Int. J. Aquat. Biol. (2025) 13(1): 1-7
ISSN: 2322-5270; P-ISSN: 2383-0956
Journal homepage: www.ij-aquaticbiology.com

Original Article

16S rRNA revealed a low rate of maternal genetic variations in *Cyprinus carpio* Linnaeus, 1758 across the southern Caspian Sea

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Abstract: Genetic diversity surveys are informative and practical tools in aquaculture and restocking programs. With its maternal inheritance, mtDNA provides direct information about the available source of genetic variations in the female brood stock. The present study investigated the maternal genetic diversity of the Caspian common carp, Cyprinus carpio, and the farmed common carp across the southern Caspian Sea using direct sequencing of 571 bp fragment of 16S rRNA. A number of eight haplotypes were identified, with an average of Hd = 0.55. A low level of population differentiation was recognized with the overall Fst = 0.01, indicating an assumption of the shared ancestry in *C. carpio* in the southern Caspian Sea. Among different regions, Anzali population was observed to be a more unique stock of common carp across the southern Caspian Sea basin. Hence, considering Anzali as a separate population is highly recommended to enrich the genetic diversity and avoid the population structure breakdown in *C. carpio*. The obtained results during the present study can be useful in the ongoing restocking activities of *C. carpio* along the southern Caspian Sea. Furthermore, rehabilitation of the main rivers to provide the natural breeding of anadromous fish species such as *C. carpio* should be considered for their future conservation.

Article history:
Received 4 May 2024
Accepted 29 June 2024
Available online 25 February 2025

Keywords:
Genetic diversity
Haplotype diversity
mtDNA
Population
Restocking

Introduction

Environmental and anthropogenic factors complexly affect the genetic population structure of fish species (Bruford et al., 2003; Zenger et al., 2007). This is particularly important in fish species under aquaculture and restocking programs (Bohling et al., 2016). Consequently, hybridization events and outbreeding crosses have resulted in a poor genetic identity in many fish species. Hence, determining and maintaining genetic population structures is the basis for long-term survival in fish communities (Thai et al., 2006). Genetic diversity has been considered a critical index in species and ecosystem conservation programs. It has been well unraveled that genetic diversity is the center of maintenance in fish populations dynamic, either for reconstruction programs or in aquaculture productivity. The importance of genetic diversity stands from its impact on growth, disease resistance, and reproduction success, in which the higher the genetic diversity, the higher the genetic health (Robledo et al., 2024). Genetic diversity is directly related to the effective population size, which can be affected by numerous factors, including overfishing, water pollution, and loss of spawning and nursery grounds. In aquaculture, the loss of genetic diversity through generations is an inhibiting factor for higher production, mostly due to the smaller number of breeds in the practice of propagation (Lind et al., 2019; Ropp et al., 2023).

The common carp, *Cyprinus carpio*, is one of the most commercially important fish species with a long history of domestication and globe-wide distribution due to human activities (Tóth et al., 2022). Due to the high economic value of *C. carpio*, many crosses and introductions occur in different hatcheries worldwide, making it more difficult to trace the origin of each

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DOI: https://doi.org/10.22034/ijab.v13i1.2184

stock. Furthermore, unwanted crosses between wild and aquaculture forms of this species through the escape of domesticated forms into the rivers is one of the threatening factors that causes population admixture structure (Kohlmann et al., 2005; Mabuchi et al., 2008).

Cyprinus carpio is a native species to the Caspian Sea, playing an important role in fishing activities across the southern coasts (Moshayedi et al., 2016). The annual capture production of this species from the southern Caspian basin shows severe reduction and field observation demonstrates yearly length and weight reduction. The restocking program of the wild populations of *C. carpio* has been performed for three decades. However, no positive signs of recovery have been observed in the catch rate (Jafari et al., 2022a). In recent years, several studies have been conducted to decipher the population structure of common carp in the southern basin of the Caspian Sea, mostly with GBA methods such as SSR markers (Ghelichpour et al., 2013; Laloei et al., 2013). The recent study by Jafari et al. (2022) for assessing the efficiency of the restocking efforts on wild populations of common carp using 17,828 binary SNPs provided new insight into the population structure of this species, reporting a high level of admixture structure.

