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Research Article

Exploring the presence of tilapia species in a central western Mexican reservoir using mitochondrial DNA control region sequencing

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ABSTRACT. *Oreochromis niloticus* is the most important aquacultural species worldwide. Several countries are interested in repopulating natural or artificial reservoirs with the fry of tilapia (*Oreochromis* sp.) to develop new fisheries or to enhance the existing ones. However, as tilapia is considered an invasive species, it is essential to check the existence of the species in the place before releasing any fry. Characterization of tilapia species with meristic is unreliable because of existing morphological similarities among species. In this work, the composition of the tilapia species of Laguna El Mastranzo (LEM), Nayarit, Mexico, was explored for the first time through a molecular method using the mitochondrial DNA control region. Three tilapia species (*Oreochromis niloticus*, *O. aureus*, and *O. urolepis*) were found in LEM, and it was confirmed that the two commercial strains (Aquamol and Spring) intended for repopulation were *O. niloticus*. Seven haplotypes of mitochondrial DNA control region sequences were obtained: three in LEM, three in Aquamol, and one in Spring strains. Before repopulating any reservoir, a molecular verification strategy is recommended to avoid spreading the species. The results of this work represent a glimpse into the genetic material of LEM, Nayarit, Mexico.

Keywords: *Oreochromis* sp.; tilapia species; molecular characterization; phylogenetic relationships; aquaculture

INTRODUCTION

Tilapia is a widely consumed freshwater fish native to Africa and the south-west Middle East. It belongs to the family Cichlidae and includes the maternal mouth-breeding genus *Oreochromis*, the paternal-maternal mouth-breeding genus *Sarotherodon*, and the substrate spawning for the genus *Tilapia* (Bhassu et al. 2004, Canonico et al. 2005). The worldwide production mainly comprises *Oreochromis* sp. (Bhassu et al. 2004). This genus is a significant group of fish species that is widely consumed in many parts of the world due to its excellent culture characteristics such as resistance to disease, easy reproduction, high tolerance to a wide range of environmental conditions, efficient use of low-protein diets, high palatability, marketability, nutrient content, and high resistance to stress and infections (Bhassu et al. 2004, Hassanien & Gilbey 2005, Omer et al. 2020). The world production of tilapia has increased by 70.2% from 2010 to 2018, exceeding 4.5 million tons produced (FAO 2020). In 2020, Mexico registered an aquaculture production of 72,596 t. In the first

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quarter of 2021, the preliminary number is 15,958 t, where 10,000 t came from aquaculture activities and the rest from fisheries in lakes, lagoons, and rivers (CONAPESCA 2021).

Since the 60s, several tilapia species have been introduced in dams and natural and artificial reservoirs of Mexico to generate new fisheries (Morales-Díaz 2003). The high adaptability, resistance, and good immunity of tilapia have been the key to its successful dispersal in different bodies of water, such as lakes, coastal lagoons, rivers, and dams, throughout the tropical zone of the country (Arredondo-Figueroa & Guzmán-Arroyo 1986, Abdelrhaman et al. 2020, Fuadi et al. 2021). Laguna El Mastranzo (LEM) is a typical freshwater reservoir of the western part of Mexico that could represent that region (Ramírez-Zavala et al. 2005). Local aquaculturists and fishermen are interested in freeing genetically improved fry of *O. niloticus* in this lagoon. Still, the population structure of naturalized tilapia is not known, which is crucial for its management, behavioral study, and utilization as an aquaculture resource (Espinosa-Chaurand & García-Morales 2016). On the other hand, two main sources of genetically improved tilapia fry in Mexico (Spring and Aquamol) are intended for repopulation purposes and are commercialized as *O. niloticus*. It would be very useful to confirm the species and determine the level of genetic variability.

