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Original Article

Molecular identification of two tilapia species of the genus *Oreochromis* from Shat Alarab River

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Abstract: In the current study, the two species of Tilapia, including *Oreochromis niloticus* and *O. aureus*, collected from Shat Alarab River in the Basrah region, South of Iraq, were identified using the nuclear *sox3* gene to confirm their genetic matching. They have been recorded since 2007 in the middle part of Shat Alarab River, Iraq based on morphometric characters; however, there is no evidence to prove how these two species were introduced into the Iraqi inland waters. After collection, morphological characteristics of specimens, including meristic measurement, were counted to identify the two species. Then, their phylogenetic relationship with other available genes from different geographical regions was constructed using maximum likelihood and neighbor-joining algorithms. The results revealed that both species did not belong to a common ancestor. Furthermore, the results confirmed that *Scan4ab* existed in *O. niloticus*, but it was not found in *O. aureus*, showing they are probably not taxonomically related.

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Introduction

Tilapia fishes of *Oreochromis niloticus* and *O. aureus* belong to the Cichlidae family and are alien fish in Iraqi inland water. Cichlid fish are tropical and can survive in warm water (Anene, 1998; Radkhah and Eagderi, 2021; Jalili et al., 2022). They can tolerate temperatures around 25-30°C but cannot survive at temperatures less than 13°C (Yousef and Goda, 1996). They were recorded in Iraqi water first in Al Musayib (Saleh, 2007). It was thought these species were accidentally introduced into Iraqi water by fish farmers who brought them from outside Iraq for cultivation. The origin of tilapia is East Africa (Tesfaye et al., 2021), but many species taxa reach 3,000 species distributed in the Middle East, Sri Lanka, Madagascar, and south of India (Snoeks, 2000; Turner et al., 2001). Tilapia has been vastly distributed outside its original geographical area (Philippart et al., 1982). The reproductive strategy of these fish species allowed them to distribute and live successfully in the water bodies that inhabit them. They are multispawner over the years and nurse their larvae in their mouth to protect them from enemies (King, 1994). *Oreochromis niloticus* is considered a commercial fish in Africa for its use in cultivation farms, and it is the source of protein for many African countries (Durr and Gonzalez, 2002).

Phylogenetic trees of some species belong cichlids were studied; for example, more than thirty tilapia species were investigated using mitochondrial DNA by Nagl et al. (2001) and Klett and Meyer (2002). *Oreochromis* sp. was at the top of the list and was analyzed from the African model (Dunz and Schliewen, 2013). *Oreochromis* species have an evolutionary pattern called adaptive radiations, which means the ability to produce rapid diversity from common ancestors, especially when the organisms are subjected to various environmental pressures. More than 1,650 species are found in the cichlid family (Fishbase, 2023).

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Table 1. The primer sequence was used for the target genes in this study

Target gene	Sequence (5'-3')	Ta (°C)	Product size
Scn4ab	F: GTCCAGCAGAATGTCAA R: CTCTTCAATAGGAACATTGAGGAG	56	261 bp
Sox3	F: GTATAACATGATGGAA R: ATGTGGGTGAGAGGTAG	56	897 bp

