IUCN-SSC Crocodile Specialist Group
***Student Research Assistance Scheme* (SRAS)**

Phylogeography and population genetics of Cuvier's dwarf caiman (*Paleosuchus palpebrosus*) in the Amazon: next-generation sequencing approach

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ABSTRACT

Cuvier's dwarf caiman (*Paleosuchus palpebrosus)* is a widely distributed crocodilian species in the Neotropics. It occurs in different biomes and watersheds, spanning a range of environments and discontinuous habitats. A preliminary genetic evaluation revealed that there are four mitochondrial lineages that substitute each other as one moves from the Amazon to the Pantanal. Each mitochondrial lineage is probably an Evolutionarily Significant Units (ESUs) or Management Units (MUs); therefore it is necessary to use nuclear molecular markers to confirm these mitochondrial lineages as ESUs or MUs. Here, I report some field observations about the threats that the crocodilians is subject in the sampled regions and the result of a population structure analysis based on a representative genome sequencing using the ddRADseq approach.

INTRODUCTION

Cuvier's dwarf caiman (*Paleosuchus palpebrosus)* is a widely distributed crocodilian species in the Neotropics. It occurs in different biomes and watersheds, spanning a range of environments and discontinuous habitats. The main conservation threats to *P. palpebrosus* are habitat loss, due to deforestation, urbanization and damming of rivers, and habitat modification due to mining pollution (Campos *et al.*, 1995; Magnusson & Campos, 2010; Campos *et al.*, 2013a; Campos *et al.*, 2015a). Despite all these threats, *P. palpebrosus* is classified as Least Concern for conservation by IUCN Red List, mainly because it is widely distributed and locally abundant in some areas (last assessment in 1996; IUCN, 2016).

Until recently, *P. palpebrosus* was considered one of the least studied species of Neotropical crocodilians (Magnusson & Campos, 2010). However, recent genetic (Muniz, 2012) and ecological studies (Campos *et al.*, 2010; Campos *et al.*, 2012; Campos & Magnusson, 2013; Campos *et al.*, 2013b; Campos *et al.*, 2015a,b; Campos & Magnusson, 2016) promise to help both conservationists to make important decisions to maintain the evolutionary potential of the species, and evolutionists to understand their evolutionary history.

Paleosuchus palpebrosus is one of the smallest species (Campos *et al.*, 2010) and with one of the most terrestrial habits among the living crocodilians (Magnusson 1985; Campos *et al.*, 1995; Magnusson & Campos, 2010). It occur in a wide variety of habitats, including: streams lined by gallery forests, palm swamps, flooded forests near the major rivers and lakes, quiet stretches of large rivers, rapids and headwater streams with rocky substratum (Magnusson, 1985; Thorbjarnarson, 1992; Campos *et al.*, 1995; Campos & Mourão, 2006; Magnusson & Campos, 2010).

All these factors, such as the occupation of a widely geographic distribution,

terrestrial habits and occupation of a wide variety of habitats, reinforced our interest in investigating whether the species was genetically structured, in the same way as the African crocodile species of the genus *Osteolaemus* (Eaton *et al.*, 2009; Franke *et al.*, 2013), whose characteristics are similar to those of *P. palpebrosus*. Thus, during his master degree, Muniz (2012) developed a population genetic study to address this question. The author sequenced the cytochrome b (cyt b) mitochondrial gene from 172 individuals from the upper Paraguay River and Amazon River basins, and discovered a strong pattern of genetic structuring in *P. palpebrosus*. This strong genetic structuring suggest long term isolation between intraspecific lineages in the *P. palpebrosus*. Population structure analysis detected four cyt b clusters and allowed him propose geographically well defined groups: "Amazon", "Madeira-Bolivia" and "Pantanal". The discovery of genetic structuring may have important implications for conservation of this species, since each lineage can be an Evolutionarily Significant Unit (ESU) or a Management Unit (MU) and both identifying and establishing the boundaries of this lineages are considered one of the first steps to make successful decisions for conservation (Funk *et al.*, 2012). However, it would be necessary to use nuclear molecular markers to confirm whether this mitochondrial lineages really represented ESUs or MUs.