Molecular techniques can verify the presence of *Oreochromis* sp. species to characterize tilapia populations in the region and compare their genetic diversity with other distinct strains of *O. niloticus*. The genetic information in the deoxyribonucleic acid (DNA) sequences can distinguish the differences between phylogenetically close species (Teletchea 2009, El-Zaeem et al. 2012, Amoussou et al. 2019) because environmental interaction can cause DNA mutations, producing genetic variations. Genetic variation allows species to adapt to environmental changes and survive (Liu & Cordes 2004, Askari et al. 2013, Maqsood & Ahmad 2017). When these changes in the DNA sequence can be detected, they can be used as markers to differentiate species, populations, and even between individuals (Liu & Cordes 2004, Askari et al. 2013, Saad 2019). In tilapia cichlid, various molecular markers have been used to differentiate between species, such as random amplified polymorphic DNA (RAPD) (Bardakci & Skibinski 1994, Ahmed 2004), PCR-RFLP of 5SrDNA (Toniato et al. 2010), microsatellites (Hassanien & Gilbey 2005, Saad et al. 2012, Gu et al. 2014), mitochondrial DNA (mtDNA) regions (Wu & Yang 2012, Gu et al. 2016), and single nucleotide polymorphisms (SNPs) (Syaifudin et al. 2019).

The rapid evolution of the mtDNA due to the faster mutation rate and strictly maternal inheritance (Liu & Cordes 2004, Liu 2007, Liaqat et al. 2012, Sukumaran & Gopalakrishnan 2019) makes it highly polymorphic, and it is suitable for studying closely related species (Sultana et al. 2016). The displacement loop (D-loop) or control region is a highly variable part of the mitochondrial genome that serves as a replication origin (Kucuktas & Zhanjiang 2007). Their polymorphism makes it applicable to genetically analyzing populations and characterizing species (Liu 2007, Liaqat et al. 2012, Wu & Yang 2012, Gu et al. 2016). It has been recommended as an effective method for examining the discreteness of different strains of *Oreochromis* sp. and developing species-specific management strategies (Kwikiriza et al. 2023).

Limited information exists about the genetic diversity of tilapia populations in Nayarit, Mexico. This study aims to begin filling this gap by using molecular techniques to identify and characterize tilapia populations in the region. The primary objective is to verify the presence of *Oreochromis* sp. species and compare their genetic diversity with two distinct strains of *O. niloticus*. It is worth noting that this is the first report of tilapia in the region, which opens the way for further studies on the tilapia species in the area.

MATERIALS AND METHODS

Study area

LEM is a 58 ha freshwater lagoon in the Mexican state of Nayarit (21°10'53"N, 105°11'26"W). It is located 5600 m west of Las Varas and 4000 m east of the community of Chacala, municipality of Compostela, Nayarit. It has an average depth of 1.5 m, a water temperature range of 26.8-33.1°C; 2.36-6.42 mg L^{-1} O_2, 5.4-8.9 pH, and 30-82 cm Secchi disc transparency (Fig. 1) (Espinosa-Chaurand & García-Morales 2016).

Collection of fish samples

A total of 43 dorsal fin clip samples were collected from 15 tilapia fish from LEM, 19 tilapias of the Aquamol strain, and 9 of the Spring strain. These were preserved in 80% ethanol and kept in the refrigerator at 4°C.

DNA extraction and PCR amplification

Genomic DNA was extracted from each sample using a Wizard® SV 96 Genomic DNA Purification Kit extraction (Promega, Madison, WI, USA), following

Figure 1. Geographic location of Laguna El Mastranzo, Nayarit, Mexico.

the manufacturer's guidelines. The DNA concentration was first estimated by spectrophotometer (NanoDrop-1000, ThermoScientific, Chicago, IL, USA). Then, the mtDNA control region (mtDNA-CR) fragment (~500 bp) was amplified by polymerase chain reaction (PCR) using ORMT-F 5'-CTAACTCCCAAAGCTAGGAAT TCT-3' and ORMT-R 5'-CTTATGCAAGCGTCGAT GAAA-3' oligonucleotides (Wu & Yang 2012, Gu et al. 2016). The PCR reaction was performed in a volume of 25 µL with 2.5 U GoTaq Flexi DNA polymerase (Promega), 1.5 mM $MgCl_2$, 1x Go Taq Flexi buffer, 0.2 mM dNTP mix (Promega), 0.2 µM of each oligonucleotide, 1 µL of DNA at 50 ng μL^{-1}, and Milli-Q water. The PCR was carried out on an Eppendorf thermal cycler (Eppendorf, Hamburg, Germany) using the following conditions: initial denaturation at 94°C for 3 min, 35 cycles at 94°C for 30 s, 54°C for 40 s, 72°C for 40 s, and a final extension at 72°C for 10 min. PCR amplifications were verified by electrophoresis on a 1% TBE agarose gel with 1x GelRed (Biotium Inc., Fremont, CA, USA), visualized using a gel documentation system (GelDoc XR+, Bio-Rad Laboratories, Hercules, USA). The PCR product was sequenced by Genewiz (Genewiz Inc., South Plainfield, NJ, USA).