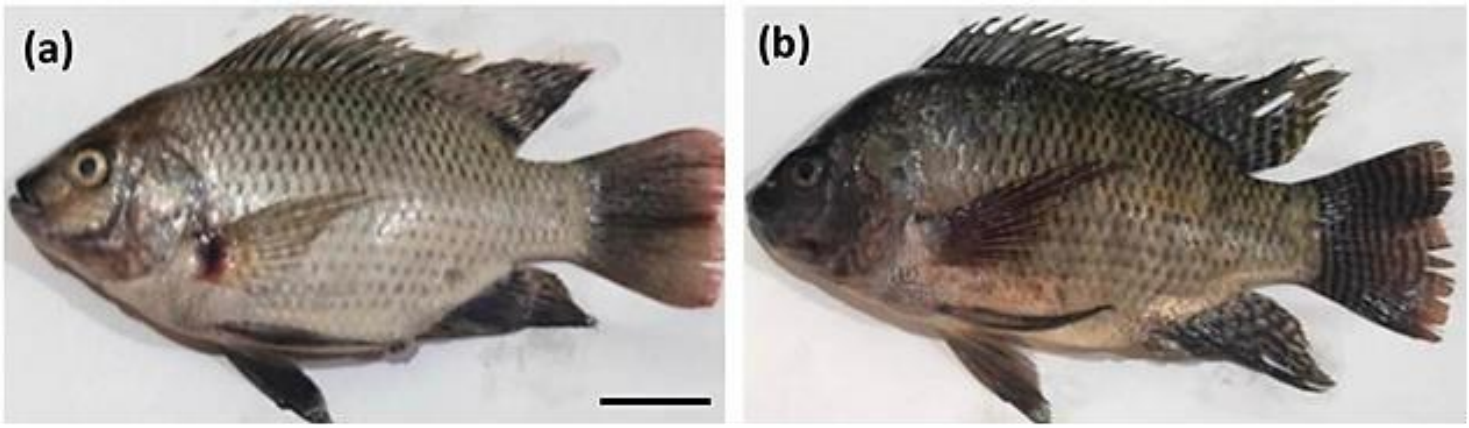

Figure 1. (a) *Oreochromis aureus,* (*b*) *Oreochromis niloticus* (Scale bar = 3 cm).

Many studies on *Oreochromis* taxonomy, including the phylogenetic tree, were based on mtDNA markers (Ford et al., 2019). However, other studies were done on phylogenetic studies based on nuclear loci (Schwarzer et al., 2009; Dunz and Schliewen, 2013; Matschiner et al., 2017). Genetic analyses are required to determine species' taxonomic status at the molecular level, especially for alien species that entered the new environment and adapted to new biosphere conditions. This, in turn, enhances our understanding of biodiversity studies and species evolution, in addition to resolving their taxonomic issues. Of course, the phenotypic classification of species is a basis for placing species within the taxonomic hierarchy; many species are phenotypically similar but are different taxonomically when examined based on DNA information. Therefore, molecular identification is the key in many cases. Therefore, this study aimed at the molecular classification of two species of tilapia, *O. niloticus* and *O. aureus* based on the nuclear loci gene (*sox3*). *Sox3* is widely studied in many species, including *Oreochromis* sp., and it can be easy to clone. We will use *scan4ab*, which is found in muscles for some fish, to detect the presence of this gene and discuss it from an evolutionary point of view.

Materials and Methods

Sampling and morphological measurements: A total of 60 specimens of *O. niloticus* and *O. aureus* (Fig. 1) were collected from Shat Al-Arab River in Basrah City, Iraq, with the help of local fishermen for morphological and molecular study. Twenty individuals of *O. niloticus* and *O. aureus* were used for morphological traits, including main key meristic characteristics as well as body coloration, using a magnifying dissecting microscope (ILE Company, China).

DNA extraction: DNA was extracted from the flesh tissues of *O. niloticus* and *O. aureus* using a DNA kit (Promega, USA). The two genes *Sox3* and *Scn4ab* were investigated to identify the studied species. The DNA extraction protocol provided by the kit company was followed.

PCR reaction: *Sox3* and *scn4ab* were amplified at the chosen domain for *Oreochromis* spp. 4 µL (60 ng) of DNA templet mixed with 25 µL (1X) master max (Promega/USA), 4 µL (10 µM·µL^{-1}) primers (Table 1), and 13 µL molecular free RNA water. The mixture was transferred to a PCR (Bioneer company, Germany) and settled at the following conditions: 94°C, 5 min initial denaturation [1X]; (94°C, 30 sec. denaturation; 56°C, 30 sec. annealing; 72°C, 1 min

Table 2. Meristic characteristics of *Oreochromis aureus* and *O. niloticus*.