The mitochondrial DNA genome includes 37 genes that encode 13 proteins, 22 tRNAs, and 2 rRNAs. mtDNA has a maternal inheritance, and its mutation rate is 10 times greater compared to the genomic DNA (Imsiridou et al., 2009; Zhang et al., 2023). The maternal inheritance of mtDNA can be a great tool in providing deeper knowledge about the female founders of the wild populations of *C. carpio* in the southern basin of the Caspian Sea. Therefore, during the present study, the 16S rRNA gene of the mtDNA was sequenced for the first time to investigate the genetic diversity of *C. carpio* across the southern coasts of the Caspian Sea.

Materials and Methods

Sampling and DNA extraction: A total of 24 specimens of *C. carpio* from Gomishan (n = 7), Miankaleh (n = 6), Anzali (n = 7), and Anzali Wetland

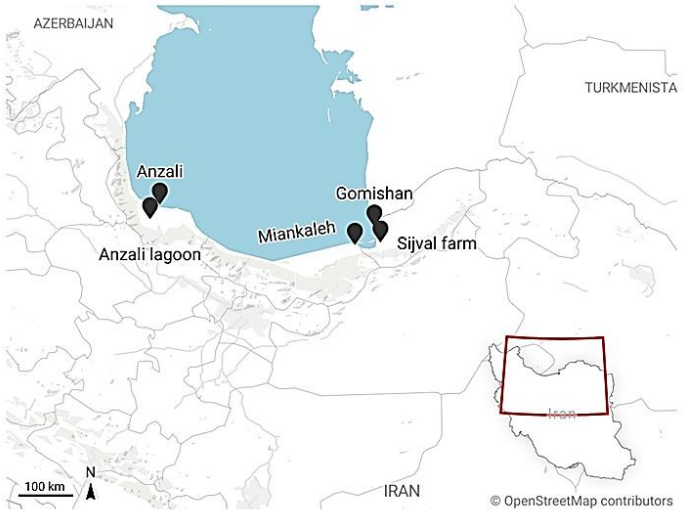

Figure 1. Sampling location of *Cyprinus carpio* across the southern Caspian Sea.

(n = 6) were caught through the commercial beach-seine fishing nets. Additionally, four *C. carpio* was sampled from the Sijuval farm in Golestan (n = 4) (Fig. 1). The caudal fin tissue of each specimen was clipped and saved into absolute ethanol for the later laboratory molecular work. DNeasy Blood and Tissue Kit (Qiagen®, Germany) was used for high-quality DNA extraction from fin tissue samples based on the protocol provided by the manufacturer. After the DNA extraction, the quality and quantity of the DNA samples were assessed through an Agarose 1% gel and Nanodrop (ND-1000) instrument.

Polymerase Chain Reaction (PCR) and Sanger sequencing: Amplification of the 571-bp fragment encoding 16S rRNA was done using universal primers, namely 16SAR (5-CGC CTG TTT ATC AAA AAC AT-3') and 16SBR (5-CCG GTC TGA ACT CAG ATC ACGT-3') (Palumbi, 1996). Each 25 µl of PCR reactions was included by 2 µl of template DNA (50 ng), 0.4 µl of Tag DNA polymerase (5 U/µl),1 µl of DNTPs (10 mµ, Fermentase®), 1 µl of $Mgcl_2$ (25 mµ, Fermentase®), 2.5 µl of PCR 10x buffer (Fermentase®), 1 µl of each primer (10 pM/lL) and double distilled water (Fermentase®). After performing PCR, the PCR products were visualized through 1.5% agarose gel. To purify the PCR products, fragments were purified using GeneJET Gel Extraction Kit (Thermo Scientific™, catalog number:

Table 1. Genetic statistics based on 16S rRNA sequences in common carp populations across the Caspian Sea.