Sequence analysis

The sequences obtained were aligned and manually edited using BioEdit software version 7.2.5 (Hall 1999). Nucleotide homology analyses were performed using BLAST searches (http://www.ncbi. nlm.nih.gov/ BLAST/) with the consensus of each sequence for the characterization of tilapia. A total of 43 nucleotide sequences of mtDNA-CR were amplified from the DNA of tilapia fish samples and the sequences of three recognized species of tilapia, including *O. niloticus* (Genbank Accession: MF385001.1, MG728070.1, MG728003.1, MG728020.1, MG728016.1, GU477624.1, NC_013663.1, MG728036.1, GU370126.1, GU4776 28.1, MG728093.1, GU477626.1), *O. aureus* (Genbank Accession: MN384753.1, MN384750.1, MN384754.1, EU430994.2, GU477629.1, MH515178.1), *O. urolepis* (Genbank Accession: EU308500.2, MN384749.1, OP 219995.1, MN384748.1), and the cichlid *Archocentrus*

centrarchus (Genbank Accession: KX227441.1, KX22 7442.1, KX227446.1) that was used as an outgroup, and were included in the analysis. Sequences were aligned using MUSCLE (Edgar 2004) with a default setting, and curated blocks were obtained using the Gblocks program (Talavera & Castresana 2007). The cured alignment was used to find the best-fit model based on the Bayesian Information Criterion (BIC) with MEGA 10.2.4 software (Tamura et al. 2013).

Molecular analysis

A phylogenetic analysis was done in MEGA, using the maximum likelihood (ML) method based on the Hasegawa-Kishino Yano model plus gamma-distributed rate heterogeneity (HKY+G). Ten thousand bootstrap replications evaluated the topological stability of the tree. The number of haplotypes (h), haplotype diversity (Hd), nucleotide diversity (Pi), and the average number of nucleotide differences (k) in each data set and between all samples were analyzed in DNASP 6.12.03 software (Rozas et al. 2017). Haplotypes for specific samples were also identified with DNASP 6.12.03 and haplotype network was visualized with the Population Analysis with Reticulate Trees (PopART) network analysis software (Leigh & Bryant 2015), using the TCS network inference method (Clement et al. 2000). The genetic distances among populations of tilapia *Oreochromis* sp. were obtained by GENEALEX (Peakall & Smouse 2012). All sequences from the wild populations of LEM were submitted in GenBank under the accession number from OQ607580 to OQ607583 by *O. aureus*, from OQ607584 to OQ607589 by *O. niloticus*, and from OQ607590 to OQ607595 by *O. urolepis*.

RESULTS

Characterization of tilapias using mtDNA-CR

A total of 43 sequences of sufficient quality were obtained successfully to perform phylogenetic analysis (15 from LEM, 19 from Aquamol, and 9 from Spring strain). These were compared with the reported tilapia fish mtDNA-CR sequences in GenBank. The results indicate that in LEM, six individuals (40%) showed high similarity with *O. niloticus*, six (40%) with *O. urolepis*, and three (20%) with *O. aureus*. All sequences from Aquamol (19) and Spring (9) strains were highly similar to *O. niloticus*. All sequences (~498 bp) showed a query cover of 100% and a maximum identity of 99.45 to 100%.

Haplotype network analysis

Seven haplotypes were defined according to all variable positions in the mtDNA-CR gene from the LEM, Aquamol, and Spring strains samples (Fig. 2). The Spring strain shows a unique haplotype (Hap_1). The Aquamol strain shows three haplotypes (Hap_2, Hap_3, and Hap_4). On the other hand, LEM shows three haplotypes, each one for each tilapia species identified: Hap_5 from *O. niloticus*, Hap_6 from *O. aureus*, and Hap_7 from *O. urolepis*. Hap_3 has a major frequency from Aquamol haplotypes, showing one mutational step of Hap_5 separating them from the rest with 20 mutational steps. Another group consists of Hap_6 of *O. aureus* from LEM separated from Hap_4 by five mutational steps, with Hap_2 derived from Hap_4 with one mutational step. Hap_7 of *O. urolepis* from LEM is separated from the other groups by 18 mutational steps, and 15 mutational steps separate Hap_1.