Meristic characteristic	*Oreochromis aureus*	*Oreochromis niloticus*
Scale counts in lateral line	12-24	17-23
Scale counts above lat. line	4	4
Scale counts below lat. line	4	4
Spine counts in the dorsal fin	15-15	17-18
Ray counts in the dorsal fin	12	11
Spine counts in anal fin	3	3
Ray counts in anal fin	10	9
Spine counts in the pectoral fin	1	1
Ray counts in the pectoral fin	5	9
Gill rakers in the first arch	27	25

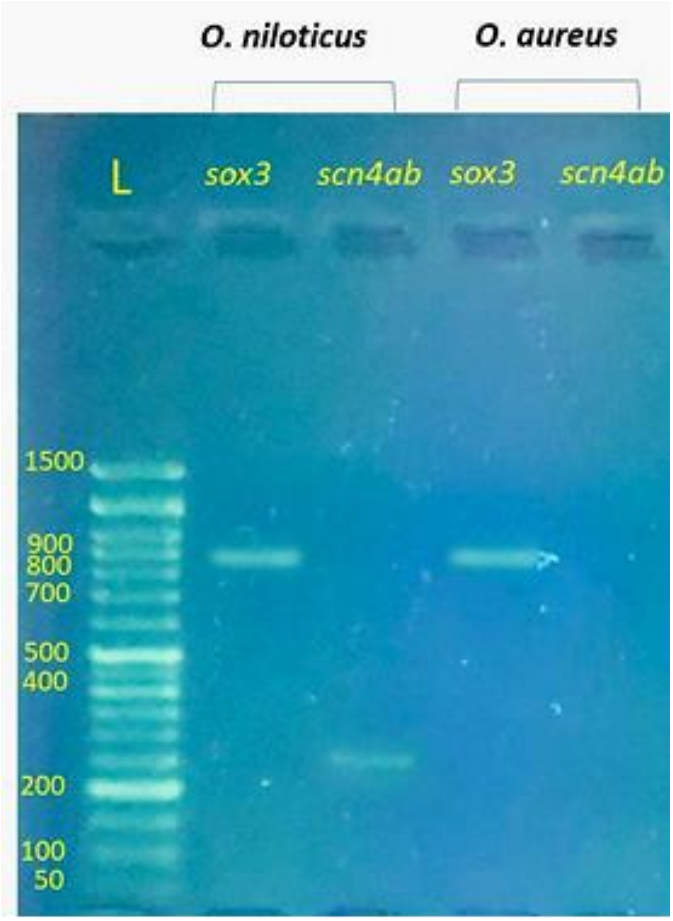

Figure 2. PCR product bands of *sox3* and *scn4ab* genes in *Oreochromis niloticus* and *O. aureus*. The PCR product of scn4ab is 261 bp and for sox3 is 897 bp, L: DNA ladder. The gel was 1.7% and the DNA dye is Red Safe (Intron, Korea). Condition of electrophoresis V: 90, Time: 45 minutes.

extension [35X]; 72°C, 5 min extension [1X]. PCR products were sequenced by Intron/Korea company. DNA bands for both genes were detected using electrophoresis.

Phylogenetic tree construction: The *Sox3* nuclear gene was used to draw a phylogenetic tree for the studied species to detect their relationship to each other. All the sequences of samples, including the detected species and the more similar identical species, were aligned using BioEdit software, and the phylogenetic tree was constructed using MegaX software.

Results

Meristic and morphological characteristics: The coloration of the body in *O. aureus* is marked by dark bluish to grey at the back and silvery at the abdomen. There are some dark spots alternate with bright spots on the dorsal and anal fins, caudal fin lined with dark lines and red edge. *Oreochromis niloticus* is distinguished by the vertical dark lines on the caudal and dorsal fins (Fig. 1). The spines in the dorsal fin are less in *O. aureus* is 15-16 vs. 17-18 in *O. niloticus*, gill rakers of the first arch in *O. aureus* is 27 and 25 in *O. niloticus* (Table 2).

