Population genetics analysis of *Podocnemis sextuberculata* (Testudines, Podocnemidae): lack of population structure in the central Amazon Basin

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ABSTRACT. The chelonians are, in general, important for the economy of the traditional populations of the Amazon region, especially as a source of animal protein. Furthermore, sub-products, such as eggs and fat, are utilized in the manufacture of cosmetics, and the plastron and carapace are used in the manufacture of adornments. The freshwater turtle species *Podocnemis sextuberculata*, locally known as "iaçá" or "pitiú", is widely distributed in the Amazon Basin in Brazil and

also in Colombia and Peru. This species is on the International Union for Conservation of Nature Red List in the category of vulnerable species. We examined the genetic variability and population structure of three populations represented by 64 individuals sampled from Reserva Federal de Abufari, Tapauá, Amazonas State; Reserva de Desenvolvimento Sustentável Mamirauá, Tefé, Amazonas State, and Terra Santa, Pará State. All of these are over 1000 km from each other. A partial 415-bp sequence of the mitochondrial gene ND1 was utilized as a molecular marker. Seven haplotypes were observed; the most common haplotype was shared by all the areas sampled, while the rarest haplotypes were represented by a single individual and were thus restricted to a single locality. The sharing of the most common haplotype, the high number of migrants (*Nm*) and the AMOVA results indicate a lack of genetic structure among the sampling localities. The levels of genetic variability observed were homogeneous among the sampling localities. These results (Θ_{ST} and *Nm*) are compatible with what is known about the ecology of this species, which has a great migratory capacity.

Key words: Chelonians; Iaçá; Amazon basin; Mitochondrial DNA; *Podocnemis sextuberculata*

INTRODUCTION

In Brazil, the genus *Podocnemis* is represented by four species, one of which is *Podocnemis sextuberculata*, known commonly as "iaçá", "pitiú" or "cambéua". This species can be found in the white-water rivers such as the Solimões, Amazonas and Branco, as well as in the lower section of clear- and black-water rivers such as the Trombetas, Tapajós, Xingu, and Negro. *P. sextuberculata* is found not only in Brazil but also in many rivers in Colombia and Peru. The female possesses yellow patches with two barbels below the mouth. In young individuals, the plastron has six projecting gray or brown points (tubercles), hence the name *P. sextuberculata*. The eggs have soft shells, and the average clutch is 20-25 (IBAMA, 1989). *Podocnemis sextuberculata* is economically important for Amazonian riverbank inhabitants, where their meat and eggs are often appreciated (SEBRAE, 1995). Furthermore, their sub-products such as fat are utilized in the manufacture of cosmetics, and the plastron and carapace are used in the manufacture of adornments.

Podocnemis sextuberculata is on the IUCN Red List in the category of vulnerable species (IUCN, 2009). The basic criterion for such classification is a decrease in census sizes of natural populations. However, according to Fachín-Terán et al. (2003), there are no quantitative biological data for this species that support such classification in any of the countries where it occurs. The lack of biological and ecological data for this species hinders the determination of its current status, and, in particular, the introduction of management and conservation practices.

The management of many threatened species is based on knowledge of their population dynamics, as specific demographic information is lacking, mainly with regard to

the chelonians, which are organisms that show longevity (Finch, 1990; Lutz et al., 2003). Population genetic data are one of the most important baseline components of any conservation and management plan (Frankham et al., 2002). Chelonian management, that is their conservation and breeding in captivity, depends on knowledge of the patterns of variability and genetic structure of the populations in different regions, combined with data on their ecology, physiology, nutrition, and reproduction.

Considering the economic importance, the history of the exploitation of *P. sextuberculata* in the Amazon region (Fachín-Terán et al., 2000), and the total lack of data on its population genetics, the central objective of this study was to characterize the variability and the genetic structure of natural populations of this species in three Amazon Basin locations, utilizing the ND1 gene of mitochondrial DNA (mtDNA).

