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ARTÍCULO ORIGINAL

EVIDENCE OF VERY LOW GENETIC DIVERSITY OF CUBAN GAR (ATRACTOSTEUS TRISTOECHUS)

Evidencia de pobre diversidad genética del manjuarí (Atractosteus tristoechus)

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ABSTRACT

The Cuban gar (*Atractosteus tristoechus*) is an endemic freshwater fish species of Cuba. Among the Lepisosteiformes, this species is one of the most threatened and has the lowest natural distribution range. It is restricted to the southwestern region of the Cuban archipelago. This research is focused on the genetic characterization of captive and wild populations of Zapata Swamp, using two mitochondrial sequences (one in the gene coding cytochrome c oxidase I and the other in the Control Region) and nine microsatellite *loci*. A total of 47 individuals were included in the study. Low genetic variability was detected: $h = 0.202 \pm 0.077$ and $\pi = 0.0002 \pm 0.00008$ for mitochondrial sequences; $H_o = 0.016$ and $H_e = 0.025$ for microsatellites. Among lepisosteids studied so far, this species showed the lowest genetic diversity. The results suggest that *A. tristoechus* had a low effective population size over a long period of time and/or the population went through a recent bottleneck. Given the recent demographic trend of this species and the low genetic diversity revealed in the present study, special attention and conservation efforts are urgently needed.

KEYWORDS: Conservation genetics, endangered species, genetic management in captivity, lepisosteid.

RESUMEN

*El manjuarí (*Atractosteus tristoechus*) es un pez de agua dulce endémico de Cuba. Entre los Lepisosteiformes, esta especie es una de las más amenazadas y ocupa la menor área de distribución natural. Está restringida al área suroeste del archipiélago cubano. Esta investigación se enfoca en la caracterización genética de las poblaciones cautivas y naturales de la Ciénaga de Zapata, mediante dos secuencias mitocondriales (gen que codifica citocromo c oxidasa I y la Región de Control) y nueve* loci *microsatélite. Un total de 47 individuos fueron incluidos en la investigación. Se detectó baja variabilidad genética: $h = 0.202 \pm 0.077$ y $\pi = 0.00021 \pm 0.00008$ para las secuencias mi-*

tocondriales; H_o=0.016 y H_e=0.025 para los microsatélites. Entre los lepisosteidos estudiados hasta el momento, esta especie muestra la menor variabilidad genética. Estos resultados sugieren que A. tristoechus *ha mantenido un pequeño tamaño efectivo poblacional durante largo tiempo y/o la población ha experimentado un cuello de botella reciente. Dada la reciente tendencia demográfica de la especie, y la baja diversidad genética mostrada en este estudio, se hace necesaria con urgencia una atención especial, así como esfuerzos para su conservación.*

PALABRAS CLAVE: *Especie en peligro, genética de la conservación, lepisosteidos, manejo genético en cautividad.*

INTRODUCTION

Biodiversity loss, including within species genetic erosion, is a global crisis threatening all ecosystems at multiple scales. Many freshwater ecosystems are undergoing this process at a faster pace than most terrestrial ecosystems (Dudgeon *et al*., 2006). Moreover, in aquatic habitats, species at higher trophic levels are more frequently lost than those at lower trophic levels (Petchey *et al*., 2004) and endemic species with very small distributions are most likely to be close to extinction (Moyle and Leidy, 1992). This is the case of the Cuban gar *Atractosteus tristoechus* (Bloch and Schneider, 1801) that is an endemic top level freshwater fish predator (primarily piscivorous). This species inhabits rivers, tidelands, channels and lagoons of freshwater of Zapata Swamp, where it is believed that the biggest populations exist, and Lanier Swamp in Isla de la Juventud, both in the southwest of Cuban archipelago (Vergara, 1992). Among the members of the order Lepisosteiformes this species has the lowest natural distribution range, restricted to an archipelago (Cuba).

The lack of information about the status of the natural populations of the Cuban gar, is the main cause for which this species has neither been evaluated for the Convention on International Trade in Endangered Species (CITES) and for the International Union for Conservation of Nature (IUCN).

