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A REVIEW ON MOLECULAR MARKERS DEVELOPMENT FOR *CHITALA CHITALA*: APPLICATIONS IN GENETIC STUDIES AND CONSERVATION

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Abstract– Human actions have highlighted the necessity of creating molecular tools for the conservation and management of aquatic species. Three major risks to freshwater fish species are overfishing, habitat degradation, and water pollution. Among these, *Chitala chitala* (Hamilton, 1822) is one of the economically valuable species in the Notopteridae family. To investigate its genetic diversity, population structure, and evolutionary links, numerous molecular markers have been produced over the last 20 years. The review covers the types of molecular markers, their uses in population genetics, phylogenetics, conservation genomics, and fisheries management, specifically in relation to *Chitala chitala* investigations. The importance of these indicators' ability to help *C. chitala* survive environmental obstacles over the long run is emphasized.

INTRODUCTION

Chitala chitala (Hamilton, 1822), the clown knifefish, is a member of the primitive order Osteoglossiformes, which is part of the Notopteridae family. *Chitala chitala* is sometimes referred to as a humped featherback because of its large anal fin, which is confluent with the caudal fin and provides the impression of feathers. *Chitala chitala* is typically found in rivers, but it can also adapt to stagnant water, thanks to modifications in its swim bladder. These adaptations allow it to survive in different types of aquatic environments. The species is recorded from Pakistan, India (Manipur, Uttaranchal, West Bengal, Assam, Tripura, Uttar Pradesh, and Bihar), Bangladesh, Nepal, and Myanmar; Indonesia, Cambodia, Malaysia, and Thailand. In India, it inhabits the Mahanadi and Ganga-Brahmaputra river basins as well as swamps and is largely popular for its flesh quality and ornamental value (Chandran *et al.*, 2020; Dutta *et al.*, 2020). Because of their rarity and delicate nature, *Chitala chitala* fish are expensive to buy for food, aquariums, and sports (Gopalan *et al.*, 2004; Sarkar *et al*., 2008). The population of *Chitala chitala* has sadly decreased a lot in Pakistan, Bangladesh, India, and Nepal due to pollution and overfishing, leading to its listing as "Near Threatened" by the International Union for Conservation of Nature (Swain *et al.*, 2001), though it was earlier categorized as endangered (Ayyappan *et al.*, 2001; Sarkar *et al.*, 2007). It has even been declared the state fish of Uttar Pradesh (Sarkar *et.al.*, 2008).

The main causes behind the decline of *Chitala chitala* are habitat degradation and fragmentation, exotic species introduction, water diversions, pollution, global climate change impacts, and overexploitation (Gibbs, 2000). The illegal fishing is also a major threat to the decline of this population, as local fishermen make the overcatch and sell it in the markets and transport it to various parts of the country. Therefore, it is needed to identify the illegal trading/selling/transportation by identifying the animals, fleshes, and their residues so that the necessary action may be taken. Populations must have genetic variety in order to be resilient to pressures imposed by humans and environmental changes. Nowadays, various methods have come into the scientific line for the identification of the animals using different molecular markers. Assisting in the development of conservation strategies, molecular markers provide important insights into the genetic diversity, structure, and evolutionary relationships of species.

OVERVIEW OF MOLECULAR MARKERS

The field of molecular genetics began in the early 1950s with the invention of the double helix, which is now the accepted model of DNA structure, by F. Crick, J. Watson, and M. Wilkins (Okumuº and Çiftci, 2003). Their discovery won the Nobel Prize (Hallerman *et al.*, 2003). Since then, information about the composition and purpose of genes and DNA has become clearer and is being used to estimate genetic diversity. Different kinds of DNA markers were established in the 1980s as a result of DNA amplification and automated sequencing, though methods like DNA cloning, sequencing, and hybridization were developed in the 1970s. These markers provide a framework for understanding the genetic makeup and evolutionary history of species. Molecular markers, or marker genes, are sequences of DNA that are used to "mark" or track a specific locus on a certain chromosome. The study of the inheritance of a characteristic or gene is made easier by the presence of a gene with a known location, a unique phenotypic expression, and detection by analytical techniques or a recognizable DNA sequence. Although there are many molecular techniques available today to analyse fish populations, they can be broadly divided into two categories: protein and DNA markers. Allozymes, mitochondrial DNA, and nuclear DNA are the three main categories of genetic markers that are frequently employed in population genetic and phylogenetic research. A lot of recent reviews have been written about them (e.g., Avise, 1994; O'Reilly and Wright, 1995; O'Connell and Wright, 1997; Parker *et al.*, 1998; Sunnucks, 2000; Hallerman, 2003).

