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Short Communication

Tocantins river as an effective barrier to gene flow in *Saguinus niger* populations

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Abstract

The *Saguinus* represent the basal genus of the Callitrichinae subfamily. Traditionally this genus is divided into three groups: Hairy, Mottled and Bare-face, however, molecular data failed to validate these groups as monophyletic units, as well as raised some subspecies to the species status. This is the case of the former subspecies *Saguinus midas midas* and *S. midas niger*, which are now considered as different species. In the present study, we sequenced a portion of the D-loop mtDNA region in populations from the East bank of the Xingu and from both banks of the Tocantins river, in order to test the effectiveness of large rivers as barriers to the gene flow in *Saguinus*. According to our results, the populations from the East and West banks of the Tocantins river are more divergent than true species like *S. mystax* and *S. imperator*. The Tocantins river may be acting as a barrier to gene flow, and consequently these very divergent populations may represent distinct taxonomic entities (species?).

Key words: primates, *Saguinus niger*, D-loop, mtDNA, Tocantins river.

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The tamarins, *Saguinus* spp., are small-bodied primates distributed from the Amazon to the northern coast of South America and Panama. They are the basal members of the Callitrichinae subfamily, which also includes the genera *Callithrix, Cebuella, Mico, Leontopithecus* and *Callimico* (Primates - Platyrrhini) (Schneider *et al.*, 1993, 1996: Schneider 2000: Rylands *et al.*, 2000).

After Hershkovitz's work (1977), few studies were carried out concerning the taxonomic relationships among *Saguinus* species. Based on variations in dental measurements, Natori and Hanihara (1992) classified the genus into two clades: one comprising *S. oedipus*, S. *geoffroyi*, *S. bicolor*, *S. midas* and *S. niger*, and the other encompassing *S. leucopus*, *S. imperator*, *S. nigricollis*, *S. fuscicollis*, *S. inustus, S. labiatus*, and *S. mystax*.

Based on DNA sequences of the first three exons and introns of the β2-microglobulin nuclear gene, Canavez *et al.,* (1999) considered *S. fuscicollis* as the first offshoot within *Saguinus*, followed by an unresolved trichotomy for *S. mystax/S. imperator*, *S. niger*, *S. midas/S. bicolor*, and *S. oedipus.* Additionally, these authors suggested that *S. midas* was more closely related to *S. bicolor* than to *S. niger*, contradicting previous studies based on biochemical (Meireles *et al.*, 1997) and mitochondrial DNA data (Cropp *et al.*, 1999), which clearly showed *S. midas* and *S. niger* to be closely related taxa, separated well apart from the *S. bicolor* -*S. martinsi* clade.

Rylands *et al.* (2000) presented a synthesis for *Saguinus* classification and suggested the occurrence of 15 species without any subdivision. These species are: *S. nigricollis*, *S. fuscicollis*, *S. graellsi*, *S. tripartitus, S. labiatus, S. inustus, S. geoffroyi, S. leucopus, S. oedipus, S. mystax, S. imperator, S. midas, S. niger, S. bicolor* and *S. martinsi.* This is the classification we adopted in the present work.

The purpose of this paper was to examine the phylogenetic relationships among taxa of the genus *Saguinus* based on the fast evolving mitochondrial D-loop nucleotide sequences, aiming to answer two questions: 1) is *S. midas* from the North of the Amazon river more closely related to *S. bicolor* than to *S. niger* from the southern Amazon river? and 2) is the Tocantins river an effective barrier to *S. niger* populations?

Part of the D-loop region of the mitochondrial DNA was sequenced for 34 *Saguinus* specimens, being 22 from three different populations of *S. niger* (East bank of the Xingu river, 3°31' S/51°58' W, Pará State, Brazil, and East and West banks of the Tocantins river, 4°19' S/49°32' W, Pará State, Brazil), four of *S. midas* from two populations (Uatuma river, 1°55' S/59°28' W, Amazonas State, Brazil,

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and Trombetas river, 1°25' S/56°25' W, Pará State, Brazil), three of *S. martinsi* (unknown origin), two of *S. bicolor* (Manaus, Amazonas State, Brazil, 3°31' S/41°58' W), two of *S. imperator* (unknown origin) and one of *S. mystax* (Acre State, Brazil, 10°03' S/67°27' W). The DNA was extracted using the standard phenol-chloroform method, followed by sodium acetate precipitation (Sambrook *et al.*, 1989). The primers used for the amplification were L15926 (5'-TCAAAGCTTACACCAGTCTTGTAAACC-3') and H00651 (5'- TAACTGCAGAAGGCTAGGACCAAACC T-3'), as described by Kocher *et al.* (1989). PCR was performed using 10 ng of genomic DNA, 10 mM Tris-HCl pH 8.85, 25 mM KCl, 5 mM $(NH_4)_2SO_4$, 0.2 mM dNTP, 50 ng of each primer and 1 U Taq DNApolymerase (Qiagen). The amplification reactions were performed in a minicycler MJ Research thermocycler with a cycling profile of 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, with an additional extension period of 72 °C for 5 min in the last cycle. The PCR products were purified using ExoSAP-IT (USB) and then sequenced using a dye terminator cycle sequencing kit in a 377ABI automatic sequencer, according to protocols supplied by the manufacturers (Applied Biosystems, Foster, CA, USA).