Location	N	P	Pi	H	Hd
Anzali	7	0	-	1	-
Gomishan	7	2	0.001	3	0.52
Miankaleh	6	23	0.01	4	0.80
Farmed	4	2	0.005	3	0.83
Anzali wetland	6	8	0.005	4	0.80

N: number of fish individuals; P: Polymorphic sites; Pi: Nucleotide diversity, H: Number of haplotypes; Hd: Haplotype diversity.

Table 2. Estimates of evolutionary divergence over sequence pairs between populations of *Cyprinus carpio*.

Location	Anzali	Gomishan	Miankaleh	Farmed	Anzali wetland
Anzali	–				
Gomishan	0.0005	–			
Miankaleh	0.0074	0.0080	–		
Farmed	0.0008	0.0011	0.00083	–	
Anzali wetland	0.0026	0.0030	0.0097	0.0032	–

Table 3. AMOVA-based Genetic diversity investigation in populations of *Cyprinus carpio*.

Source of variation	df	ss	Component variance	% variations
Between populations	4	5.752	0.01953	1.46
Within populations	25	33.048	1.3219	98.54
Total	29	38.8	1.3414	100
F_{st}			0.01456	

K0691) based on its provided manual. The purified PCR products were double-checked using 1.5% agarose gel and consequently sent to Macrogen Inc., South Korea, for sequencing on the ABI 3730 automated DNA Sequencer.

Sequence data analysis: Quality control and trimming of the AB1 files was done using Chromas Ver 2.6.2 (Technelysium, Australia), and the generated fasta outputs were then fed into the alignment tool, Mega 11.0 (Tamura et al., 2011). Genetic diversity (in terms of haplotype and nucleotide diversity) and the number of haplotypes and polymorphic sites were calculated using DnaSP version 5.0 (Librado and Rozas, 2009). Nucleotide composition and genetic distances were analyzed using an uncorrected genetic distance of DNA evolution, including 1000 bootstraps in MEGA 11.0 (Tamura et al., 2011). An AMOVA was done using Arlequin version 3.1, with populations grouped into the clades identified in the phylogenetic analysis. Also, the fixation index (F_{st}) between populations was estimated to be 10000 permutations (Excoffier et al., 2005). In the end, an individual-based clustering through a heat map over the observed genetic distances was illustrated in R (R Core Team, 2013).

Results

In total, 571 bp from the 5' end of 16S rRNA was sequenced. The nucleotide composition of 16S rRNA included (A: 26.56%, T: 26.56%, C: 23.44%, and G: 23.44%). Among 571 sites, 27 positions were identified as segregating sites (4.72%), out of which 21 sites were singleton and 6 contained parsimony information. Across 30 sequences, eight different haplotypes with the overall mean values of Hd = 0.55 and Pi = 0.004 were identified. While the Miankaleh and Anzali wetland regions showed the highest number of haplotypes (H = 4), the Anzali population was recognized to have the least with one haplotype (Table 1).

Nei unbiased genetic distances between populations are shown in Table 2. The highest distances were observed in Miankaleh vs. Anzali wetland (0.19) and Anzali (0.15) populations. AMOVA dedicated most of the variance to the within-population variations (98.54%), while between populations accounted for 1.46% (Table 3). The overall value for the F_{st} was calculated as $F_{st} = 0.014$.

