis arachnoides* subspecies. However, more data are needed to study the possible relationship of different carapaces and the adaptation to different habitats. Nevertheless, in comparaison with the giant Galapagos radiation where morphologically named subspecies and genetic data shown discrepancies (CACCONE et al. 1999a, 2002; CIOFI et al. 2002), in *Pyxis arachnoides* genetic data confirm the existence of three separate *P. arachnoides* entities (identified as subspecies). These entities deserve separate conservation actions to preserve their genetic diversity.

Moreover, from our data we did not observe any haplotype sharing between the three subspecies nor any strong substructuring within each of them, probably due to the lack of resolution with the cytochrome *b* marker (no variation within each population). PALKOVACS et al. (2002) found that *Pyxis* appears to have an accelerated rate of mtDNA sequence evolution compared with the other tortoises examined in their study. This has been suggested to be related to the short generation time and the small body size of this genus (PALKOVACS et al. 2002). Despite that, the cytochrome *b* does not seem to be a good marker for population studies in this genus. It does not present enough variation within the same subspecies. It is assumed that turtles have a slower evolution rate in mtDNA than the rate in mammals (AVISE et al. 1992, but see SEDDON et al. 1998). More genetic data is necessary to assess the population structure within each subspecies and to check the existence of gene flow between subspecies in their contact zones. More field work is needed in order to assess the status of extant populations, which can be combined with genetic data in order to define conservation priorities for this species.

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