There is no information about the population size of this species. Nevertheless, the species was granted the status of vulnerable in the Meeting of Conservation, Analysis and Planned Handling (Pérez *et al*., 1999). More recently, the species was recognized in danger and included in the Red Book of the Vertebrates of Cuba (Ponce de León *et al*., 2012). In spite of these efforts, the Cuban gar future is highly compromised according to recent observations that populations had disappeared or diminished drastically in number of individuals, despite the fact that the capture of this species is forbidden by law (Decree-Law 164 of 1996 of the Ministry of Fishing Industry). In this context, the captive population of Cuban gar living in the Center for Indigenous Ichthyofauna Reproduction (CIIR) represents an insurance in the face of possible wild population extinction.

Our knowledge of genetic diversity in many gar species is limited with respect to others fishes (Moyer *et al*., 2009; Sipiorski, 2011; Solomon, 2012; Wright *et al*., 2012; Bohn *et al*., 2013) and it is completely lacking for the Cuban gar. The aim of this study is to genetically characterize Cuban gar biggest population in order to contribute to the conservation and management of the species.

MATERIALS AND METHODS

Forty-seven *A. tristoechus* fin clips were collected from Zapata Swamp; thirty-four were unrelated founders (also the only

breeders) from the captive population and thirteen from the wild (Fig. 1). Fin clips were preserved in 96 % ethanol at -20 °C. Tissue was digested with Proteinase K and DNA was extracted following Sambrook *et al.* (1989) protocol and using Phase Lock Gel Heavy tubes (Eppendorf).

Two mitochondrial DNA sequences (535 bp within cytochrome c oxidase I gene (COI) and 464 bp within the Control Region (CR)) were amplified using PCR with primers reported in previous studies (COIf and COIr, Hebert *et al.*, 2003; L16594 and H22, Sipiorski, 2011). PCR mix was as follow: 25 mM $MgCl_2$, 10 mM dNTPs, 10 µM of each primer, 6.0 µL of 5x GoTaq® Flexi Buffer (Promega), 0.2 U of GoTaq® DNA polymerase (Promega), 20 – 100 ng of template DNA and water to a 30 µL final volume. Polymerase chain reactions (PCR) conditions consisted in an initial denaturation at 95 °C (5 min) followed by 8 cycles of 92 °C (45 s), 53 °C (45 s), 72 °C (45 s), 30 cycles of 92 °C (45 s), 58 °C (45 s), 72 °C (45 s). A final elongation step at 72 °C (10 min) ended the cycle. Amplified PCR products were cleaned using a PCR clean-up Nucleospin® Extract II kit (Macherey-Nagel). Purified PCR products were used as templates for Big Dye (Applied Biosystems) cycle sequencing, using the primers used for PCR. Sequencing reactions were run on an ABI 3100 automated sequencer.

Sequences were aligned with Mega 7.0 (Kumar *et al.*, 2016) and chromatograms were checked in Finch TV 1.4.0 (Geospiza Inc.). Haplotype networks were computed with Network 5.0 (Fluxus Technology) using concatenated mitochondrial sequences (999 pb). Nei´s (1987) haplotype diversity (h) and nucleotide diversity (π) were estimated using DnaSP v5 (Librado and Rozas, 2009). Additionally, three COI sequences published by Wright *et al.* (2012) (GenBank accession numbers: JN853329, JN853330, JN853331) were compared with the haplotypes obtained in our study.

Primers designed to amplify ten microsatellite *loci* in other gar species, and described in Moyer *et al.* (2009), were tested with *A. tristoechus* DNA (*Locus*-GenBank accesion number: Asp007-EU625547, Asp035-EU625549, Asp040-EU625550, Asp057-EU625553, Asp066-EU625554, Asp122- EU625559, Asp159-EU625560, Asp341-EU625564, Asp031-EU625570, Asp046-EU625574). All but one, *locus* Asp122, could be amplified.