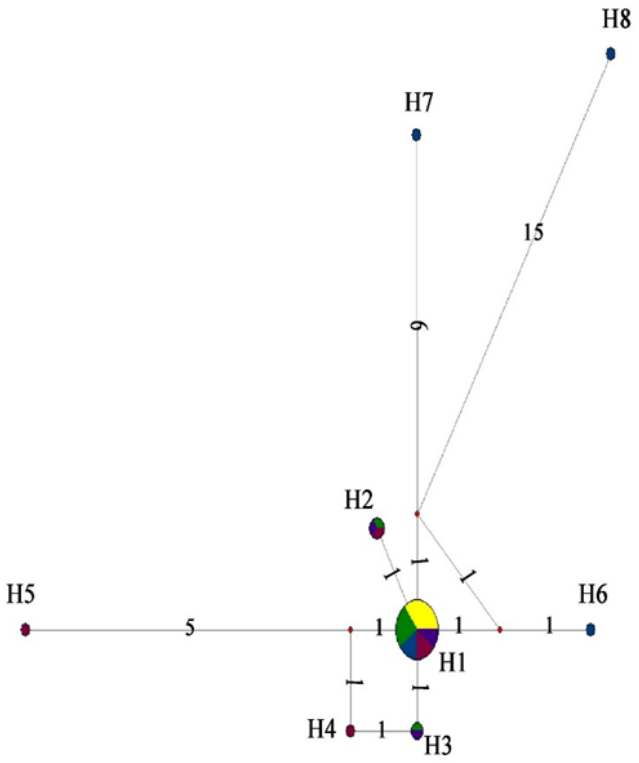

Figure 2. Phylogenetic relationship among different haplotypes of *Cyprinus carpio* (wild and farmed form) across the southern Caspian Sea (The numbers above the connecting branches reflect the number of mutational steps joining the haplotypes. Circles represent haplotypes and the size of each circle is proportional to the number of haplotypes).

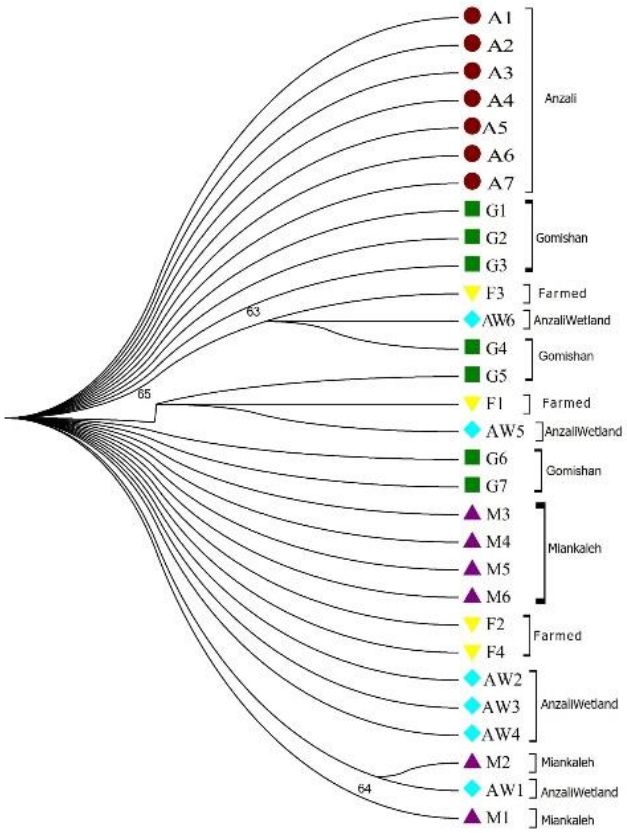

Figure 3. UPGMA tree of *Cyprinus carpio* across the southern Caspian Sea based on 16S rRNA sequence.

Totally eight haplotypes were identified in *C. carpio* among which the haplotype H1 was contained by 20 of the sequences. All Anzali population sequences were observed in the H1 haplotype (Fig. 2). The phylogenetic tree using maximum parsimony (Fig. 3) and UPGMA population clustering based on genetic distances (Fig. 4) classified all *C. carpio* individuals into two main clusters.

Discussions

During the present study, the genetic health of *C. carpio* in terms of genetic diversity was investigated across the southern Caspian Sea using a 571 bp fragment length of mtDNA 16S rRNA. Based on the results, a severe admixture pattern and a mediocre level of genetic diversity were deciphered. Furthermore, 16S rRNA variations of *C. carpio* in our study showed a degree of admixture between the Caspian and the European-farmed strain of common carp.

