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Genetic structure of the invasive Nile tilapia *Oreochromis niloticus* (Perciformes, Cichlidae) and molecular identification of tilapia species in Panama using barcode

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Abstract: Genetic diversity and genetic structure of the invasive Nile tilapia (*Oreochromis niloticus*) were studied using partial sequences of cytochrome oxidase I gene from samples collected in Gatun, Bayano, Alajuela, and Barrigon lakes, Republic of Panama. *O. niloticus* sequences from Bayano included in the analysis corresponded to samples from previous studies conducted in this lake. *O. niloticus* showed low genetic diversity ($\pi = 0.00010$, $Hd = 0.059$) and exhibited only two haplotypes. Haplotype 1 was common in all areas whilst, haplotype 2 was present uniquely in Bayano Lake. Pairwise F_{ST} and AMOVA results showed reduced genetic differentiation among areas ($F_{ST} = 0.07$ and percentage of variation between populations of 7.76% respectively). Reduced values of genetic diversity and absence of genetic differentiation among locations suggest the existence of a single population for *O. niloticus*. This research also provides the first molecular list for other four tilapia species (*O. mossambicus*, *O. aureus*, *O. urolepis* and *Coptodon rendalli*) and one molecular hybrid (*O. niloticus* x *O. aureus*) reported in Panama. This information will allow managers to trace local demos of *O. niloticus* and other invasive species populations along Panama and Central America.

Key words: genetic diversity, invasive species, barcode, traceability, hybridization.

Estructura genética de la tilapia invasora del Nilo *Oreochromis niloticus* (Perciformes, Cichlidae) e identificación molecular de otras especies de tilapia en Panamá utilizando código de barras. Resumen: La diversidad y estructura genética de la tilapia del Nilo (*Oreochromis niloticus*) fue estudiada utilizando un segmento del gen Citocromo oxidasa I de muestras colectadas en los lagos Gatún, Bayano, Alajuela y Barrigon en la República de Panamá. Las secuencias de *O. niloticus* de Bayano corresponden a muestras colectadas en estudios previos realizados en este lago. *Oreochromis niloticus* mostró una baja diversidad genética ($\pi = 0.00010$, $Hd = 0.059$) y únicamente se encontraron dos haplotipos. El haplotipo 1 fue común para todas las áreas; , mientras, el haplotipo 2 únicamente se encontró en el lago Bayano. El valor de F_{ST} por pares obtenido y el reducido porcentaje de variación entre poblaciones calculado por AMOVA ($F_{ST} = 0.07$ y 7.76 % respectivamente) indica una baja diferenciación genética entre poblaciones y la existencia de una población. Los resultados proporcionan la primera lista de identificación molecular de otras especies de tilapia (*O.*

mossambicus, *O. aureus*, *O. urolepis* y *Coptodon rendalli*) y un híbrido molecular (*O. niloticus* x *O. aureus*) presentes en Panamá. Esta información permitirá a las autoridades realizar la trazabilidad de demos locales de de *O. niloticus* y otras poblaciones de invasores presentes en Panamá y Centroamérica.

Palabras clave: diversidad genética; especies invasoras; código de barras, trazabilidad, hibridización.

Introduction

Cichlid Fish species belonging to the genus *Oreochromis* sp, *Tilapia* sp and *Saroterodon* sp., are commonly known as “tilapias”. These species are common to the rivers and lakes of tropical and subtropical parts of Africa and Madagascar (Trewavas, 1983; Arredondo & Guzman, 1986). *Tilapia* species have been introduced worldwide to increase protein-based food sustainability (De Silva *et al.*, 2004; Brinez *et al.*, 2011) and as a biological control for aquatic weeds and insects (Canonico *et al.*, 2005). In the Americas, they have been successfully introduced into natural aquatic ecosystems of Central and South America since the 1940s (Morales, 1991; Zambrano *et al.*, 2006; Grammer *et al.*, 2012). Their success is attributed to a capacity to tolerate changes in salinity, temperature and oxygen concentration. In particular, *Oreochromis niloticus*, leads world aquaculture production over carp (*Ctenopharyngodon idella*) and silver carp (*Hypophthalmichthys molitrix*) with 3,197 million tons of annual yield representing a commercial value of 5.3 billion dollars (FAO, 2017). In Panama, *O. niloticus* is considered the main commercial aquaculture product and main protein source for some rural communities and one of the main exportation products (Allard, 2018). *Tilapia* harvest from aquaculture in Panama represents the main exportation aquatic product right after the shrimp industry; around 400 tons per year of *tilapia* are produced by year (Allard 2018). Nonetheless, due to their broad distribution, great adaptability, fitness, and success in aquaculture, *tilapias* including *O. niloticus* have raised concerns about their impact on community structure and freshwater biodiversity.