Microsatellite *loci* were amplified in two multiplex reactions (primer mix A: *Loci* Asp 046, Asp057, Asp066, Asp 341; primer mix B: Asp031, Asp035, Asp040, Asp057, Asp159) using PCR conditions described in Moyer *et al.* (2009) and PCR mix as follow: 2 µM of primer mix, 5.0 µL of Platinum Multiplex PCR Master Mix 2X (Applied Biosystems), 20 – 100 ng of template DNA and water to a final 9.0 µL volume. Microsatellite alleles were visualized using an ABI PRISM 3130 (Applied Biosystems) with a GeneScan-500 LIZ size standard (Applied Biosystems) and scored using Peak Scanner Software 2 (Applied Biosystems).

Observed (H_o) and expected (H_e) heterozygosity were estimated. F_{IS} (Wright, 1965; modified by Weir and Cockerham, 1984) and its significance using Bonferroni correction (Rice, 1989) were computed to test for Hardy-Weinberg equilibrium deviation. All the estimates were obtained using FSTAT 2.9.3.2 (Goudet, 2001).

RESULTS

Mitochondrial sequences

The alignment of concatenated mitochondrial DNA sequences of 47 individuals

Table 1. Mitochondrial haplotype diversity of lepisosteids using COI and CR sequences. Excepted *A. tristoechus*, COI diversity was computed with data of Solomon (2012) and CR diversity was computed with data of Sipiorski (2011).

Species	Haplotype diversity (h)	
	COI (n)	CR (n)
A. tristoechus	0.04 (47)	0.31 (47)
A. spatula	-	0.79 (17)
L. oculatus	0.39 (22)	0.96 (23)
L. platyrhincus	0.00 (3)	0.83 (4)
L. platostomus	-	0.91 (19)
L. osseus	-	0.96 (52)

showed only three polymorphic sites among 999 bp (*i.e.* four haplotypes; Fig. 2): two transitions (one in COI and one in CR) and one transversion in CR. The derived allele in COI was founded in a captive individual (Haplotype B). Each derived allele in CR was founded in two wild population individuals (Haplotype C' in Laguna del Tesoro and Finca Campesina individuals; Haplotype D' in Guanal Grande and Finca Campesina individuals; see Fig. 1). Haplotype diversity was low ($h = 0.202 \pm 0.077$; for each sequence separately, see Table 1) as well as