The overall mean value of haplotype diversity as an index of genetic diversity was 0.55, including eight haplotypes. Five of the haplotypes contained one individual, while the main haplotype (H1) contained 66 percent of the all-investigated specimens (20 fish individuals). *Cyprinus carpio*, with globe-wide distribution and commercial importance, is a native species to the Caspian Sea, showing a severe reduction in the southern basin. In our previously conducted study using genome-wide data, SNP markers identified three common carp populations; however, a considerable rate of crosses between geographically separate populations was observed (Jafari et al., 2022b). One of the most threatening factors for the wild populations is the mixing events between wild and aquaculture forms during the introduction of exotic strains into natural habitats due to domestication and translocation (Mabuchi et al., 2008). The present study observed a small degree of

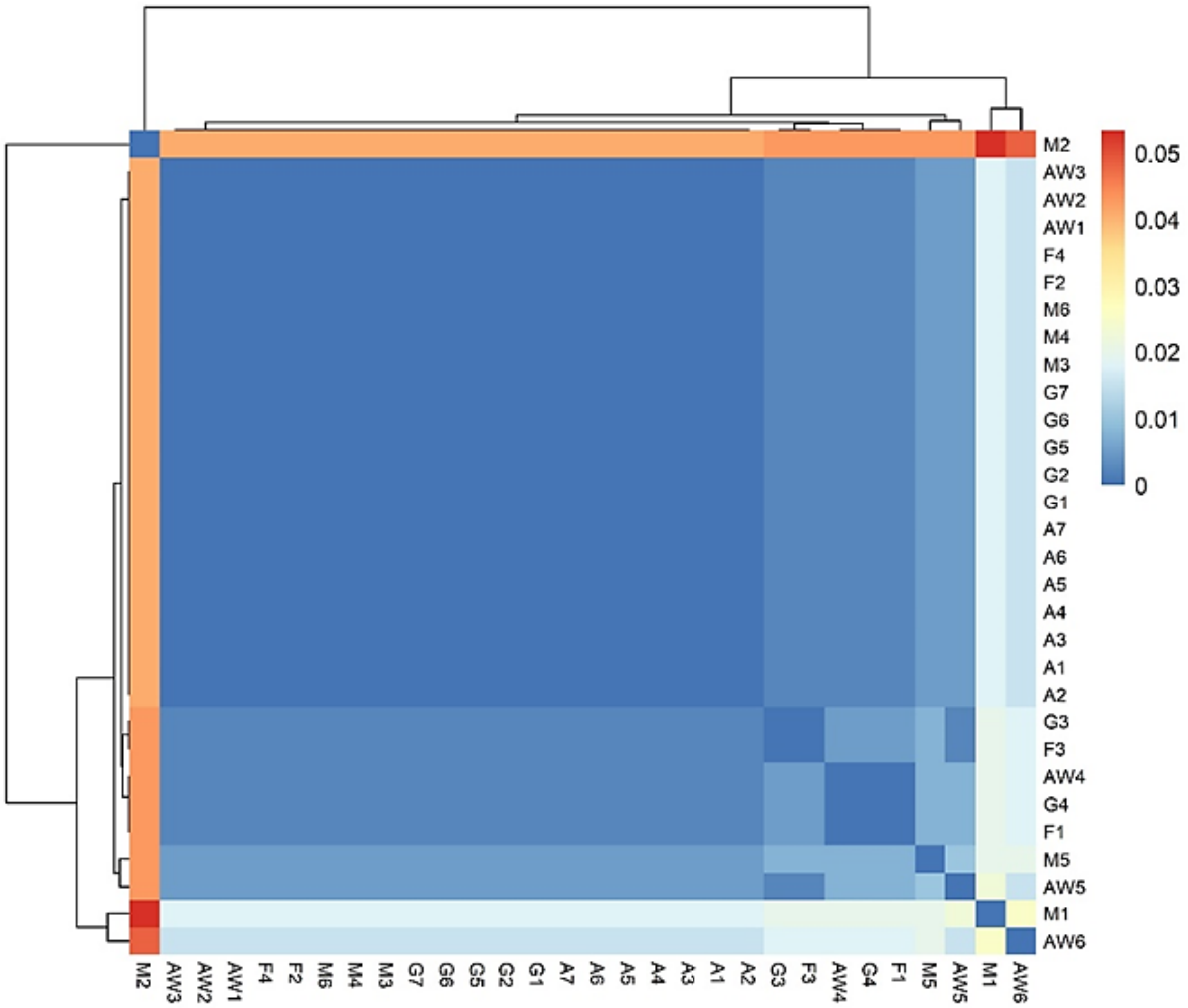

Figure 4. Heat map show the genetic clusters of *Cyprinus carpio* based on the pairwise genetic distances.

genetic divergence between Caspian and farmed forms of common carp. The observed level of admixture in the current study through mtDNA 16S rRNA supports the theory of mixing events through the escape of aquaculture forms into the rivers running to the Caspian Sea based on genome-wide SNP data. On the other hand, the presence of a captive form in the H1 haplotype can be due to the historical distribution of wild common carp from the Caspian Sea towards the Aral Sea, East Asia, and the Black Sea after the glacial stage (Balon, 1995, Chistiakov, and Voronova 2009).

In our study, the common carp sequences showed a degree of separation based on their geographic origin. Still, clear and affirmative clustering based on region is impossible using 16S rRNA data. These results align with the results from the study of mtDNA genes' sequences on Hungarian strains of common carp, stating these strains cannot clearly be classified based on their geographical location (Tóth et al., 2022). Maternal genetic diversity is an excellent tool in aquaculture and restocking programs, providing insightful vision about the effective population size of female breeds (Napora-Rutkowski et al., 2017; Shuli et al., 2023). The low genetic divergence and medium level of haplotype diversity in the Caspian Sea variety of *C. carpio* may indicate a small