Phylogenetic analysis

The phylogenetic analysis is presented in Figure 3. With the haplotypes obtained, it was possible to create phylogenetic relationships between all sequences obtained in the present study and published sequences in the GenBank database. First, the analysis separated all the tilapia fish from the cichlid *A. centrarchus*, which served as the outgroup. Subsequently, the analysis created two large groups where one of them relates all the nine sequences of *O. niloticus* of Spring, with six sequences of *O. urolepis* of LEM, and related three sequences of *O. aureus* of LEM with eight of *O. niloticus* of Aquamol. The second group was composed only of *O. niloticus* sequences, which related six of LEM with 11 of Aquamol.

Genetic distance

Table 1 shows the genetic distances between strains of *Oreochromis* sp. from different populations. The longest distance (0.555) found was between *O. urolepis* and *O. aureus* of LEM, followed by *O. aureus* and *O niloticus* of Spring (0.496) and of LEM (0.495) populations. *O. niloticus* from Aquamol showed an intermediate genetic distance of 0.303 and 0.340, with *O. niloticus* and *O. urolepis* from LEM, respectively. The lowest distance of the Aquamol population of *O. niloticus* was with the same species (0.079) and with *O. aureus* (0.180) from LEM.

Genetic variability

The genetic variability analysis of mtDNA-CR shows one haplotype for *O. niloticus*, one for *O. aureus*, and one for *O. urolepis* identified from LEM, three haplotypes for Aquamol, and one for Spring strain. The highest genetic variability was found in the Aquamol

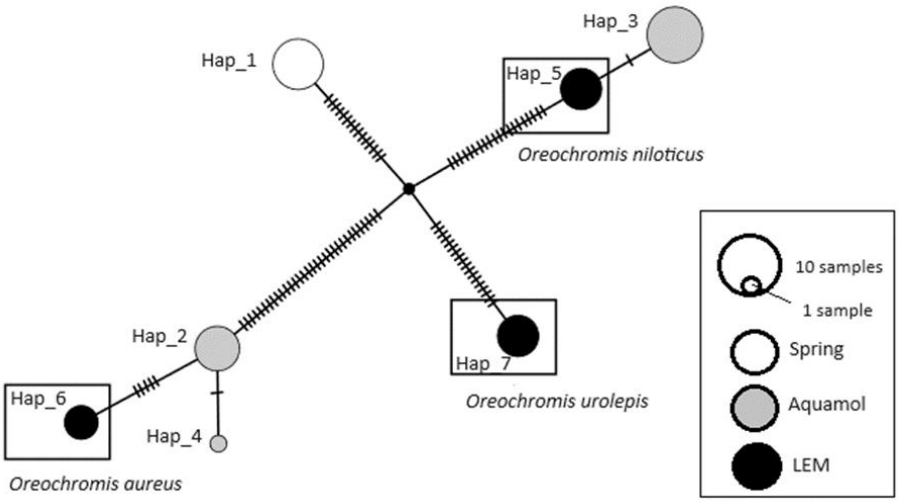

Figure 2. TCS haplotype network of mitochondrial DNA control region sequences of tilapia from Laguna El Mastranzo (LEM), Nayarit, México, Aquamol, and Spring strains. Squares show the haplotypes corresponding to three species of LEM tilapia. The lines represent mutational steps that separate haplotypes, and the black circle in the center of the lines represents an additional mutational change. The size of the circle is proportional to the frequency of the haplotype.

strain, with a Hd of 0.556, a Pi of 0.061, and k of 26.8. The genetic variability of Spring was null (zero) because these were characterized by only one haplotype.