Genes of mtDNA have quite often been utilized in genetic studies of freshwater turtles, sea turtles, and tortoises. Population genetics as well as the genetics of the reproductive system have been studied in the Amazon River turtle *P. expansa* (Sites Jr. et al., 1999; Pearse et al., 2006a,b). More recently, *P. lewyana*, a species endemic to northern Colombia, has been genetically characterized using the cytochrome *b* gene (Vargas-Ramírez et al., 2007), and the mating system of *P. unifilis* has been studied with microsatellite DNA markers (Fantin et al., 2008). In the study of *P. expansa* from the Amazon Basin, Pearse et al. (2006a) utilized mitochondrial and microsatellite markers in individuals drawn from various hydrographic basins. The authors found a lack of population structure in tributaries of the same hydrographic basin, but genetic differences among basins. Ecological data have suggested that *P. sextuberculata* shows a high capacity for dispersion (Fachín-Terán et al., 2006), which may lead to a lack of population differentiation within the Amazon Basin, similar to that observed with *P. expansa*. In this study, we tested the null hypothesis of panmixia of the populations of this species among Amazon Basin locations that are separated by distances over 1000 km, using the mtDNA gene ND1.

MATERIAL AND METHODS

Sample collection, DNA extraction and polymerase chain reaction (PCR)

The samples were collected at Reserva Biológica Federal do Abufari, Tapauá Municipality (Middle Purus River), Amazonas State; at Reserva de Desenvolvimento Sustentável Mamirauá (RDS Mamirauá), Tefé Municipality (between Solimões and Japurá Rivers), Amazonas State, and at Terra Santa (Lower Amazon River), Pará State in a chelonian management area of the "Pé-de-pincha" project (Figure 1). A total de 64 individuals were collected, 29 from the Reserva Biológica Federal do Abufari, 11 from RDS Mamirauá and 24 from Terra Santa. Total genomic DNA was extracted from 0.5 mL blood per individual following the phenol/chloroform protocol (Sambrook et al., 1989) with few modifications.

The partial mitochondrial ND1 gene was amplified by PCR using the primers 16SF.2 and MetR.1 (Hrbek et al., 2002). PCR was carried out in a 25-µL total volume and consisted of 1 µL DNA (approximately 100 ng DNA), 2 µL 2 µM of each primer, 2.5 µL 10X buffer (200 mM Tris-KCl, pH 8.5), 3 µL 25 mM MgCl$_2$, 2.5 µL 10 mM dNTP, 0.2 µL 5 U/µL *Taq* DNA polymerase and 11.8 µL H$_2$O. PCR was run in a Perkin
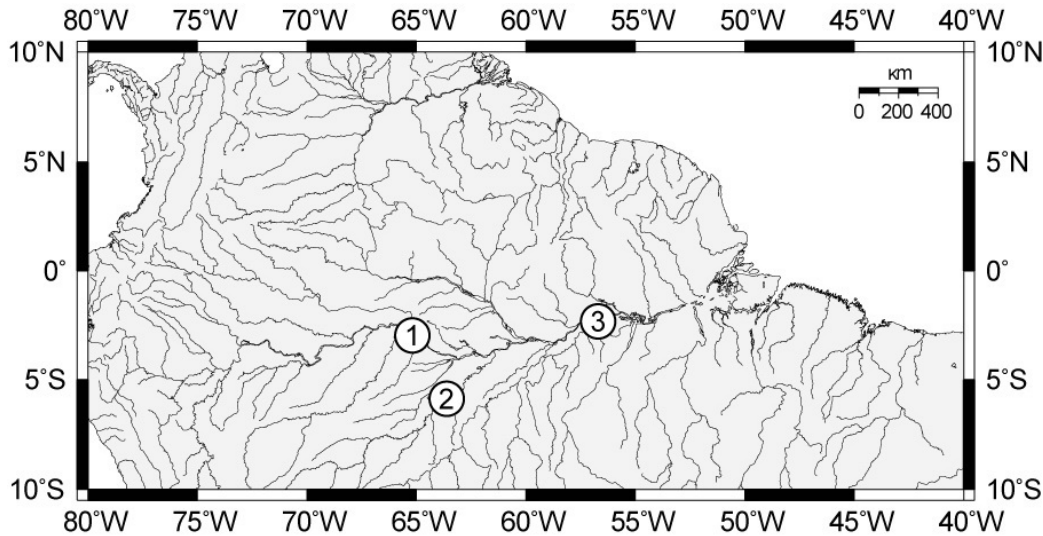
Figure 1. Sample locations. **1.** RDS Mamirauá (AM). **2.** Abufari (AM). **3.** Terra Santa (PA).