number of female breeds during restocking programs. While restocking is the main activity in rehabilitating this species throughout the southern Caspian Sea basin, if it is not based on genetic considerations, the negative consequences are unavoidable (Tanya and Kumar, 2010; Duong et al., 2019; Cossu et al., 2021). In a study conducted by Fallahbagheri et al. (2013), the

genetic diversity of common carp inhabiting the Anzali wetland was investigated through a 420 bp fragment of mtDNA control region and reported a low degree of haplotype diversity (Hd = 0.13). There are two forms of wild *C. carpio* in the southern Caspian basin; the first form belongs to the anadromous type, in which mature adults migrate to the rivers for breeding annually. The second refers to the permanent habitats of *C. carpio* in the Anzali Lagoon, which thoroughly complete their life cycle in the lagoon. However, as findings through SNP data suggested in our previous study (Jafari et al., 2022b), based on the UPGMA tree and cluster analysis, it seems some human-mediated introductions from the sea form have occurred in the Anzali wetland population. While Gomishan is the main restocking center for common carp in the southern Caspian Sea, the introductions may have originated from that population. After all, it is highly suggested that the Caspian Sea variety of common carp in the Anzali should be considered a unique stock during restocking activities, and it is crucial for maintaining genetic diversity in the common carp populations in the Caspian Sea.

Conclusion

To the best of our knowledge, this is the first study in the maternal genetic diversity of *C. carpio* using the 16S rRNA sequence of mtDNA in the southern Caspian Sea. However, combining genes such as COI, D-loop, and Cytb in a concatenated approach can also provide a privilege in population study and fisheries management of *C. carpio* in the Caspian Sea. Based on the current results and also bearing in mind the published results in other studies, it seems that avoiding mixing and enlarging the number of breeds during restocking are crucial to keep the *Cyprinus carpio* away from the loss of population structure in the southern Caspian Sea.

Ethics Statement

All procedures involving animals were followed according to the Ethics Committee of Agricultural Biotechology Reasearch Institute of Iran (NO. 014-05-05-006-94006).

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