A promising approach to delimiting ESUs and MUs is via Next Generation Sequencing (NGS). Using approaches such as ddRADseq (double digest RAD sequencing protocol; Peterson *et al.*, 2012), that partially sample the nuclear genome, it has been possible to study any non-model organism, including crocodilians. The NGS approach also produces large quantities of robust information at greatly reduced cost per locus compared to microsatellites (Funk *et al.*, 2012). The use of ddRADseq also allows reconstruction the actual evolutionary history of the lineages and not only the representative tree story of a gene.

How many ESUs and MUs of *P. palpebrosus* are there in the entire Amazon Basin? What are their limits? Which ESUs and MUs are the priority for conservation? These are

questions that remain unresolved and that motivate my doctorate. Thus the aim of my doctoral study as a whole is to (1) Evaluate the spatial distribution of genetic variability of *Paleosuchus palpebrosus* in the Amazon and Pantanal; (2) Delimit ESUs and MUs in *P. palpebrosus*; (3) Establish priorities and propose actions for the conservation of the identified lineages, especially those most susceptible; (4) Propose and test hypotheses of the diversification of the evolutionary lineages found.

The Student Research Assistance Scheme (SRAS) allowed me to pursue some of the objectives of my doctoral studies; these results I discuss in this report. Here, I report some field observations about the threats affecting the crocodilians in the regions in which the collects were supported by the Student Research Assistance Scheme (SRAS), and also the result of a population structure analysis based on a representative genome sequencing using the ddRADseq approach.

METHODS

As part of this proposal, we carried out several field excursions in the Amazon basin, in order to extent the geographic area to be genetically evaluated for *Paleosuchus palpebrosus*. The excursions were performed with the aid in the field of Dr Zilca Campos, Reginey Gomes and Msc Pedro Senna. We collected individuals in areas with important biogeographical breaks for several taxa. Two of theses field trip were supported by the Student Research Assistance Scheme (SRAS) of the Crocodile Specialist Group (CSG), one in June 2016 to Guiana Shield, in the state of Amapá, Brazil, and another in December 2016 to upper Negro River, in the Amazonas state, Brazil (Fig. 1). We collected tissues from *P. palpebrosus* individuals and from other species of crocodilians that occurred in both localities and deposited them in the Animal Genetics Tissue Collection (CTGA) at the Federal University of Amazonas (UFAM), Amazonas, Brazil.

We conducted nocturnal spotlight surveys to obtain census of *P. palpebrosus* populations and of other simpatric caiman species (*P. trigonatus*, *Caiman crocodilus* and *Melanosuchus niger*). We surveyed the edges of the rivers at night during the new moon phase (darkest nights of the month) by using canoeing, totaling seven different localities searched, six localities in the Amapá state and one in the upper Negro River (Fig. 1). We also searched during an additional night in the lake "Redondo" (01º47'N and 50º49'W), but we did not conduct a formal survey.