***Sox3* and *scn4ab* genes:** The nuclear genes of *sox3* and *scn4ab* were used to identify the relationship between the two tilapia species. The results revealed the existence of the *scn4ab* gene in *O. niloticus*, whereas it is absent in *O. aureus* (Fig. 2). *Sox3* is a generally conserved gene, so it is used in a phylogenetic tree to detect the common lineage of *Oreochromis* spp.

Phylogenetic trees: The maximum likelihood phylogenetic tree and the neighbor-joining phylogenetic tree were used to exhibit the relationship between species. Both trees put *O. aureus* in a cluster, separating *O. niloticus* from *O. aureus* (Figs. 3, 4).

Discussions

Recently, methods based on genetic variation are the

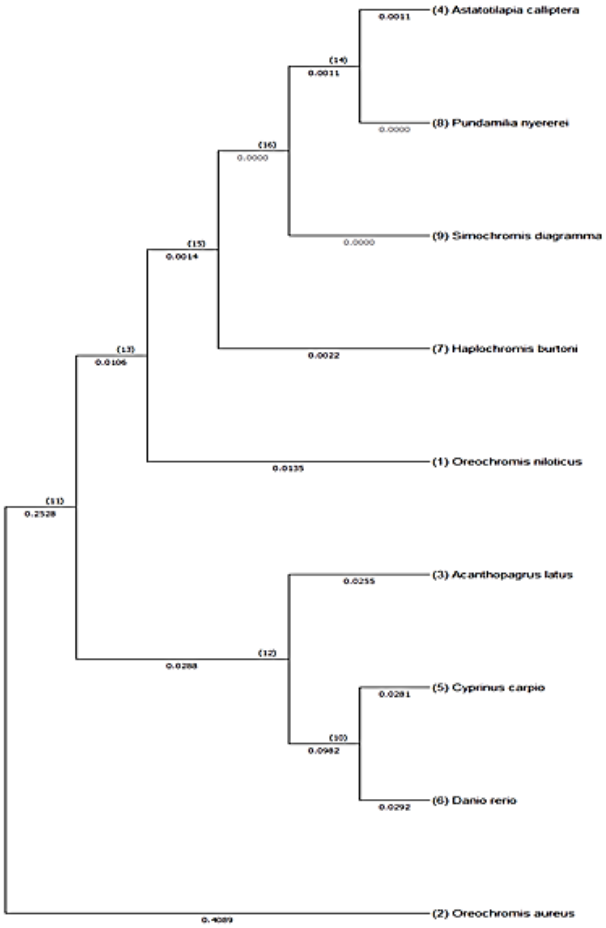

Figure 3. Maximum likelihood phylogenetic tree of *Oreochromis niloticus* and *O. aureus* with another species have similar morphological features based on *sox3* variants.