The introduction of invasive species changes community structure in freshwater ecosystems leading to the extinction of indigenous species and are considered one of the main global threats to biodiversity (Vicente & Fonseca-Alves, 2013). Since their introduction in the Americas, the *Oreochromis* species complex displaced native ichthyofauna and significantly the environment of freshwater ecosystems and has become a threat in several Latin American countries. (Perez *et al.*, 2004; Zambrano

et al., 2006). In particular, lentic ecosystems such as lakes are prone to species loss during the invasion process (Ricciardi & Rasmussen, 1998).

Invasive species management requires understanding the biology and natural history of a species and its potential impact on the invaded ecosystem. Together this information supports surveillance and species population control measures. *Tilapias* and other cichlids achieve high colonization success due to character traits such as aggressive behavior, environmental tolerance, diverse diet, and high reproduction and survival rates (Philippart & Ruwet, 1982; Njiru *et al.*, 2004). Such traits have received much attention from evolutionary biologists (Agostinho *et al.*, 2007) and geneticists as fitness is closely related to the individual genetic profile, population genetic structure, and genetic diversity. Furthering invasive species success is frequently related to an elevated hybridization and genetic introgression rates (Watanabe, 1991; Angienda *et al.*, 2011; Wu & Yang, 2012).

Therefore, understanding *tilapia*'s genetic diversity and genetic structure provides a two-fold benefit. First, species management plans based on knowing effective population size and invasive potential can prevent the expansion of species into other areas (Rutten *et al.*, 2004). Second, genetic profiles inform commercial purposes and product traceability, which contribute to genotypes selection for aquaculture assistance management programs.

Tilapia were first introduced to Panama in 1940 when *Oreochromis mossambicus* was added to Alajuela and Gatun lakes for recreational angling. In 1976, the Nile *tilapia* (*O. niloticus*) was introduced to Alajuela, Gatun, and Bayano lakes (Morales, 1995). An additional entry of farm cultivated Nile *tilapia* occurred accidentally following tropical storm-induced floods in the 90s. Currently, *tilapia* species are the main protein source in Eastern Panama and the Canal area. Among *tilapia* species, Nile *tilapia* leads national production. Bayano Lake by itself produced nearly 3.9 tons of Nile *tilapia* in 2017; 10% of this production was exported to the

US, making of this lake the country's primary continental fishery area (FAO, 2017).

Seven decades after the introduction of the tilapia species to Panamanian lentic ecosystems, their success supports the invasive species paradox. The paradox explains how species with reduced effective populations and low genetic diversity in the initial stages of invasion, eventually succeed and colonize new environments (Frankham, Ballou & Briscoe, 2002; Allendorf & Lundquist, 2003; Roman & Darling, 2007). Herein, we present the first assessment of genetic diversity and genetic structure of established *O. niloticus* populations in lentic ecosystems of the Republic of Panama (i.e., lakes Gatun, Alajuela, Bayano and Barrigon). Additionally, the study provides the first molecular identification of four other *Oreochromis* species in the country. Information presented here is also a reference for historical connectivity and demographic history of the species using genetic differentiation (FST) and Tajima's D values respectively. The genetic data constitute a baseline to understand ecology, invasion history, hybridization process, and genetic structure of *O. niloticus* and other tilapia species. Furthermore, genetic data are essential for developing breeding programs, and management and traceability plans. Our results contribute to the prevention and control of invasive species expansion and colonization into other freshwater ecosystems of Panama and Central America.