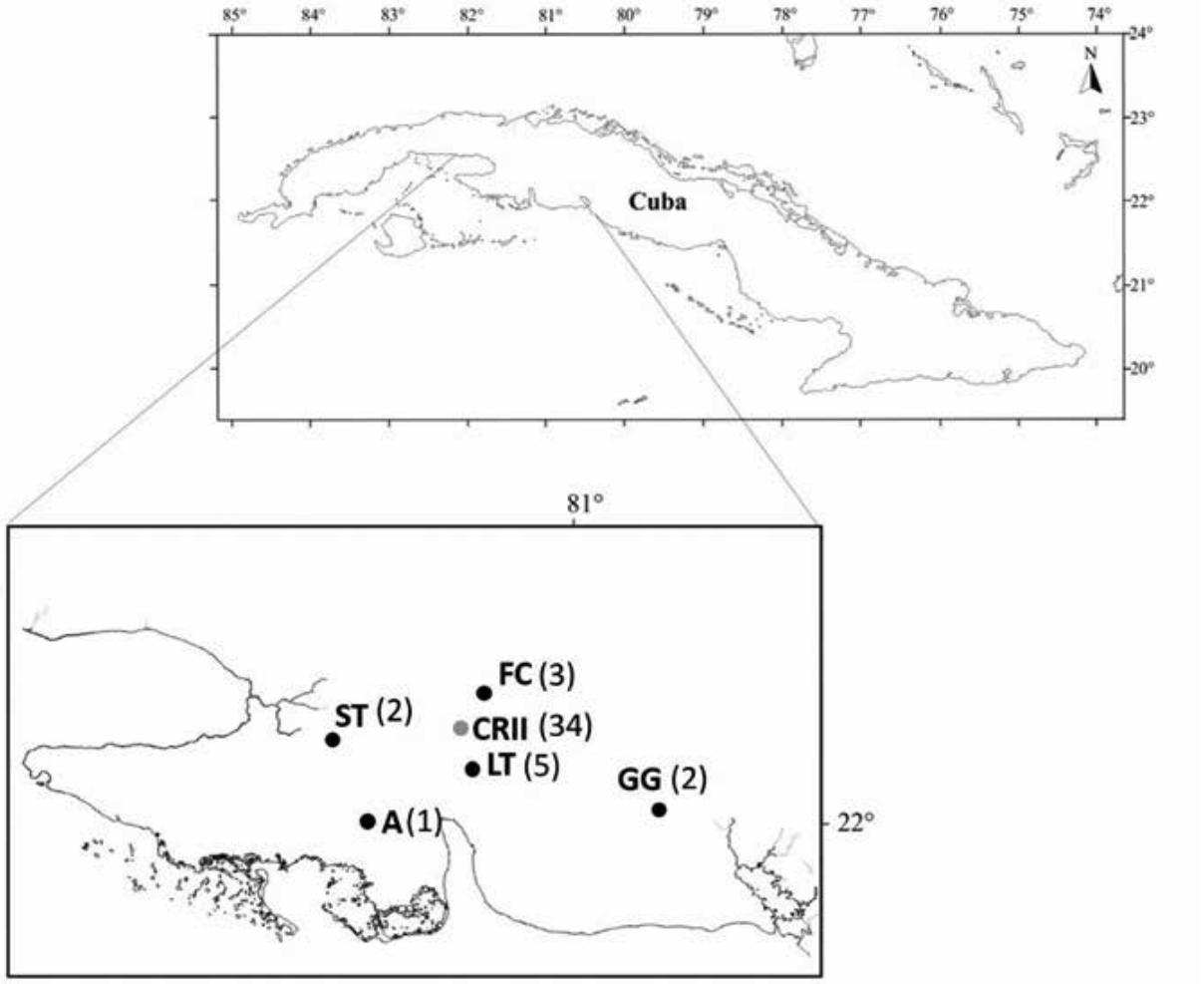

Fig. 1. Sampling sites of *A. tristoechus* in Zapata Swamp. Numbers represent the sample size in each locality. Black dots are wild populations and the gray dot is the captive population. A: Arroyones, CRII: Center for Indigenous Ichthyofauna Reproduction, FC: Fiesta Campesina, GG: Guanal Grande, LT: Boca de Guamá-Laguna del Tesoro, ST: Zanja de Santo Tomás.

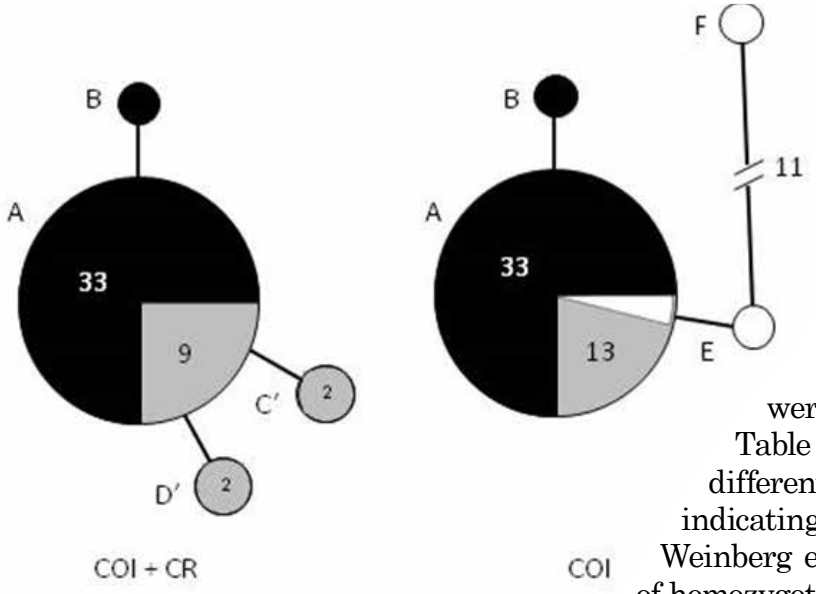

Fig. 2. Networks constructed using concatenated COI and CR sequences and COI sequences only. In both networks colours represent a particular locality or origin: black (Center for Indigenous Ichthyofauna Reproduction), gray (natural populations) and white (from Wright *et al.* 2012). Figures inside the circles indicate the number of individuals. Only numbers higher than 1 are shown. Lines are one change except otherwise indicated. See text for haplotypes IDs

nucleotide diversity ($\pi = 0.00021 \pm 0.00008$). Among the three COI sequences published by Wright *et al.* (2012), one match with the COI sequence of the most common haplotype (A) and the two others represent two different haplotypes (E and F).

