Indian J. L. Sci. 8 (1): 01-05, 2018
ISSN: 2277-1743 (Print)
ISSN: 2278-7879 (Online)

Analysis of Genetic Variation in Mitochondrial DNA of *Cyprinus carpio* Using Restriction Enzymes

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ABSTRACT

The study involved isolating mitochondrial DNA (mtDNA) from common carp (*Cyprinus carpio*) collected from regions near Pondicherry and Tamil Nadu to assess genetic variation. Restriction digestion analysis revealed variability in mtDNA sequences among fish from different locations, indicating molecular-level genetic divergence. These differences are likely influenced by environmental and geographical factors. The detection of distinct mtDNA haplotypes suggests that the carp populations may originate from separate gene pools shaped by varying agro-climatic conditions. These findings highlight the genetic diversity within regional carp populations and may be useful in selecting robust and adaptable fish stocks for sustainable aquaculture practices.

KEYWORDS: Mitochondrial DNA, Genetic variation, Environment, Carp species, Restriction digestion

Genetic variation at the molecular level forms the foundation of modern population genetics and phylogeography, providing insights into how populations evolve, exchange genes, and maintain fitness. However, due to genetic drift and inbreeding, this variation is commonly assessed through metrics such as allelic diversity and heterozygosity and tends to decline in small or isolated populations (Ferguson & Taggart, 1995). Detecting and interpreting such genetic changes is therefore crucial for reconstructing evolutionary history and assessing a population's adaptive potential. Among molecular markers, mitochondrial DNA (mtDNA) has proven especially valuable. Its high mutation rate, maternal inheritance, lack of recombination, and conserved gene order make it particularly sensitive to demographic events such as bottlenecks, range shifts, and recent divergence (Avise et al., 1987; Moritz, 1994). Moreover, because mtDNA has a smaller effective population size than nuclear DNA, it can reveal fine-scale phylogenetic patterns that may be obscured in biparentally inherited markers. In fisheries genetics, mtDNA variation has significantly advanced stock identification, DNA fingerprinting, QTL mapping, and the monitoring of transgenic lines (Billington and Hebert, 1991; Carvalho and Hauser, 1994; Carvalho and Hauser, 1998).

Freshwater ecosystems present unique challenges to gene flow. The dendritic and fragmented nature of rivers, lakes, and wetlands creates natural barriers to dispersal, while anthropogenic influences such as dams, pollution, and habitat degradation further limit connectivity (Slatkin, 1987; Vrijenhoek, 1998). These constraints often promote allopatric divergence, leading to pronounced genetic structuring even over short geographic distances. Experimental studies support these expectations, for example, it was observed that greater genetic differentiation in freshwater and anadromous fish species compared to marine counterpart (Ward, 1995; Ward et al., 2005). Similar patterns have been documented in *Galaxias platei*, *Percichthys trucha*, and *Oncorhynchus mykiss* in fragmented river systems (Ruzzante et al., 2006; Pavlova et al., 2017). These findings align with the theoretical model of isolation by distance (Wright, 1943; Avise, 2000). Because mtDNA captures both historical isolation and recent gene flow, its inclusion in population genetic analyses offers critical insights for conservation. Mitochondrial lineages are frequently used to define evolutionarily significant units and management units, supporting strategies aimed at preserving genetic diversity and local adaptations (Billington and Hebert, 1991; Moritz, 1999; Palsbøll et al., 2007). Maintaining this diversity is essential: reduced variability can compromise long-term viability (Gilpin, 1991; Lande, 1995), whereas high genetic diversity enhances a population's capacity to respond to environmental change (Barrett and Kohn, 1991). Anthropogenic activities in freshwater ecosystems, such as industrial discharge and agricultural runoff, introduce heavy metals that pose serious environmental stress (Zhou et al., 2008; Cannino et al., 2009). The mtDNA highly sensitive to oxidative damage, undergoes mutations and copy number variations under such exposure. These alterations can impair cellular function and serve as effective biomarkers for assessing the impact of stress on freshwater biodiversity (Cannino et al., 2009; Srivastava et al., 2016).

In the present study, we assessed the genetic diversity of *Cyprinus carpio* (common carp) populations to understand their evolutionary relationships and population structure. Samples were collected from multiple freshwater habitats across different regions. Restriction enzyme-based analysis revealed clear

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patterns of genetic differentiation among populations, suggesting local adaptation to distinct environmental conditions.

MATERIALS AND METHODS

Sample collection

Live specimens of common carp (*C. carpio*) were collected from various locations, including Thookkanambakkam near Pondicherry, Kuriyamangalam, and Udhagai (Tamil Nadu), and transported to the laboratory under live conditions. The fish were maintained in water tanks until ovarian tissues were dissected and collected for analysis.