Material and Methods

Study sites: This research was conducted in the largest artificial lakes of the Republic of Panama: Gatun, Bayano, Alajuela and Barrigon. Gatun Lake was created in early 1900's and has a surface area of 436 km². Bayano Lake, with a surface area of 350 km², was created by damming the Bayano River in 1976 (PREPAC-OSPESCA, 2007). Alajuela Lake has a surface area of 50 km²; this lake was created by damming the Chagres River in its upstream section in 1935. Barrigon Lake is the smallest lake with a surface area of 2.74 km² and was built at the Barrigon creek bed.

Sample collection: Tilapia sampling was conducted in aforementioned lakes, between January and April 2016 (Table I). An additional fifteen samples collected at Bayano Lake in previous years were also included in the analysis. Fish were captured using a stationary gill net of 60 m length by 2 m high with a net light of 4 inches. The net was placed on collection sites for 48 h before fish were

captured. Other methods like harpoon and fishing rod were also used in some areas. From each fish, 25 mg of muscle tissue were taken and preserved in 95 % absolute alcohol using 1.5 ml collection tubes.

DNA extraction: Total genomic DNA was isolated from preserved tissues using a commercial DNA extraction kit, DNeasy® Blood and Tissue (QIAGEN, Inc., Valencia, CA). DNA was stored in 1.5 ml tubes at 4 °C. DNA was quantified and its quality was checked using the Nanodrop™ 2000 spectrophotometer (Thermo Scientific).

COI amplification and sequencing: Isolated DNA was amplified by PCR using a pair of Fish COI universal primers designed by Ward *et al.*, (2005). The PCR reaction was performed under the following conditions: an initial denaturation for 1 min at 95 °C, followed by 5 cycles of denaturation at 95 °C for 30 s, hybridization at 55 °C for 40 s and extension to 72 °C for 1 min. After 35 cycles of denaturation at 95 °C for 30 s, hybridization at 55 °C for 40 s and extension at 72 °C for one min and amplification was completed by an extension step of 72 °C for 5 min. Positive amplification of COI gene was confirmed by PCR product visualization through electrophoresis in 1 % agarose gel containing GelRed (Biotium).

PCR products were purified using the QIAquick Purification Kit (QIAGEN, Inc., Valencia, CA) following the recommendations of the commercial company (USB Corporation, USA). Sequencing was performed using the commercial kit Big® Dye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and the parameters used were the following: 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min (Macrogen, Corporation, USA). The chromatograms were visualized using the Sequencing analysis v 5.2 (Applied Biosystems, USA) program.

Molecular identification of *Oreochromis niloticus* and other *Oreochromis* species: Obtained sequences were edited with the software MEGA v 6.0 (Tamura *et al.*, 2013) and exported as Fasta files to other programs for further analysis. Molecular identification was conducted by comparing focal species sequences with referenced sequences deposited in Genbank through BLASTn program (Altschul *et al.*, 1997). Percentage of similarity and percentage of query cover was verified on each sequence.

Analysis of genetic diversity and connectivity: Haplotypic diversity (Hd) and nucleotide diversity (π), total number of haplotypes (H) and number of polymorphic sites (NS) were calculated overall and

Table I. List of tilapias species identified at molecular level by site, common name, total number of individuals collected by site and GenBank accession numbers.