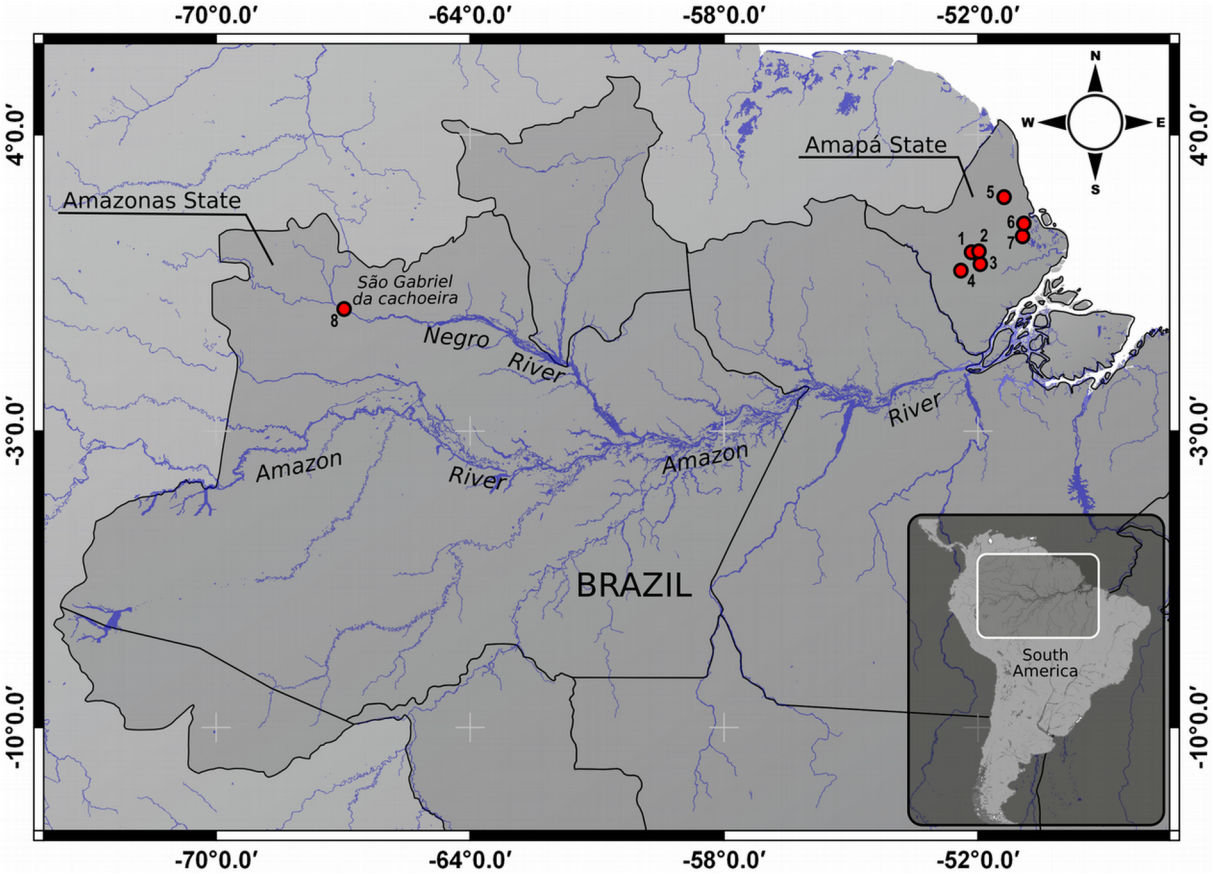


Figure 1. Sample map showing eight localities surveyed in the Amapá state and in the upper Negro River surrounding São Gabriel da Cachoeira. Numbers represent each waterbody researched as follows: (1) Wilhame stream; (2) Cupixi River; (3) Cupixizinho River; (4) Amapá stream; (5) Agamim stream; (6) Redondo Lake; (7) Tartarugal River; (8) Acatunum stream.

We attempted to capture all caiman to obtain tissue samples by using a steel loop attached to a stick and immobilizing by a homemade catch all. Biometric measurements were taken from captured individuals, including snout-vent length (SVL), total length (TL), head

length (HL) and weight (W); as well as date and geographic coordinates. Sex was also determined, but only for individuals of total length > 40 cm. Opportunistically we also collected sample tissues from specimens hunted by local hunters, and whenever possible we also collected all biometric measurements. The hunted specimens were photographed and we also took note about the conditions of the capture and purpose of hunting.

From all specimens one or more caudal scutes were removed and stored in small tubes containing 96% ethanol. In the laboratory, the total DNA of each individual was extracted following the protocol of Green & Sambrook (2012). The mitochondrial gene cytochrome b (cyt b) was amplified with specific primers and subsequently sequenced in ABI3500 (Applied Biosystems).