Elmer GeneAmp PCR System 9600 and had the following steps: an initial denaturation step at 92°C for 1 min, followed by 35 cycles of annealing at 55°C for 1 min, and extension at 72°C for 1 min. The PCR products were purified according to manufacturer recommendations using the Concert Nucleic Acid Purification System (Life Technologies).

DNA sequencing and statistical analysis

Sequencing reactions were performed according to manufacturer recommendations using the Terminator Cycle Sequencing kit (GE-Healthcare), and resolved on a MegaBACE 1000 automated sequencer (GE-Healthcare). Partial ND1 nucleotide sequences were edited using the BioEdit Version 5.0.6 program (Hall, 1999) and aligned by eye. The haplotype network was reconstructed using the TCS version 1.13 program (Clement et al., 2000), which implements the algorithm described by Templeton et al. (1992). The DnaSP v5 program (Librado and Rozas, 2009) was used to estimate number of haplotypes, segregating sites (a site that is polymorphic in the data), haplotype diversity (probability that two randomly chosen haplotypes are different in the sample; Nei, 1987) and nucleotide diversity (average number of nucleotide differences per site between any two DNA sequences randomly chosen from the sample population; Nei and Li, 1979). The mutation-drift genetic equilibrium of mtDNA alleles was tested using Tajima's D test (Tajima, 1989), and Fu's Fs test (Fu, 1997), which are based on the infinite-site model under the hypothesis of selective neutrality and population equilibrium. Population subdivision and structure were examined using analysis of molecular variance (AMOVA) (Excoffier et al., 1992), and pair-wise population θ_{ST} (Cockerham and Weir, 1993) as implemented in the ARLEQUIN v. 3.0 program (Excoffier et al., 2005).

RESULTS AND DISCUSSION

The total alignment of the partial ND1 gene consisted of 415 bp. The average nucleotide base proportions were 27.69% cytosine, 32.05% thymine, 29.40% adenine, and 10.85% guanine, providing evidence of a bias against G and an A-T bias (61.45%), typical of vertebrate mitochondrial genes (Zhang and Hewitt, 1996).

In the three populations of *P. sextuberculata*, a total of 7 polymorphic sites were found (Table 1; Figure 2), 6 arising from transitions and 1 from a transversion. The low levels of genetic variability found for the partial sequence of the ND1 gene in *P. sextuberculata* (Table 2) are consistent with those suggested by Avise et al. (1992) for the low rate of molecular evolution of mtDNA in turtles compared with other taxa. However, such a suggestion may be more frequent for coding genes, since non-coding regions, as observed in ribosomal genes and mitochondrial control region, have been broadly used for analyses of population level (Seddon et al., 1998; Sites Jr. et al., 1999; Pearse et al., 2006a). Protein-coding genes, such as cytochrome *b*, have been characterized as relatively invariable in *E. marmorata* (Janzen et al., 1997) and *P. lewyana* (Vargas-Ramírez et al., 2007), although they have shown sufficiently high variation for phylogeographic analyses in other species, such as in North American softshell turtles *Apalone* spp (Weisrock and Janzen, 2000). Similar high levels of genetic variability were observed for the segments of ND4 in *Emys marmorata* (Spinks and Shaffer, 2005). Such results suggest that protein-coding genes and non-coding genes may display patterns of different levels of genetic variability in different turtle taxa.

Table 1. List and frequency of haplotypes found in the three different locations sampled for *Podocnemis sextuberculata*.

Haplotype	Abufari	RDS Mamirauá	Terra Santa
02Abuf - H1	24	10	22
03Abuf - H2	1		
11Abuf - H3	2		1
14Abuf - H4	1		
21Abuf - H5	1		
43RDSM - H6		1	
72TSan - H7			1
	29	11	24

Table 2. Main genetic patterns for *Podocnemis sextuberculata*.