Lake	Tilapia species	Common name	N	GenBank Accession numbers
Gatun	<i>O. niloticus</i>	Nile	23	MT418326-MT418348
	<i>Coptodon rendalli</i>	Redbreast	3	MT418502-MT418504
Bayano	<i>O. niloticus</i>	Nile	11	MT418315-MT418325
	<i>O. mossambicus</i>	Mosambique	3	MT418254-MT418256
	<i>O. urolepis h</i>	Wami	1	MT418350
Alajuuela	<i>O. niloticus</i>	Nile	1	MT418349
	<i>O. mossambicus</i>	Mosambique	3	MT418251-MT418253
	<i>O. niloticus</i> * <i>O. aureus</i>	Hybrid	1	MT433998
Barrigon	<i>O. aureus</i>	Blue tilapia	15	MT418461-MT418475

by Lake using DNAsp v 6.12.03 for *O. niloticus* sequences (Rozas *et al.*, 2017). Genetic connectivity among sites was examined using paired F_{ST} values through Arlequin v 3.5 (Excoffier *et al.*, 2005). Also, a molecular variance analysis (AMOVA) was performed to determine whether or not sequences were geographically homogeneous or genetically differentiated.

Demographic history and neutrality test: Values of Tajima D and Fu F_s statistic were determined (Tajima, 1989; Fu & Li, 1993) to understand demographic history using the software DNAsp v 6.12.03. In addition, distribution of frequencies of nucleotide differences between haplotypes was determined by DNAsp v 6.12.03. This distribution is commonly known as mismatch distribution (Rogers & Harpending, 1992).

Results

Molecular identification of *Oreochromis niloticus* and other cichlid species: A total of 61 sequences were obtained from collected fish tissue. Analysis and comparisons to reference sequences deposited in the GenBank allowed us to identify five tilapia species (S) and one molecular hybrid (Table I). Identified species were the following: *Oreochromis niloticus*, *Oreochromis mossambicus*, *Oreochromis urolepis*, *Oreochromis aureus*, *Coptodon rendalli* and the molecular hybrid between *Oreochromis niloticus* x *Oreochromis aureus*. The number of sequences by species, common name and GenBank accession numbers are presented in Table I.

Genetic diversity and connectivity: Genetic diversity was estimated through the analysis of COI sequences of *O. niloticus* populations with more than 10 samples per location for lakes Gatun and Bayano. Two haplotypes of *O. niloticus* were

observed. Haplotype 1 was common in all areas and occurred in 34 out of 35 sequences. Haplotype 2 was only reported in Bayano Lake. Overall haplotypic diversity (both populations, all samples) showed a value of $H_d = 0.059$ and overall nucleotide diversity showed a value of $\pi = 0.00010$ (Table II). Among lakes, Bayano demonstrated higher values of genetic diversity $H_d = 0.1818$ (Table II). Historical genetic connectivity showed reduced genetic differentiation between Gatun, Alajuuela and Bayano base on F_{ST} pairwise values ($F_{ST} = 0.07258$) and percentage of variation between populations calculated by AMOVA (7.26 % of variation among *O. niloticus* populations) (Table III).

Demographic history and neutrality test: Tests of neutrality and mismatch distribution (paired differences) were calculated in order to understand deviations of mutations from expectations in a group of sequences. Values of Tajima's D and Fu F_s values were negative and not significant ($D = -1.1378$ and $P > 0.10$, $F_s = -1.315$ and $P > 0.10$). Distribution of paired differences (mismatch) in the set of analysed sequences showed a non-significant variation in the observed and expected paired differences (Fig. 1).

Discussion

Several fish molecular identification studies have been conducted to solve taxonomic uncertainties (Hebert *et al.*, 2003; Zhang & Hanner, 2011). However, for Panama in particular, no sequences have been deposited in GenBank nor molecular studies been conducted so far.

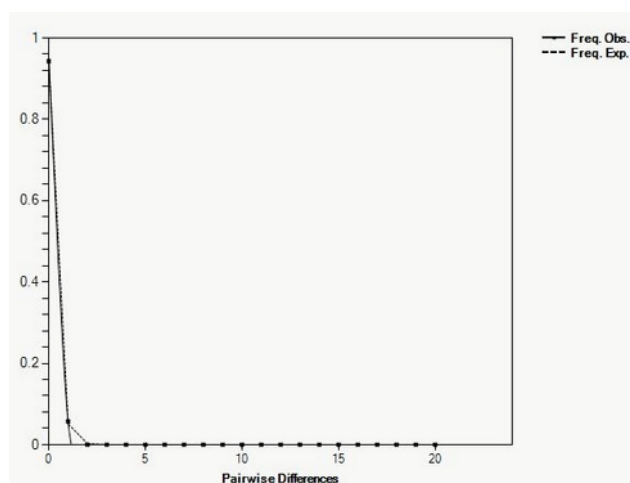
There are more than 1000 cichlids species in the tropics. A vast majority of these species including tilapias are well known for their ability to