Mitochondrial DNA isolation

The mtDNA was isolated from developing or ripe ovaries. The ovaries were carefully vivisected, and the tissues were finely minced before being transferred into ten volumes of homogenizing buffer [0.25 M sucrose, 10 mM Tris-Cl (pH 7.6), 5 mM Na_2EDTA]. Homogenization was performed at low speed using a mechanized glass-Teflon homogenizer to gently disrupt the cells. To purify mitochondria, the homogenate was diluted with an equal volume of 15% sucrose in TEK buffer [50 mM Tris-Cl (pH 8), 10 mM Na_2EDTA, and 20 mM KCl] and centrifuged at 5000 rpm for 10 minutes at 4°C. The resulting supernatant was then centrifuged at 14,000 rpm for 30 minutes at 5°C to pellet the mitochondria.

The mitochondrial pellet was resuspended in resuspension buffer [50 mM glucose, 20 mM Tris-Cl (pH 8.0), and 10 mM Na_2EDTA] and incubated on ice for 10 minutes. To lyse the mitochondria, a freshly prepared lysis solution (0.2 M NaOH and 1% SDS) was added at twice the resuspension volume, followed by immediate incubation on ice for 5 minutes. Subsequently, 1.5 volumes of ice-cold neutralization solution (3 M potassium acetate in 2 M acetic acid) were added. The mixture was gently inverted to mix and incubated on ice for an additional 10 minutes. The lysate was centrifuged at 14000 rpm for 15 minutes at 4°C, and the supernatant was sequentially extracted once with phenol-chloroform and once with chloroform. Mitochondrial DNA was then precipitated by adding two volumes of ethanol and incubating the mixture overnight at −20°C. The resulting DNA pellet was washed with 70% ethanol and dissolved in TE buffer. To remove any residual RNA, the DNA solution was treated with DNase-free RNase. The purified mtDNA was stored at −20°C.

Restriction digestion of mtDNA samples

Restriction digestion of DNA samples was performed using standard protocols. For single enzyme digestion, the reaction mixture consisted of 1 µl of restriction enzyme, 2 µl of 10X buffer, bovine serum albumin (BSA) as required, 6 µl of DNA, and distilled water to make a final volume of 20 µl. The reaction was incubated at 37 °C for 1.5 hours. After incubation, the digested DNA was separated on a 1% agarose gel via electrophoresis, and the resulting fragments were visualized under a UV transilluminator.

Agarose Gel Electrophoresis

Agarose gel was prepared by boiling the desired amount of agarose in 1X TAE buffer, based on the required gel percentage. Once the solution cooled to approximately 60°C, ethidium bromide was added to a final concentration of 0.5 µg/ml. The solution was then poured into a casting tray fitted with a comb and allowed to solidify. After setting, the comb was carefully removed, and the gel was placed in an electrophoresis tank containing 1X TAE buffer. For standard DNA separation, a 0.6% agarose gel was prepared by dissolving 0.15 g of agarose in 24 ml of distilled water and adding 1 ml of 50X TAE buffer. The solution was boiled until fully dissolved, partially cooled, and then supplemented with 0.5 µg/mL ethidium bromide. DNA samples were mixed with 1X tracking dye and loaded into the wells of the gel. Electrophoresis was performed at a constant voltage of 7 V/cm using a gel tank (Biotech Laboratories) filled with 1X TAE buffer. Upon completion of the run, the gel was visualized under a UV transilluminator, and images were captured using a Bio-Rad gel documentation system. Band analysis was carried out with the Quantity One software.

RESULTS AND DISCUSSION

The mtDNA was extracted from fish ovary tissue using a standard alkali-lysis protocol (Figure 1).

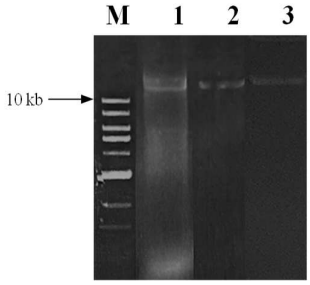

Figure 1: Isolation of mtDNA from fish samples collected from three locations: Udhagai (Well No. 1), Thookkanambakkam (Well No. 2), and Kuriyamangalam (Well No. 3).

To evaluate mtDNA variability, four restriction endonucleases, *Hae*III (blunt-end cutter), *Pst*I and *Bgl*I (3′-overhang cutters), and *Sal*I (5′-overhang cutter), were

tested. Single-enzyme digests using *Pst*I and *Sal*I each produced a single 16.5 kb fragment across all three species, indicating monomorphic patterns and rendering them uninformative for population-level screening (Figure 2). *Hae*III digestion yielded no detectable bands, likely due to complete digestion or fragment sizes falling below electrophoretic resolution.