DISCUSSION

Genetic characterization of tilapia species in natural reservoirs and farms located in the same place is the key to preserving genetic resources (Taniguchi et al. 1985) because individuals can exchange between natural reservoirs and farms due to accidental leaks, floods, or water withdrawals from the natural environment, which affects gene flow between different species (Anane-Tababeah et al. 2019). mtDNA sequences is one of the methodologies to identify and characterize tilapia species. Using mtDNA-CR sequences, Wu & Yang (2012) identified seven distinctive tilapia species: *O. aureus*, *O. mossambicus*, *O. niloticus*, *O. urolepis*, the hybrid of *O. niloticus* × *O. mossambicus*, *Coptodon rendalli*), and *Sarotherodon melanotheron*, in the wild and cultivated populations in Hawaii. They mentioned that this mtDNA region had a higher variability than other mtDNA markers, such as cytochrome oxidase I (COI). Likewise, Gu et al. (2016) identified five species of tilapia in the rives of South China: *O. niloticus*, *Coptodon zillii*, *O. aureus*, *Sarotherodon galileus*, and hybrids of *O. mossambicus* × *O. niloticus*. Fatsi et al. (2020) employed the same mtDNA-CR region for the population genetics analyses of wild tilapia in Japan, finding 52 distinct haplotypes and detecting significant differences within all populations analyzed. They identified *O. mossambicus*, *O. niloticus*, *O. aureus*, *C. zillii*, *O. aureus* × *O. niloticus* hybrids, and *O. mossambicus* × *O. niloticus*. Later in 2022, Yamasaki et al. (2022) used a mtDNA-CR to identify the presence of *O. niloticus* and its hybrids in natural environments in Hawaii, providing information that can be used to inform management strategies for tilapia populations in the islands of Molokai, Maui, as well as Hawaii.

In 2023, other authors using genotyping of the mtDNA-CR to examine the impact of aquaculture spillage on the establishment and colonization of non-endogenous tilapias, genetically identified non-native tilapias from a Pagani River and three aquaculture facilities nearby (Chuhila et al. 2023). Recently, the complete mitochondrial genome has been used for investigating the genetic structure and diversity of Mango tilapia (*Sarotherodon galilaeus*), just as they established the phylogenetic relationship between *O. niloticus* from two lakes in Nigeria (Fiteha et al. 2023). Finally, Kilawati et al. (2023) discuss identifying mtDNA sequences of captive and wild tilapia species in Lake Ranu Klakah, Lumajang. COI sequences identified *O. niloticus* in the wild and captivity, the Srikandi tilapia, locally known as *O. niloticus* × *O. aureus*.

In the present study, a focused exploration of the tilapia species in the LEM, Nayarit, was carried out.

This allowed us to infer the biogeographical presence of three tilapia species in the study area with