The sequences were aligned using the BIOEDIT sequence editor (Hall, 1999) and ClustalW (Thompson *et al.*, 1994) by constructing a common alignment of 489 bp. The sequences were deposited in GenBank under accession numbers DQ241218-DQ241250 A divergence matrix was constructed using the parameters and evolutionary model selected by the MODELTEST program version 3.07 (Posada and Crandall, 1998). The phylogenetic reconstructions were made using PAUP* version 4b10 (Swofford, 2003) for maximum parsimony and neighbor joining, and Treefinder (Jobb *et al.*, 2004) for maximum likelihood. Saturation test was performed using the DAMBE program (Xia and Xie, 2001). The confidence of tree topologies was assessed by bootstrapping of 1000 pseudo-replicates. Genetic differentiation within, between and for the entire population was evaluated by computing genetic diversities in Mega version 3.1 (Kumar *et al.*, 2004).

The alignment obtained showed 120 variable sites (87 parsimony-informative), defining 33 distinct haplotypes among the tamarins (*Saguinus*). Considering only *S. niger* populations, 64 variable sites were observed generating 21 distinct haplotypes, five from the East bank of the Tocantins, nine from the West bank of the Tocantins, and seven from the East bank of the Xingu river. The haplotype diversity and standard error for each population were: East Tocantins = 1.0 ± 0.126; West Tocantins = 1.0 ± 0.052 and East Xingu = 0.89 ± 0.11. In addition, the estimates of nucleotide diversities showed that East Xingu (Pi = 0.0213 ± 0.006) displayed the highest diversity, followed by East Tocantins (Pi = 0.017 ± 0.004) and West Tocantins (Pi = 0.012 ± 0.003) with similar nucleotide diversities.

Table 1 shows the pairwise genetic distances estimated between all the taxa investigated, based on the HKY model (Hasegawa *et al.*, 1985), assuming among-site variation to follow a gamma distribution with parameter alpha equal to 0.7539, a transition transversion ratio of 3.9555, and the proportion of invariant sites being equal to 0.5317. The divergence between *S. niger* populations separated by the Tocantins river was 0.086 ± 0.007. This value is in the range of the differences found between *S. niger* and *S. midas* (0.079 to 0.105) populations; it is similar to those differences found between *S. bicolor* and *S. martinsi* (0.075 ± 0.003) or even *S. imperator* x *S. mystax* (0.094 ± 0.001), which are all considered as valid species. The genetic distances between *S. midas* from the Uatumã and Trombetas (0.049 ± 0.010) were, to some extent, higher than those between *S. niger* populations from the East Xingu and West Tocantins. Interestingly, the Trombetas population seems to be as related to *S. niger* from East Tocantins (0.048 ± 0.009) as it is to *S. midas* from Uatumã (0.049 ± 0.010). However, since only two specimens from each site were studied, these results should be interpreted with caution, but they certainly deserve special attention in future studies. The analysis of mean diversities comparing the three

Table 1 - Estimates of genetic distances among *Saguinus* lineages based on HKY85 model assuming heterogeneous substitution rates among lineages and rates among sites following a gamma distribution.

	S. niger TE	*S. niger* -TW	*S. niger* - XE	*S. midas* 1-2	*S. midas* 3-4	*S. bicolor*	*S. martinsi*	*S. imperator*
S. niger-TW	0.086 ± 0.007							
S. niger-XE	0.089 ± 0.011	0.039 ± 0.007						
S. midas 1-2	0.079 ± 0.015	0.105 ± 0.007	0.101 ± 0.011					
S. midas 3-4	0.048 ± 0.009	0.056 ± 0.005	0.059 ± 0.005	0.049 ± 0.010				
S. bicolor	0.2024 ± 0.021	0.164 ± 0.009	0.186 ± 0.012	0.223 ± 0.019	0.169 ± 0.027			
S. martinsi	0.185 ± 0.159	0.163 ± 0.006	0.170 ± 0.007	0.187 ± 0.010	0.149 ± 0.019	0.075 ± 0.003		
S. imperator	0.2761 ± 0.043	0.298 ± 0.032	0.289 ± 0.031	0.315 ± 0.048	0.267 ± 0.052	0.231 ± 0.029	0.201 ± 0.021	
S. mystax	0.260 ± 0.006	0.266 ± 0.006	0.236 ± 0.027	0.254 ± 0.027	0.228 ± 0.028	0.201 ± 0.002	0.188 ± 0.004	0.094 ± 0.001