Table II. Levels of genetic diversity calculated for *O. niloticus* populations.

Location	n	H	H1	H2	(π)	(Hd)	(NS)
Gatun	23	1	23		0	0	0
Alajuela	1	1	1		0	0	0
Bayano	11	2	10	1	0.0003	0.1818	1
Total	35	2	33	1	0.00010	0.059	1

Table III. Molecular Analysis of Variance (MANOVA) showing the overall genetic differentiation and the percentage of variation between populations. **n** = number of samples, **H** = number of haplotypes, (π) = nucleotide diversity, (**Hd**) = haplotypic diversity, (**NS**) = number of polymorphic sites, **H1** = Haplotype 1, **H2** = Haplotype 2.

Source of variation	d.f	Sum of squares	Components of variance	Index of fixation FST	Percentage of variation
Between population	1	0.061	0.00222 Va		7.26
Within population	34	0.909	0.02841 Vb		92.74
Total	35	0.971	0.03063	0.07258	

**Figure 1.** Distribution of paired differences (mismatch) for Cytochrome oxidase I sequences of *O. niloticus*.

hybridize as invasives in captivity and within their natural distribution range (Daget & Moreau, 1981; Pouyaud & Agnès, 1995). Cichlid hybridization and phenotypic plasticity make taxonomic identification difficult, especially for tilapias. Thus, taxonomic identification has become a challenge for ichthyologists, and the use of molecular methods has become an essential tool when morphological differences are not conspicuous for taxonomic identification (Berra, 2001).

Molecular identification of *Oreochromis niloticus* and other *Oreochromis* species: In this study, 61 fish sequences were obtained and compared with GenBank referenced sequences. Results showed that

35 out of 61 sequences corresponded to *O. niloticus*, 15 out of 61 to *O. aureus*, and four out of 61 to *O. mossambicus*. In addition, three sequences out of 61 corresponded to *Coptodon rendalli*, one out of 61 to *O. urolepis hornorun* and one to the molecular hybrid between *O. niloticus* and *O. aureus*. These molecular findings were consistent with previous taxonomic studies where *O. niloticus*, *O. mossambicus* and *O. aureus* were listed for Bayano and Alajuela lakes (Morales, 1991, Morales, 1995; Maturel, 1984). Herein, for the first time *Coptodon rendalli* and *Oreochromis urolepis* were confirmed at the molecular level and added to the list of species in Panamanian aquatic ecosystems. Likewise, the molecular hybrid detected provides evidence of hybridization between *O. niloticus* and *O. aureus*.

Hybridization between these two tilapia species was previously reported in African and Israeli ecosystems (Rognon & Guyomard, 2003; Shirak *et al.*, 2009). The existence of a molecular hybrid in Alajuela Lake (*O. niloticus* x *O. aureus*) suggests that hybridization either occurred right after the introduction of pure individuals from both species to Alajuela Lake or it was caused by introgression of ancestral lines of some mitochondrial gene sequences in the past. Agnès (1997), explained hybridization in tilapias using two hypotheses. The first one suggests that these species shared an ancestral haplotype before a speciation process separated them into two different ones. Second, the mitochondrial DNA of one species could have been transferred and established in the DNA of the other.