We chose 20 individuals, five from each mitochondrial lineage, to generate a partial representational genome sequencing using the double digest RAD sequencing protocol (ddRADseq) (Peterson *et al.*, 2012). We extracted 200 ng of genomic DNA of each individual and digested with SdaI and Csp6I restriction enzymes (Fermentas) and the IonTorrent P and A adapters were linked to the digested fragments. The fragments were enriched via PCR. The A adaptor contains a unique molecular barcode for identification of individuals. The fragments were then size selected on Pippin Prep (Sage Science), and fragments between 320 and 400 bp were used for sequencing. Genome size of alligatorids is approximately 2.2 x 10^9 bp (based on sequenced genomes of *Alligator mississipiensis* and *A. sinensis* deposited in Genbank), resulting in an expectation of ~ 15,000 ddRAD fragments in the range of 300 to 400 bp. The complete protocol is available on GitHub (https://github.com/legalLab).

All reads were processed using the PyRAD pipeline (Eaton, 2014). During de novo assembly, we used a minimum Phred Score of 20 and coverage of 5x. We performed an analyzes on the platform STRUCTURE (Pritchard *et al.*, 2000) to infer the genetic population structure based on this ddRADseq data and compared with the cyt b analysis. For

STRUCTURE analysis we collected 110,000 samples from the MCMC chain of which the first 10,000 were discarded as burn-in and the remaining 100,000 steps were used for this analysis. The number of groups (K) varied from 1 to 6, with 10 independent runs for each K value. The convergence between independent runs was assessed via examination of α values and profile of posterior probabilities. The Q values from each of the 10 independent runs for each K scenario were extracted using the program STRUCTURE HARVESTER 0.6.92 (Earl & VonHoldt, 20121) and summarized in the program CLUMPP 1.1.2 (Jakobsson & Rosenberg, 2007). Results were visualized in the program DISTRUCT 1.1 (Rosenberg, 2003). The most likely number of clusters (K) was inferred using the method of Evanno *et al.* (2005).

RESULTS

Field observations

During eight nocturnal spotlight surveys we covered a total of 65.8 km of streams and rivers habitats across the two regions searched, Amapá state and the upper Negro River (Amazonas state). Both areas are covered by many portions of pristine habitats with dense forest along the rivers, mainly within indigenous areas. Contrary to expectations we found very few caiman during the spotlight surveys, only 19 caimans were counted in the total (Table 1), of which only two was of the target species of this study, *Paleosuchus palpebrosus*. We also found five *Paleosuchus trigonatus*, four *Melanosuchus niger* and two *Caiman crocodilus*, as well as four individuals that in the field could not be assigned to a species (Table 1). Furthermore, we found caiman only in three of the seven sites surveyed (42,8 %), suggesting low rates of encounter.

Table 1. Rivers sampled in both surveyed regions, the Amapá state and the upper Negro River. Coordinate, survey distance and number of specimens encountered in each locality are also shown. The numbers before each name of locality have correspondence with the points on the map in figure 1.

Sampled locality	Coordinates	Survey distance (km)	Species (nº detected/ nº captured)	Species (Specimens hunted)
Amapá state				
1. Wilhame Stream	00º48'N 51º53'W	7.9	0	0
2. Cupixi River	00º39'N 51º43'W	12.4	0	0
3. Cupixizinho River	00º49'N 51º45'W	18.4	0	0
4. Amapá Stream	00º35'N 52º19'W	9.3	*P. trigonatus* (07/02)	*P. trigonatus* (03)
5. Agamim Stream	02º26'N 51º24'W	2.2	*P. trigonatus* (01/01)	0
6. Redondo Lake	01º47'N 50º49'W	Not registered	Not registered *P. palpebrosus* (01/01)	*P. palpebrosus* (02) *P. trigonatus* (01)
7. Tartarugal River	01º34'N 50º48'W	12.6	*P. palpebrosus* (01) *Melanosuchus niger* (04) *Caiman crocodilus* (02) Unidentified (04)	0
upper Negro River				
8. Acatunum stream	00º05'N 67º07'W	3.0	0	*Caiman crocodilus* (02) *P. palpebrosus* (01)
TOTAL	**---**	**65.8**	**19**	**09**

We captured two *Paleosuchus trigonatus* in the Amapá Stream (00º35'N 52º19'W), a tributary of the Cupixi River, and another in the Agamim Stream (02º26'N 51º24'W), a tributary of the Calçoene River. We also captured one *P. palpebrosus* during an additional night in the "Redondo" Lake (01º47'N and 50º49'W), in which we did not carried out formal survey.