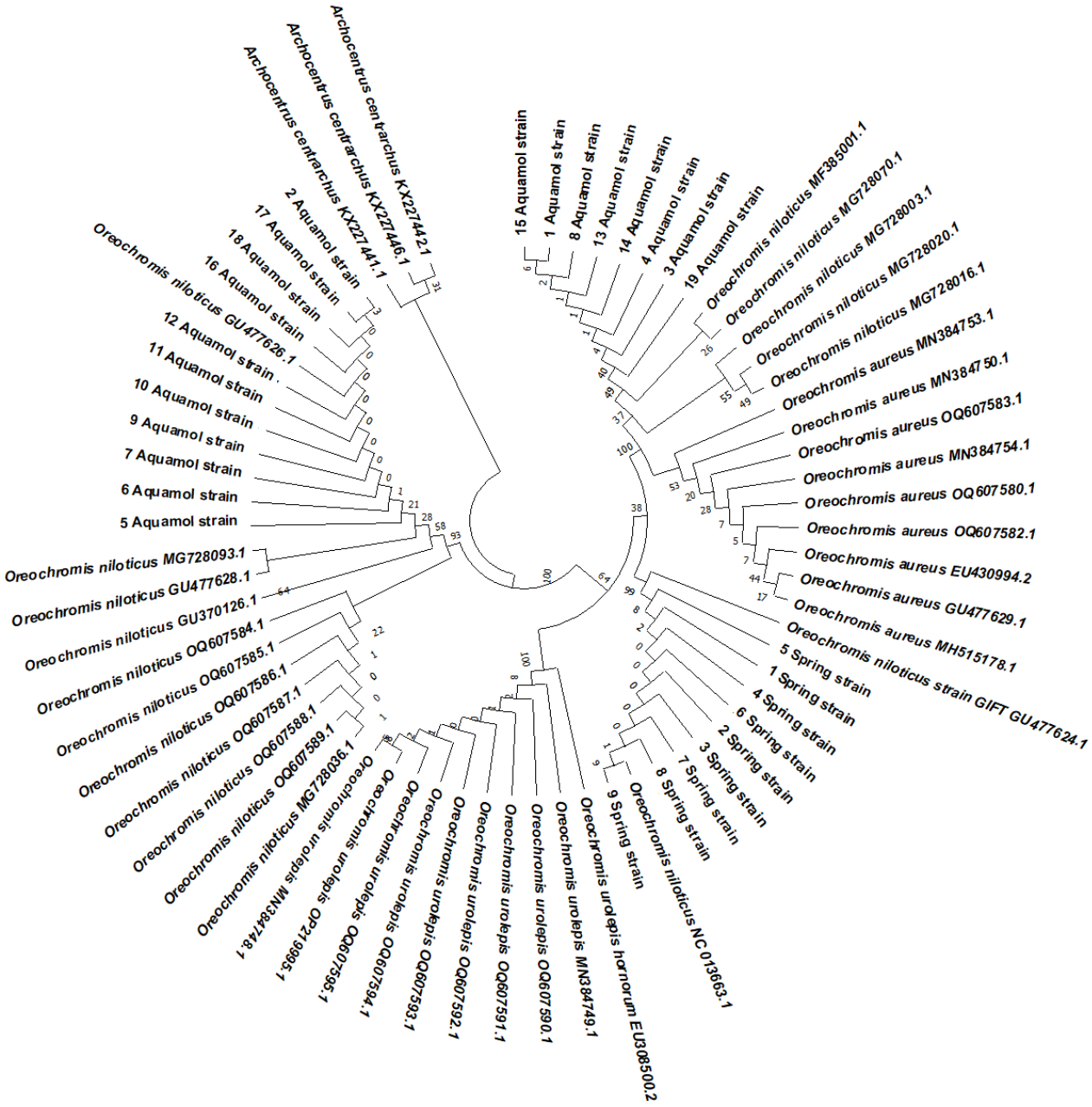

Figure 3. Phylogenetic relationships tree inferred by the neighbor-joining method with a total of 43 nucleotide mitochondrial DNA control region (mtDNA-CR) sequences of tilapia *Oreochromis* sp. from the Laguna El Mastranzo (LEM), Aquamol strain, Spring strain, and *Archocentrus centrarchus* as an outgroup.

the mtDNA-CR method: *O. niloticus*, *O. aureus*, and *O. urolepis*. We confirmed that the species from both commercial strains of fry (Aquamol and Spring) were *O. niloticus*.

From the mtDNA-CR data, the phylogenetic tree separated the outgroup *A. centrarchus* from the rest. At the same time, *Oreochromis* sp. was separated into two big groups: the *O. niloticus* group and the group where three species (*O. niloticus*, *O urolepis*, and *O. aureus*) were closely related. In the latter group, *O. aureus* from LEM was genetically related to *O. niloticus* from Aquamol. Some studies mention a high degree of synteny between *O. aureus* and *O. niloticus* (McConnell et al. 2000), and it has been shown that the mtDNA sequences of both species can ultimately be the same (Rognon & Guyomard 2003). Hybridization between

Table 1. Genetic distances (*) between different populations of tilapia *Oreochromis* spp., based on mitochondrial DNA control region (mtDNA-CR) sequences. *mtDNA-CR genetic distances between populations were obtained by Genealex. LEM: Laguna El Mastranzo.

Species	Populations	*O. niloticus*			*O. aureus*	*O. urolepis*
		Aquamol	Spring	LEM	LEM	LEM
O. niloticus	Aquamol	0.000				
O. niloticus	Spring	0.303	0.000			
O. niloticus	LEM	0.079	0.334	0.000		
O. aureus	LEM	0.180	0.496	0.495	0.000	
O. urolepis	LEM	0.340	0.305	0.341	0.555	0.000

O. aureus and *O. niloticus* species has been observed under natural conditions (Bakhoum 2002). *O. niloticus* has been documented as one of the extensively used aquaculture species for hybridization (Mtaki et al. 2022).