TE = Tocantins river, East Bank; TW = Tocantins river, West Bank; XE = Xingu river, East Bank. *S. midas* 1-2 = Uatumã river; *S. midas* 3-4 = Trombetas river.

populations of *S. niger* showed that 78% of the total diversity is ascribable to differences between the East and West banks of the Tocantins river, while only 36% is due to differences between the Xingu and Tocantins West banks (data not showed).

Maximum parsimony, maximum likelihood and neighbor joining analyses showed that populations of *S. niger* from the East bank of the Xingu and the West bank of the Tocantins rivers are more closely related to each other (bootstrap results equal to or greater than 97%) than populations from distinct banks of the Tocantins river. Intriguingly, in spite of the non-significant bootstrap values (73 to 88%), the population of the East Tocantins seems to be more related to *S. midas* from the North of the Amazon river than to *S. niger* from the West Tocantins. Finally, as expected, *S. bicolor* and *S. martinsi* were found to be monophyletic (bootstrap equal to 100% - Figure 1) and very closely related to each other (bootstrap support equal or greater than 97%).

To test the real effectiveness of the Tocantins river as a barrier for *S. niger* populations, we estimated the maximum-likelihood under two different assumptions, one constrained, *i.e.*, forcing *S. niger* from both banks of the Tocantins to be part of the same monophyletic group, and the other unconstrained, as the alternative hypothesis. The likelihood of the unconstrained tree was -1967.08126, but when Tocantins populations from both banks were constrained to be a monophyletic assemblage, a highly significant drop of 44.99627 ($p < 0.01$) in the likelihood was observed, as revealed by the SH-test (Shimodaira and Hasegawa, 1999). These results strongly suggest that *S. niger* populations from different banks of the Tocantins river have been isolated for a long time, and are consequently undergoing a process of differentiation driven by geographic isolation.

A second very significant conclusion of this D-loop analysis was that *S. midas* and *S. niger* indeed belong to a monophyletic group, strongly supported by bootstrap values of 99-100%. This finding contradicts the results of Canavez *et al.* (1999), based on nuclear DNA sequences, that indicated a closer relationship between *S. midas* and *S. bicolor*. Again, constraining *S. midas* to be sister group of *S. bicolor*, as suggested by Canavez *et al.* (1999), produced a highly significant drop in the likelihood of 77.18821 ($p < 0.01$), rejecting the proposal of these authors. Previously, Tagliaro *et al.* (2005) using ND1 mitochondrial DNA data, reached the same conclusions as those of our D-loop analysis, and attributed that incongruity to the conservative nature of the nuclear DNA sequence (900 base pairs from exons 1, 2 and 3 of the β2-microglobulin gene) used by Canavez *et al.* (1999).

A third and unexpected finding was the separation in the phylogenetic tree of *S. midas* from the Uatumã (State of Amazonas, Brazil) of those from Trombetas (State of Pará, Brazil) banks, which are about 200 km away from each