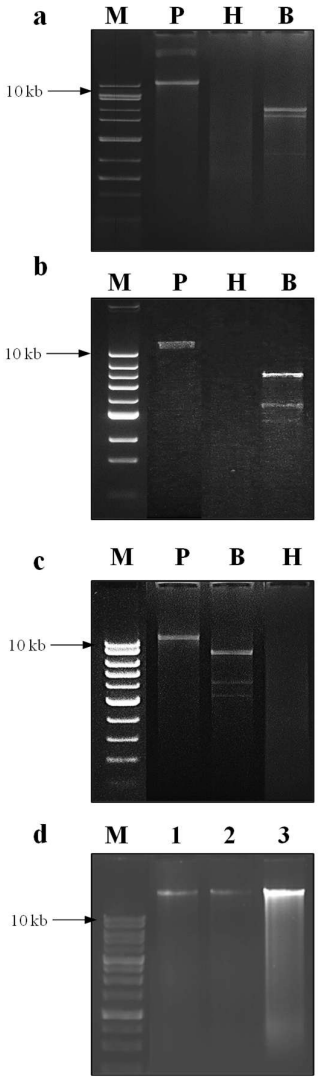

Figure 2: Restriction digestion of mtDNA samples using four restriction endonucleases: Digestion patterns using *Pst*I (P), *Hae*III (H), and *Bgl*I (B) from samples collected at (**a**) Udhagai, (**b**) Thookkanambakkam, and (**c**) Kurunchipady; (**d**) *Sal*I digestion profiles from all three locations, Udhagai, Thookkanambakkam, and Kuriyamangalam.

In contrast, *Bgl*I digestion produced clear, multi-band patterns that varied among species (Figure 2), highlighting its utility for detecting interspecific mtDNA variability. Further restriction analysis with *Bgl*I revealed distinct restriction site differences across populations, likely arising from sequence-level mutations that either created or abolished *Bgl*I recognition sites. This resulted in the identification of three distinct haplotypes, indicating substantial nucleotide divergence and potential population structuring. These findings align with a report that identified 27 haplotypes in *Brycon opalinus*, demonstrating significant intraspecific mtDNA variation (Hilsdorf et al., 2002).

As noted by Meyer (1994), restriction enzymes with six-base recognition sites are generally more effective for detecting evolutionary divergence among species, while four-base cutters are suited for resolving variation within populations (Meyer, 1994). Supporting this, four mtDNA fragments were identified in *C. carpio* using each of the enzymes HindIII and EcoRI, with distinct banding patterns observed among carp from different geographic regions, suggesting their origin from separate gene pools (Mohindra and Ponnaiah, 1998).

PCR-RFLP studies on *C. carpio* further illustrate the versatility of restriction enzymes beyond the traditionally used *Hae*III. Zhou et al. (2003) amplified a 2.4 kb ND5/6 fragment from three carp subspecies (*C. carpio*, *C. haematopterus*, and *C. rubrofuscus*) and showed that *Dde*I, *Taq*I, and *Mbo*I generated subspecies-specific RFLP patterns, which now serve as cost-effective markers for stock certification and selective breeding (Zhou et al., 2003). Additional studies have further demonstrated the utility of various restriction enzymes for mtDNA analysis across fish species. For instance, *Hae*III, known for its sensitivity to single-base substitutions (Knox and Verspoor, 1991), to differentiate wild from farmed Atlantic salmon (*Salmo salar*) in Norway, revealing aquaculture-specific banding patterns. *Pst*I has proven effective in detecting population-level variation, as demonstrated in *Thunnus albacares* (yellowfin tuna), where 34 haplotypes were identified across Pacific and Atlantic populations (Scoles and Graves, 1993). *Bgl*I has also proven effective, as it was used to identify 27 haplotypes in *Xiphias gladius* (swordfish), indicating a genetically diverse population (Grijalva-Chon et al., 1994), while in another application, *Bgl*I was used alongside other restriction enzymes to distinguish five *Astyanax* species, demonstrating its effectiveness in detecting interspecific variation (Moysés and Almeida-Toledo, 2002). Although *Sal*I is often monomorphic, it has been valuable in certain species for identifying length polymorphisms and heteroplasmic variants in the mitochondrial DNA control region. This was observed in

Alosa sapidissima, where *Sal*I digestion revealed informative variation, highlighting its potential for phylogeographic studies (Bentzen et al., 1988).

Overall, the effectiveness of a restriction enzyme is both species and region specific. Blunt-end cutters such as *Hae*III are typically ideal for detecting fine-scale polymorphisms; however, in the present study, it did not reveal any detectable variation. Although *Sal*I may appear uninformative in some taxa, it can be particularly valuable for identifying heteroplasmy and variation within the mitochondrial control region. Enzymes that produce overhangs, such as *Pst*I and *Bgl*I, are better suited for detecting larger sequence differences and structural rearrangements. These findings highlight the importance of employing a strategic combination of restriction enzymes to enhance the resolution and accuracy of mtDNA-based population genetic analyses in fish.

Conflicts of Interest: The authors declare no conflicts of interest.

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