We observed numerous instances of subsistence and commercial hunting in both regions, Amapá state (Fig. 2) and upper Negro River, Amazonas state, (Fig. 3). In many cases the hunters had to travel large distances (> 60 km) for obtain prey. During the two field expeditions we recorded a total of nine hunted specimens, four of which were hunted for subsistence and five for commercial reasons.



Figure 2. Records of *Paleosuchus* specimens hunted, done during the field trip to the Amapá state. (A) Three specimens of *P. trigonatus*, who were hunted for subsistence in the Amapá stream, stored in a freezer. (B) Part of the head of *P. trigonatus* specimen hunted for commercial purpose in the Flexal community. (C) Fresh *P. palpebrosus* head of specimen hunted to order in the previous night, in the nearby of the Breu community. (D) Frozen *P. palpebrosus* head of specimen sold by residents of the Breu community. Photographs: Zilca Campos.

The four cases of subsistence hunting were recorded in the Amapá Stream (03-*P. trigonatus;* 00º35'N 52º19'W) and Cupixizinho River (01-*P. trigonatus*; 00º49'N 51º45'W). The hunters in these localities also reported that sometimes they hunt for people who have ordered in advance. The five specimens hunted for commercial propose were recorded in the Flexal (01- *P. trigonatus*; 01º43'N 50º55'W) and Breu (02- *P. palpebrosus*; 01º45'N 50º52'W) communities, in the Amapá state, and in São Gabriel da Cachoeira (01- *P. palpebrosus* and 02- *Caiman crocodilus*; 00º07'S 67º05'W), in the upper Negro River. In both surveyed regions local people reported their preference for eating caiman meat. We also registered caiman sold openly at markets in São Gabriel da Cachoeira (Fig. 2).



Figure 3. Records of specimens hunted for commercial purpose during the field trip to São Gabriel da Cachoeira. (A) One fresh *P. palpebrosus* head of a specimen bought by one resident of São Gabriel da Cachoeira who let us to register. (B) Parts of one *C. crocodilus* smoked specimen being sold at one of the city fairs. (C and D) One specimen of *C. crocodilus* along with fish, being freely displayed in a different fair in the city. Photographs: Zilca Campos.

Hunters reported that the most frequently used method to catch caiman is to bait a hook and wait. The price paid for fresh caiman meat in the Amapá state was approximately US$ 3.06 (R$ 10.00) per kilo, and in São Gabriel da Cachoeira varied from US$ 5.50 to US$ 7.64 (R$ 18.00 to R$ 25.00). In addition to fresh meat, the caiman (*C. crocodilus* or *P. palpebrosus*) in São Gabriel da Cachoeira are sold smoked or salted, but with no change in the price.

Population structure analysis using ddRADseq

Next generation sequencing produced approximately 3.6 million usable reads after processing in PyRAD. On average, 17 to 21 thousand loci in the 300-400 bp range with an

average coverage of 3.5x were observed for each individual. Filtering loci to a minimum of 5x coverage, on average 2-3 thousand loci per individual with an average coverage of 7.5-8.5x coverage were retained. In the final dataset, where a locus had to be present in at least 50% of the individuals, 722 loci were retained, of which 532 were unlinked SNPs.

The STRUCTURE analysis using ddRADseq markers confirmed the genetic structuring of the *P. palpebrosus*, previously showed by the cyt b mitochondrial marker. This analysis inferred three genomic clusters (K= 3; LnProb = -374.95) as the result of best partition of the data (Fig. 3). The second best partition was K= 2 (LnProb = -393.84).