Sampling site	N	No. of haplotypes	Gene (haplotype) diversity	Nucleotide diversity (per site)	Tajima's *D* test	Fu's *Fs* test
RDSM	11	2	0.182 ± 0.14	0.00044	-1.1285	-0.410
Abufari	29	5	0.318 ± 0.01	0.00096	-1.5761	-3.195
Terra Santa	24	3	0.157 ± 0.09	0.00058	-1.733	-1.404
All	64	7	0.235 ± 0.07	0.00074	-2.003*	-6.570*

RDSM = Reserva de Desenvolvimento Sustentável Mamirauá. *Significant at the 5% level.
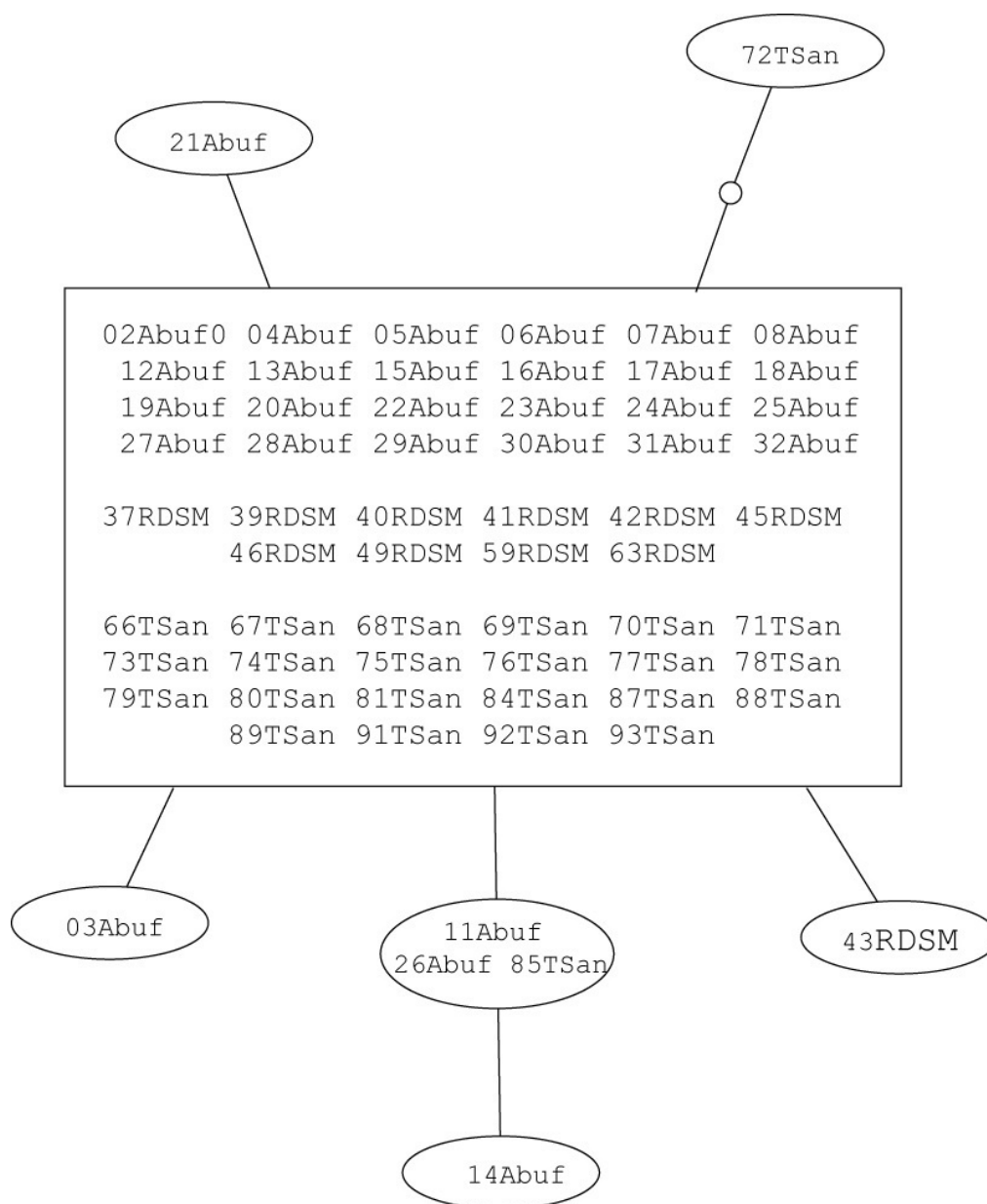
Figure 2. Haplotype network found in the partial ND1 gene sampled in 64 individuals of *Podocnemis sextuberculata* from Reserva de Desenvolvimento Sustentável Mamirauá (RDSM), Abufari (Abuf), and Terra Santa (TSan).