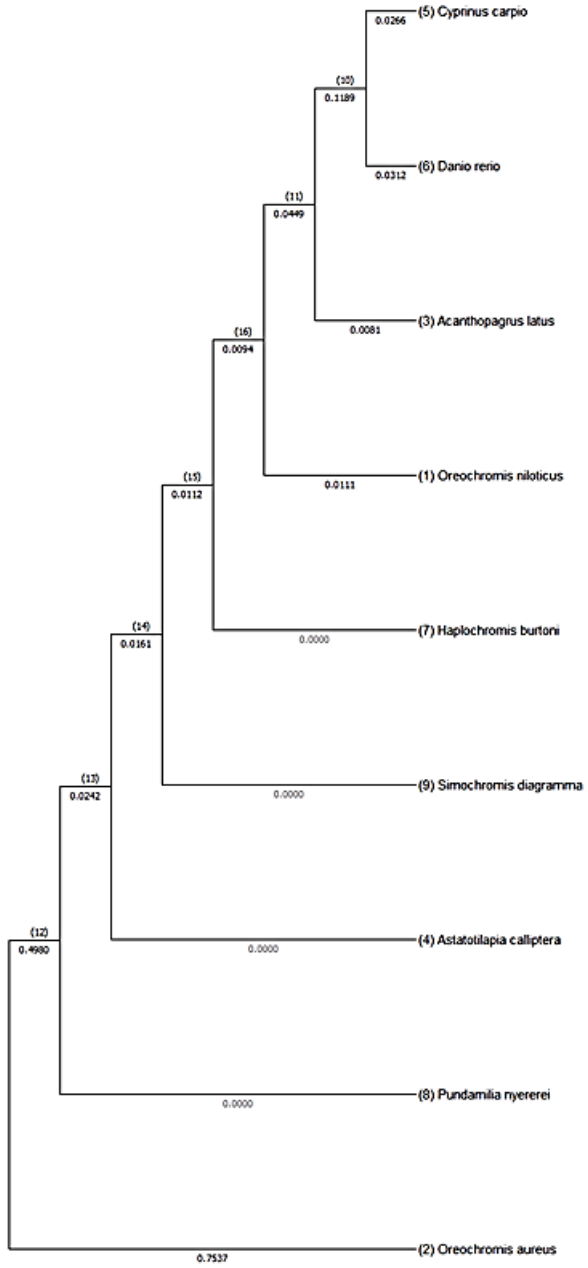

Figure 4. Neighbours joining the phylogenetic tree of *Oreochromis niloticus* and *O. aureus* with another species have similar morphological features based on *sox3* variants.

most widely used for taxonomy and finding relationships between species. Here, we used two genes to detect the relationship between *O. aureus* and *O. niloticus*. In general, *scn4ab* is a gene that is found in some species; although it is not conserved among species, but it is important for revealing the species' evolutionary history and origin. For example, *Danio rerio* has *scn4ab* in its genomes (Novak et al., 2006), whereas it is not found in medaka, *Oryzias latipes* (National Center for Biotechnology Information). *scn4ab* was not found in *O. niloticus* according to the provided data of its genetic map. However, the current study discovered this gene in its genome.

We have used two different phylogenetic trees. The first one is the maximum likelihood, which is the algorithm maximizing the probability of the data, and the second one is the neighbor-joining, a distance-based method, that minimizes the total branch length between pairs of neighbors (Challa and Neelapu, 2019). Generally, each phylogenetic algorithm provides an appointed impression of the affinity among species through their genetic analysis (Saud and Alshami, 2021). In both phylogenetic trees, *O. aureus* did not share the same clade with *O. niloticus*, suggesting genetic differences between the two studied species.

In addition to morphological study, there were differences in color and line pattern on the fins

between the two studied tilapia species; it is clear to distinguish the vertical dark lines on the caudal and dorsal fins in *O. niloticus* in addition to differences in the number of lateral line scales, spines, and rays counts in their fins. The morphological and morphometric characteristics of fish are reliable features, but they are affected by environmental parameters (Triantafyllidis et al., 2011; Zhang and Hanner, 2011). DNA information is crucial to identify the correct species (Singh et al., 2014). Therefore, the taxonomic status of many species was revised and transferred to new genera or taxonomic families that were different from previous classification characteristics. The transfer of some species to other genera or families remains an issue that is subject to discussion and deep research and requires more genetic studies. One of the problems that the researchers face is the lack of a whole genome reference for many species. Therefore, this leads to dependence on the close species. Consequently, we will rely on species close to each other taxonomically in which their reference genome is available.

Conclusions

The present work aimed to study the origin of two species of Tilapia, *O. niloticus* and *O. aureus*, using phylogenetic tree reconstruction based on the *sox3* gene. The results showed that they do not have a close common ancestry.

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