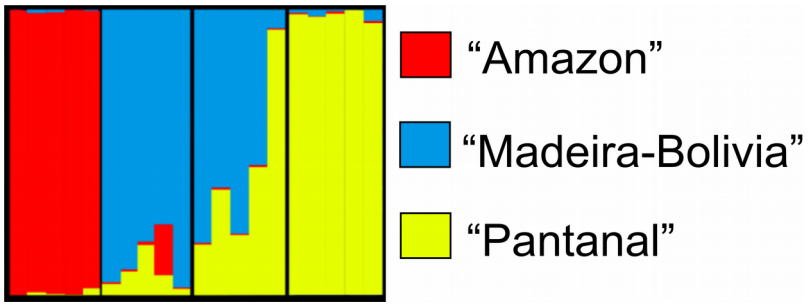


Figure 3. STRUCTURE analysis barplot. The colors represent the groups to which each analyzed individual belongs. The bars with two colors indicate individuals with genomic composition of two different groups and therefore the result of interbreeding.

The “Amazon” and “Pantanal” geographic groups were maintained as independent groups in the STRUCTURE analysis (Fig. 3), similar to what was found in the cyt b gene analyzes. However, the groups previously identified by the cyt b analyzes as separately (“Madeira” and “Bolivia”) were grouped into a single genomic cluster “Madeira-Bolivia”. This analysis suggest that not all individuals were genetically pure, e.g. some individuals are of admixed “Madeira-Bolivia” and “Pantanal” ancestry and another individual appears admixed between “Madeira-Bolivia” and “Amazon”.

Lastly, STRUCTURE analysis revealed that not all individuals of a particular genomic cluster were geographically restricted, e.g. an individual of the “Pantanal” genomic cluster was found in the area of distribution of the “Madeira-Bolivia” genomic cluster, i.e. in the Bolivian Basin.

DISCUSSION

Caiman hunting

There is relatively few and less-intense human activities that directly impact the caiman in the Amazon basin. Urbanization, damming, agriculture, pollution and deforestation are some of these less-intense activities in the Amazon. One of the exception is the mining pollution which are widely present in portions of the Amazon, mainly in the Guiana and Brazilian Shields, although almost everywhere the impacts of this activity on the caiman are unknown. The hunting is another human activity in the Amazon harmful to caiman (Da Silveira & Thorbjarnarson, 1999). In the Brazilian Amazon, more than half a century of intense commercial hunting for the caiman skins almost extirpated populations of *Melanosuchus niger* and *Caiman crocodilus* (Fittkau, 1970; Magnusson, 1979; Rebelo & Magnusson, 1983). It is widely believed that both species of the genus *Paleosuchus* are subject to little pressure from hunting, mainly due to having well-developed osteoderms in the body and thus a skin worthless commercially (Magnusson & Campos, 2010).

During eight nocturnal spotlight surveys across the two regions searched we found very few caiman (19 individuals) in only three of the seven sites surveyed (42.8 %). Furthermore, we recorded a total of nine specimens hunted for subsistence or commercial reasons. The number of individuals hunted was high considering the few days of survey. In addition this number could be greater, since we noticed the existence of other cases of hunting which could not be confirmed, because the hunters were frightened and hid their catch. Moreover, we registered that the preference of local people for eating caiman meat is such that the demand is capable of sustaining continuous trade, especially in São Gabriel da Cachoeira, where the hunted caiman are sold openly at markets.

The low encounter rates of the species in both regions surveyed may be indicative of

unsustainable exploitation (Webb *et al.*, 2000). This, taken together with the many cases of hunting recorded in the short period of time we made our observations, leads us to suspect that subsistence and illegal commercial hunting is probably the human activity that most affects caiman density in these regions, mainly because other direct threats caused by humans are much less intense in these areas.

We could not fail to notice that the indigenous people of the upper Negro River have preference for eating *Paleosuchus* meat, preparing and consuming the whole individual. We noticed the same preference in the Amapá state, where hunters reported receiving most requests for *Paleosuchus*. Additionally, they reported that people working in the illegal mines in the highlands of the Amapá state are practicing heavy subsistence hunting of caiman, mainly individuals of the genus *Paleosuchus* that occur preferentially in this region.

Almost whole upper Negro River and most of Amapá state, in Brazil, are indigenous reservation who have almost all the forest preserved. In contrast, probably these regions have not caiman populations as preserved as might be expected.