Considering these aspects of mtDNA genes, it was not our aim to relate the low levels of genetic variability observed with the effects of pressure *P. sextuberculata* has suffered from

being hunted, as such would require more variable markers. However, the partial fragment of the ND1 gene proved to be a good marker to demonstrate the lack of population structure.

Lack of population structure

The partial ND1 sequence was shown to be a good molecular marker to provide us with a tool for detecting genetic differentiation within this species. There were seven haplotypes found in the three populations sampled (Table 1, Figure 2), the most common haplotype being 02Abuf, shared by individuals from the three populations, and present in 87.5% of the individuals sampled. This haplotype was shared among individuals from all the locations sampled, where it was found in 82.75% of the individuals from Abufari, 90.9% of those from RDS Mamirauá, and 91.66% of those from Terra Santa. Singletons and rare haplotypes were also observed. The distribution of the haplotypes did not indicate any correlation with the geographic location of the individuals sampled, suggesting the lack of geographic structure.

The high level of haplotype sharing observed among the three areas was also confirmed by the observed high gene flow, where the smallest number of migrants per generation was $Nm = 35.37$ (Table 3), with the greatest gene flow found between Abufari and Terra Santa. According to Kimura and Maruyama (1971), when Nm assumes values greater than four between localities, the localities can be considered belonging to as a single panmictic population, whereas Nm less than one indicates that the localities are differentiated. The high Nm observed among the localities sampled would indicate that *P. sextuberculata* has a high migration or dispersion rate, and confirms what the ecological data had already suggested, that this species has a high capacity for dispersion (Fachín-Terán et al., 2006).

Table 3. Gene flow between the three sampling locations of *Podocnemis sextuberculata*.

Location	Abufari	RDSM	Terra Santa
Abufari	-	-	-
RDSM	∞	-	-
Terra Santa	35.37	68.27	-

RDSM = Reserva de Desenvolvimento Sustentável Mamirauá.

According to Gibbons (1986), many chelonian species move great distances in response to seasonal changes, but the response to adverse conditions can vary within or among species. In the RDS Mamirauá, *P. sextuberculata* moves in response to water level, the opposite of *P. unifilis*, where some individuals remain in the reserve. Fachín-Terán et al. (2006), observing *P. sextuberculata* in the RDS Mamirauá, demonstrated by means of radio-telemetry the migration from the feeding places to breeding areas on the Japurá and Solimões Rivers. One individual of *P. sextuberculata* marked in RDS Mamirauá was also found in the Middle Juruá River, about 300 km away from the RDS Mamirauá (Andrade P, personal communication).

The AMOVA results ($\Theta_{ST} = 0.00876$, P > 0.05) suggest that there is no genetic differentiation among the groups of individuals from the three areas sampled (Table 4). In interpreting the Θ_{ST} numbers (Table 3) according to Wright (1931), a value less than 0.05 can be

Table 4. Results of analysis of molecular variance (AMOVA).

Source of variation	Variance components	Percentage of variation
Among population	0.00311	0.88
Within population	0.35195	99.12
Total	0.35506	

$\theta_{ST} = 0.00876$ (P = 0.24536 ± 0.01442).

considered an indicator of low genetic differentiation. The lack of genetic structure between great geographic distances and extensive gene flow between the localities studied corroborate observations in field work. Fachín-Terán et al. (2006), utilizing the mark-recapture method, observed that the distances covered by individuals of *P. sextuberculata* varied greatly, and evidence of territoriality that was not found in the RDS Mamirauá. The authors suggest that the population of *P. sextuberculata* is open, as there is a very low recapture rate (3.6%), indicating the dispersion of a high number of marked individuals. Such results indicate that *P. sextuberculata* individuals make seasonal migrations. At the beginning of the dry period, to avoid unfavorable conditions, they leave the flooded forests and lakes and head towards the main river channels (Fachín-Terán et al., 2006).

Despite the lack of population genetic structure observed over the large distances between the three collection locations (the largest distance being approximately 1300 km between the Terra Santa and RDS Mamirauá locations), individuals from other areas of the Amazon Basin need to be investigated to confirm panmixia among all the tributaries of the Amazon Basin, where the species occurs. It may also be necessary to study other genetic markers to elucidate possible population differences to help with any management plan.

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