Genetic diversity and connectivity of Oreochromis niloticus: Despite of the higher numbers of individuals observed, Tilapia populations from Gatun and Bayano lakes showed low values of genetic diversity. The obtained genetic diversity values ($H_d = 0.059$; $\pi = 0.00010$) were similar to results obtained from captive tilapia populations (most of them are zero) using the mitochondrial DNA control region and COI. Low variability in invasive species can be explained by the existence of inbreeding caused by a limited number of founder individuals (small effective population size) that promotes the loss of alleles and haplotypes by genetic drift (Ryman and Utter, 1987). Also, lack of new and highly variable introductions (introduction of new alleles) could be responsible for the observed values of genetic diversity (Wu & Yang, 2012). The invasive species paradox establish that even low diversity introductions can avoid the negative impact of diversity loss (Roman & Darling, 2007). In commercial or fishery important species we have also to consider overexploitation (fishery pressure) to be responsible in some cases of bottlenecks that reduce effective population size, strengthened the genetic drift and consequently promote loss of genetic diversity (Vidal et al., 2010). In contrast, natural populations of tilapias studied in Africa showed higher levels of genetic diversity; i.e., nine haplotypes and haplotypic diversity values (H_d) between 0.77 and 0.81 were observed (Agnès, 1997).

Genetic connectivity was determined as an indirect measure of genetic similarity or differentiation between populations (pairwise F_{ST}) (Díaz-Ferguson et al., 2010, 2012; Butterfield et al., 2015). In this regard, overall value of genetic differentiation between populations ($F_{ST} = 0.07$) suggest lack of differentiation and historical connectivity. Nonetheless, in the case of this invasive species, similarity between populations probably respond to the introduction of the same genetic lines. This result is consistent with the AMOVA that showed a reduced percentage of variation between populations (7.26 %) and reveal the existence of a single population of *O. niloticus* in Panama. This finding also corroborates that population founders come from the same ancestral line and it is confirmed by the existence of a common haplotype within the three sampling areas. Most likely, the founder event in Panama for Nile tilapias occurred during the 70's (first introduction in the country). On the other hand, the existence of a private haplotype in Bayano Lake corroborates a

second introduction of tilapias to the lake during the 90's (Morales, 1995). Multiple introduction events are the key for invasive species success and haplotype frequency pattern have proved to be helpful to identify source of population, expansion and introduction events (Díaz-Ferguson & Hunter, 2019; Hunter et al. 2021).

Compared to other population studies, our results were similar to a genetic variation study that used microsatellites to reveal low to moderate differentiation among *O. niloticus* populations ($F_{ST} = 0.074$) in Lake Volta (Mireku et al., 2017). Although, Swain et al., (2014) mention that lack of genetic differentiation could be related to a shared set of ancestral genes and haplotypes or to intense gene flow between populations driven by natural events or anthropogenic intervention.

Demographic history and neutrality test: Results from Tajima's D ($D = -1.1378$) and Fu F_s ($F_s = -1.315$) (negative and non-significant values) failed to demonstrate population expansion in Panama Nile tilapia. In contrast, obtained values and results from observed mismatch paired distribution (Fig. 1) evidence neutrality and demographic equilibrium. These features are common in populations with a constant population size (N_e) (Mousset, Derome, & Veuille, 2004; Ewers & Wares, 2012).

Final remarks: In general, a small fraction of the recorded species introduced into tropical ecosystems have been genetically analyzed (Rius et al., 2014) despite tilapia studies of the controlled aquaculture systems from Mexico to Argentina (Zambrano et al., 2006). Genetic data for tilapias in Latin America are only available for cultivated populations of Pernambuco, Brazil (Lupchiski et al., 2011). Therefore, this is the first study to present tilapia genetic data collected from the natural ecosystems of the region. Furthermore, the Nile tilapia data presented here contribute to understanding their genetic diversity, fitness, connectivity, and invasion history and the evolutionary forces that shape these processes. In addition, our research provides the first invasive cichlid species molecular list for Panama and Central America. The findings presented here are essential for aquaculture, breeding program establishment, stock management, traceability and invasive species population control and surveillance.

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Ethics statement

Collection of fish and sample tissue were conducted following all applicable ethical regulations regarding experimentation with animals.

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