Microsatellite *loci*

Nine microsatellite *loci* could be amplified with all 47 DNA samples. However, only one *locus*, Asp341 (GenBank accesion number: EU625564), was polymorphic (two alleles: 203 bp and 206 bp). Among 13 individuals captured in the wild, one was heterozygote at this *locus* and 12 were 206/206 homozygotes. Among the captive individuals, 23 were 206/206 homozygotes, seven were heterozygotes, and four were 203/203 homozygotes.

Heterozygosity estimates were low ($H_o = 0.016$; $H_e = 0.025$; Table 2) and F_{IS} was significantly different from zero (0.377, $p = 0.01$), indicating a deviation from the Hardy-Weinberg equilibrium due to an excess of homozygotes.

DISCUSSION

Analyses of genetic diversity provide information on important aspects of species biology that should be taken into account in management programs (Frankham *et al.*, 2010). Here we provide the first analysis of the genetic diversity of the Zapata Swamp population of Cuban gar that is the largest population in the archipelago.

The analysis of 47 individuals from different localities (including CIIR´s population founding parents) using mtDNA sequences and microsatellite *loci* have

Table 2. Microsatellite diversity of lepisosteids. *A. spatula, L. oculatus* and *L. osseus* heterozygosities were computed with data of Moyer *et al.* (2009) and *A. tropicus* heterozygosities were computed with data of Bohn *et al.* (2013).

Species (number of locus used)	A	Ho	He
A. tristoechus (9)	1.11/ 1*	0.01/ 0*	0.02/ 0*
A. spatula (9)	4.55/ 4.25*	0.49/ 0.51*	0.5/ 0.48*
A. tropicus (7)	2.14/ 1.5*	0.22/ 0.14*	0.23/ 0.16*
L. oculatus (4)	6.25	0.68	0.61
L. osseus (4)	7.00	0.77	0.72

revealed a very low genetic diversity. Only four mitochondrial haplotypes and only one variable microsatellite with two alleles were detected. This genetic diversity is the lowest among lepisosteids studied so far, using the same mitochondrial and nuclear markers (Table 1 and Table 2). It is also lower than the genetic diversity observed within heavily overexploited fish populations (*i.e.*: acoupa weakfish (*Cynoscion acoupa*), $h = 0.892$ and $\pi = 0.003$; Rodrigues *et al.*, 2008).

Two COI haplotypes (E and F) described in CIIR individuals by Wrights *et al.*, (2012) were not found, providing some support for an erosion of the genetic diversity in the breeding pool. It could also suggest that the genetic diversity in the species is higher than the genetic diversity we found in our sample of wild and captive individuals. However, the analysis of the sequence of haplotype F showed that among 12 differences with respect to the most common haplotype (A), most of them were non-synonymous mutations (eight). Although it could suggest the existence of a relatively divergent haplotype, the fact that most of the nucleotide substitutions were non-synonymous suggests that this haplotype is either the sequence of a pseudogene (such as a nuclear mitochondrial DNA segment), or this sequence contains many sequencing errors.

Low genetic diversity is often found in endangered species with small population sizes (Méndez *et al.*, 2014; Sato *et al.*, 2014). The decline of Cuban gar in locations where it was common before (A. Hurtado, unpublished observation) and the likely concomitant loss of genetic diversity are both strong indicators of its vulnerability.

The genetic markers used in this study are insufficient to assist selection of parents for *ex situ* reproduction and reintroductions, and for a more accurate estimation of the structuration of the genetic diversity in the wild. More extensive research is needed, but these results clearly show how low is the genetic diversity of the Cuban gar. The low levels of genetic variability observed here and the restricted distribution of the species may compromise the evolvability of *A. tristoechus.* It suggests that Cuban gar is at present the most threatened lepisosteids species. These findings indicate an urgent need for a careful monitoring and the development of a management program of the species.

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