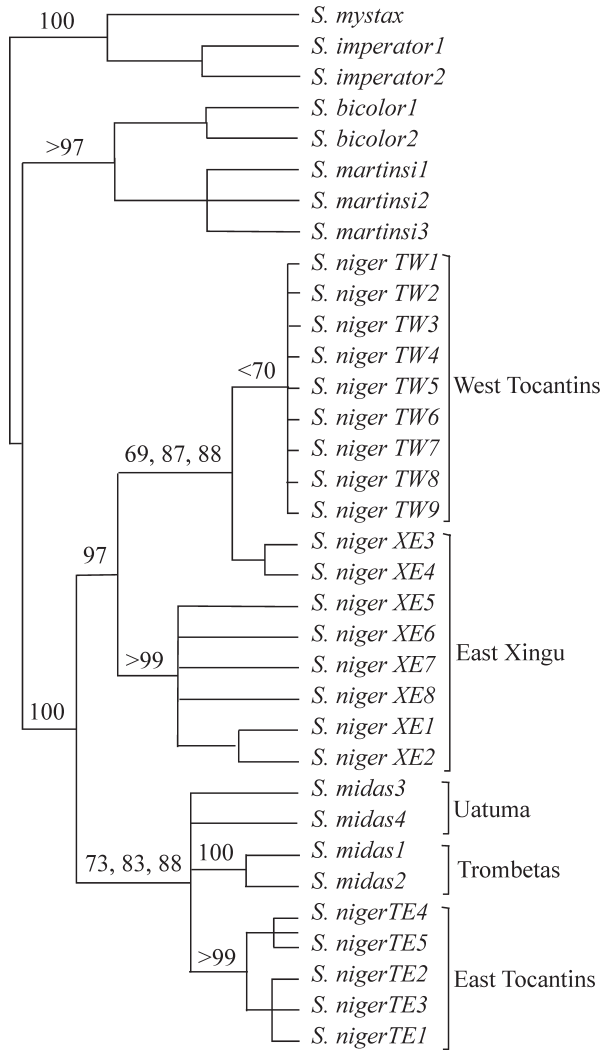

Figure 1 - Phylogenetic consensus tree depicted by bootstrapping 1000 pseudoreplicates. Numbers at nodes mean bootstrap support levels for maximum parsimony (MP), minimal evolution (ME) and maximum likelihood (ML), respectively. When bootstrap values of MP, ME and ML were similar the signal greater than (>) was used.

other, almost the same distance that separates *S. niger* populations of the Tocantins and Xingu rivers. The genetic distances between populations of *S. midas* from Uatumã and Trombetas (0.049) are of the same magnitude as those between Trombetas and Tocantins East bank (0.048). Conversely, the genetic distance between *S. midas* from Uatumã and *S. niger* from Tocantins East bank is 0.079.

It is obvious that description of biodiversity has a great importance to wildlife conservation. However, as it implies the definition of species, it has been a matter of great and unsolved debate (see Groves, 2004, for a good review on Primates). Distance methods have been proposed as a mean of objectively identifying species. The idea is that genetic similarity is greater within than between species (Nei, 1972). Some authors caution against this approach (see Vogle and DeSalle, 1994 for details). Alternatively, a

number of authors have advocated the use of the concept of evolutionarily significant units (ESUs) to characterize useful units for conservation purposes (Ryder, 1986; Cracraft, 2002). According to Moritz (1994), in order to be identified as ESUs, the populations should be reciprocally monophyletic for mtDNA alleles and also show divergence of allele frequencies at nuclear loci. Yet, Vogle and DeSalle (1994) favor a definition of ESUs based on patterns of variation. According to them, "in the theoretical framework of the phylogenetic species concept, conservation units are delimited by characters that diagnose clusters of individuals or populations to the exclusion of other such clusters". Another important concept is the management unit (MU), also proposed by Moritz (1994), which represents populations that do not show reciprocal monophyly, but have diverged in allele frequency. However, Hudson and Coyne (2002) observed that we must be cautious about recognizing species using only mitochondrial DNA, since this DNA becomes monophyletic more rapidly than does a single nuclear gene, and far more rapidly than does a sample of several nuclear genes. Therefore, the use of mtDNA alone is not a good strategy for assessing reciprocal monophyly, unless the population divergence is very ancient, which in the present study does not seem to be the case. According to the phylogenetic species concept (Hennig, 1966), species can be identified as the smallest aggregation of populations or lineages diagnosable by a unique combination of character states. It is important to note that our phylogenetic tree shows *S. niger* from the West and East banks of the Xingu and Tocantins rivers, respectively, as belonging to a monophyletic clade (bootstrap supports equal to or greater than 97%), excluding *Saguinus* populations from the East bank. This eastern population tends to form a moderately well supported group (bootstrap support 73% to 88%) with *S. midas*, populations which are separated not only by a large distance but also by a huge barrier, represented by the Amazon river. So, following the interpretation of Vogle and DeSalle (1994) "in the theoretical framework of the phylogenetic species concept", *Saguinus* from different banks of the Tocantins river should be considered as distinct ESUs, if these results were confirmed by other molecular markers. On the other hand, using the criterion of genetic distances, they also can be considered as distinct entities, such as *S. bicolor* and *S. martinsi* or *S. midas* and *S. niger*, for example. Regarding the XE and TW populations, they fit the criteria of management units (MU), as they are not reciprocally monophyletic and present a discrete percentage of nucleotide divergence (0.039 ± 0.007).

The present study reinforces the existence of two divergent populations of *Saguinus niger* separated by the Tocantins river, it corroborates the monophyletic status of the *S. midas*-*S. nige*r group and it calls attention to the divergence of *S. midas* from the northern Amazon, indicating that much has still to be done in order to clarify all aspects of the biological diversity of New World primates.

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