Genetic distance is a measure of the genetic divergence between two populations, and it is calculated based on the number of genetic differences between them (Holsinger & Weir 2009, Jiang et al. 2019). This study found the highest genetic distance between *O. aureus*, *O. urolepis*, and *O. niloticus* from LEM (0.495-0.555). These values of genetic distances and their separation in clusters in phylogenetic analysis indicate high genetic differentiation between them, suggesting that they are different taxa (Tesfaye et al. 2021), including *O. aureus* from LEM and *O. niloticus* from Spring, which show a genetic distance of 0.496. However, the genetic distance between *O. niloticus* from LEM and *O. niloticus* from Aquamol (0.079) and *O. niloticus* from Aquamol and *O. aureus* from LEM (0.108) means that there is a moderate level of genetic differentiation between them. In the case of the two populations of tilapia, a low genetic distance suggests some genetic diversity between the two populations, but they are not very different (El-Zaeem et al. 2012).

In the present work, the genetic diversity of *Oreochromis* sp. species was determined by calculating haplotype diversity (Hd) and nucleotide diversity (Pi). The values reported here show that Aquamol has greater genetic diversity (Hd = 0.556) and nucleotide diversity (Pi = 0.061) than the LEM or Spring strain organisms (Hd = 0; Pi = 0). These wide ranges of values (Hd = 0-0.556), indicating low to high genetic variability, have been reported in other studies. Jiang et al. (2019) report haplotype Hd values of 0.234 to 0.826 and Pi values of 0 to 0.060 in seven populations of *Oreochromis* sp., including the GIFT strain.

Hubert et al. (2021) report a mitochondrial genetic diversity of the GIFT *O. niloticus* strain of Hd = 0.539-0.575, while that of *O. niloticus* from other locations in Madagascar, the genetic diversity values ranged from Hd = 0.243 to 0.785.

The use of wild strains for hybridization in tilapia aquaculture is a topic of interest. For example, Matthew et al. (2016), Fagbemi et al. (2021), and Moses et al. (2021) determined the growth performance of different wild strains of *O. niloticus* in fresh and brackish water environments to use them as breeders.

Wild tilapia must be used with care since the unintentional introduction of alien species to aquaculture or wild populations can have negative consequences, such as the loss of genetic variability (MacKinna et al. 2010).

Various authors recommend using a combination of mtDNA and nuclear markers, such as microsatellites, SNPs, or ribosomal DNA regions, for species identification (Toniato et al. 2010, Syaifudin et al. 2019, Cermakova et al. 2023). However, mtDNA is a powerful tool for species identification due to its maternal inheritance, ease of isolation from low-quantity or degraded DNA samples, and simplification of interpretation (Wu & Yang 2012, Gu et al. 2016, Kilawati et al. 2023). However, it has limitations in hybridization and introgression studies (Toniato et al. 2010, Syaifudin et al. 2019). In contrast, nuclear markers provide additional information and can overcome these limitations (Cermakova et al. 2023). In the present work, using the mtDNA-CR has allowed for a focused exploration and inference of tilapia species in an unexplored region, providing a more accurate and robust method than morphological identification and meristic.

CONCLUSIONS

In this study, we successfully amplified the mtDNA-CR to investigate the presence of three different tilapia species (*O. niloticus*, *O. aureus*, and *O. urolepis*) in a natural reservoir, LEM, in Nayarit, Mexico. Our results

revealed that the fry from the hatchery strains (Aquamol and Spring) were *O. niloticus*, with varying levels of genetic variability. With high and low genetic variability, these strains could be suitable for repopulation purposes in LEM. It is strongly recommended that the methodologies outlined in this study, mtDNA and nuclear markers, be followed when seeking to repopulate any reservoir. This approach helps prevent the spread of tilapia species in areas where they do not naturally exist.

Credit author contribution

R.M. Morelos-Castro: conceptualization, validation, methodology, formal analysis, writing-original draft; B. Aparicio-Simón, R. García-Morales, D. Espinosa-Chaurand & R. Garza-Torres: field work, methodology and data curation; P. Cruz-Hernández & R. Campos-Ramos: methodology, formal analysis, review; A.N. Maeda-Martínez: funding acquisition, project administration, supervision, review, and editing. All authors have read and accepted the published version of the manuscript.

Conflict of interest

The authors declare no potential conflict of interest in this manuscript.

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