Genetic population structure

The concept of the Evolutionarily Significant Unit (ESU) has been developed in order to provide an objective method for identifying below-species-level 'units' for conservation purposes (Ryder, 1986). The main operational criterias used to identify an ESU are: reproductive isolation (Waples, 1991), absence of gene flow (Waples, 1991; Moritz, 1994; Frazer & Bernatchez, 2001), reciprocal monophyly in mitochondrial DNA (mtDNA) and significant differences in allele frequencies of nuclear loci (Avise, 1994; Moritz, 1994) and ecological and genetic nonexchangeability (Vogler & DeSalle, 1994; Crandall *et al.*, 2000). ESUs could have followed semi-independent evolutionary trajectories for different periods of time and also be genetically structured in a hierarchical level, generally recognized as

Management Units (MUs; sensu Moritz, 1994). Frazer & Bernatchez (2001) proposed the Adaptive Evolutionary Conservation (AEC) integrative framework that uses the strengths, either alone or in combination, of various operational criteria for delimiting ESUs.

We first discovered a strong pattern of genetic structuring in *P. palpebrosus* by using the gene cyt b, thus we used this previous results to test if the mitochondrial lineages are Evolutionarily Significant Units. For this, we performed a historical population structure analysis in the STRUCTURE program by using ddRADseq dataset. The geographical pattern found using the genomic marker was mostly concordant with the geographical distribution of the cyt b clusters. Thus, this analysis allowed us to identify three genomic clusters ("Amazon", "Pantanal" and "Madeira-Bolivia") sufficiently differentiated to be considered ESUs according the multiple criteria summarized by the AEC framework proposed by Frazer & Bernatchez (2001).

The "Amazon" and "Pantanal" ESUs form reciprocally monophyletic mitochondrial groups, and display no evidence of admixture with another genomic cluster. However, the "Madeira-Bolivia" ESU is comprised of two moderately divergent clades in cyt b analysis (high F_{ST} value) with a contact zone in the upper Madeira River characterized by haplotype sharing in three adjacent localities (see Muniz, 2012). Genomic analysis in the STRUCTURE indicates that individuals from the Madeira River and the Bolivian basin comprise just one biological group, despite the high genetic structuring in the cyt b gene. Therefore, the "Madeira-Bolivia" ESU is best viewed as comprising two MUs, "Madeira" and "Bolivia", according to the original definition of Moritz (1994).

Animals from the Bolivian basin also showed evidence of substantial nuclear admixture with animals from the Pantanal, while animals from the Madeira River showed small amount of nuclear admixture with animals from the Pantanal and Amazon. The discordance between both cyt b and ddRADseq markers in detect genetic structure among

populations from Madeira and Bolivia can probably be attributed to differences in the evolutionary rates of mitochondrial and nuclear markers.

We performed more detailed analyzes using the ddRADseq data and we discuss the results and implications of these analyzes in a manuscript submitted for the journal "Animal Conservation", entitle: “Identifying three Evolutionarily Significant Units in Cuvier’s Dwarf caiman: implications for its conservation from Amazon to the Pantanal”

ACKNOWLEDGEMENTS

I would like to thank Crocodile Specialist Group (CSG) from IUCN for granting the Student Research Assistance Scheme (SRAS) and Fundação Pantanal Com Ciência from Embrapa Pantanal, both who helped to support field excursions. I also thank all grants that supported this Project: CNPq/CT-Amazon Project no. 575603/2008-9 awarded to IPF, CNPq Project no. 482662/2013-1 to TH, and CNPq Project no. 470383/2007-0 to ZC. This work would have been impossible without the people who helped with the field collections: Zilca Campos, Reginey Gomes and Pedro Senna. These project was approved by Embrapa ethics committee under the Permit no. 009/2016 and the caiman were captured under License no. 13048-1 granted by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA). This study is part of my PhD thesis in the Genetics, Conservation and Evolutionary Biology program of INPA/UFAM. I’m support by a grant from FAPEAM.

DESCRIPTION OF THE RESOURCES USAGE

The resource of SRAS was used for help to support two short field trips with expenses in accommodations, transport, fuel and fast canoe rental. The first field trip was performed to Amapá state in June 2016 and the second one was performed to upper Negro River in December 2016.

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