The following are the molecular markers that are most frequently used

Allozymes: Protein markers that identify genetic variation at the level of individual proteins. This approach was developed in the 1960s and was widely used up until the early 1990s. The classic molecular technique of allozyme electrophoresis is used to investigate genetic variation at co-dominant Mendelian hereditary loci. Allozymes are codominant Mendelian traits (both alleles are independently expressed in a heterozygous individual) that are inherited in a predictable way from parent to child (May, 2003).

- Mitochondrial DNA (mtDNA): A maternally inherited marker useful in studying phylogeny and population structure. The first population genetic studies based on mitochondrial DNA analysis appeared in the early 1980s (Avise *et al.*, 1979). The major characteristics of mtDNA are as follows: a) It is typically a single haploid molecule that is inherited maternally; b) the entire genome is transcribed as a unit; c) it is not subject to recombination and gives homologous markers; d) it is primarily selectively neutral and occurs in multiple copies in each cell; e) replication is continuous, unidirectional, and symmetrical without any apparent editing or repair mechanism; f) it is optimally sized, lacking introns (Billington, 2003).
- Microsatellites (Simple Sequence Repeats or SSRs): Short, repeating sequences of DNA that are highly polymorphic and used for fine-scale population genetics. Simple sequence repeats (SSRs) with a tandem arrangement and a base pair length ranging from one to six make up microsatellites (Tautz, 1989; Litt and Luty, 1989).
- Random Amplified Polymorphic DNA (RAPD) Markers: PCR-RAPD (Random Amplified Polymorphic DNA) involves the amplification of random segments of genomic DNA through the polymerase chain reaction (PCR) utilizing a single brief primer of arbitrary sequence. This methodology allows for a more random examination of the genome compared to traditional techniques (Teletchea, 2009).
- Single Nucleotide Polymorphisms (SNPs): Single base pair variations in DNA that provide high-resolution genetic data. The term single nucleotide polymorphism (SNP) refers to polymorphisms resulting from point mutations that produce distinct alleles with differing bases at a specific nucleotide position within a locus (Chouhan and Rajiv, 2010).

This review will delve into the specific molecular markers developed for *C. chitala* and their applications in genetic and conservation research.

Molecular Markers in *Chitala chitala*

Fishing and aquaculture both make considerable use of molecular markers. A marker's type selection is determined by the purpose for which it was created. Combinations of markers usually yield the best results; no one molecular marker is better than any other. These days, fish genetics is using more and more markers that were created and examined using next-generation sequencing technology. Studies on genetic stock identification are increasingly using microsatellites and SNP markers.

Allozymes

Allelic variations of enzymes, or allozymes, were among the earliest molecular markers employed in the investigation of *C. chitala* genetic diversity. Based on differences in genes that code for enzymes, these protein markers can be found using electrophoretic methods. Using allozymes, early research aimed to determine genetic variation both within and across *C. chitala* populations throughout several river basins. Allozymes limited fine-scale assessments of genetic diversity, but they were helpful in identifying historical gene flow and population bottlenecks. Protein electrophoresis techniques were used to produce allozyme markers in *C. chitala* in order to distinguish and identify isozyme variants from different organs, such as the liver and muscle. *Chitala chitala* enzyme systems were analyzed using vertical polyacrylamide gel electrophoresis, with most resolved on 10x8 cm gels. *Notopterus notopterus* was used to test electrophoresis conditions. Allozyme profiles from the Satluj and Brahmaputra rivers were optimized, with liver being optimal. Tris-borate EDTA provided superior resolution, and optimal extract volume and running time were established for each enzyme system (Mandal *et al.*, 2009). Lal *et al.* (2006) investigated allozymes and RAPD profiles, identifying markers that distinguish between *Chitala chitala* and *Notopterus notopterus.*

Challenges: Allozyme analysis's shortcomings include low levels of polymorphism and the inability to identify silent mutations, or changes that do not alter protein function, even if it has produced useful data on genetic diversity and population differentiation (Kemp, 1991; Glenn *et al.*, 1998; Zhang *et al.*, 2002). In contemporary conservation genetics, allozymes are not as useful as DNA-based markers due to their low mutation rates and inability to accurately capture neutral genetic diversity. Thus, in the most recent investigations of chitala, allozymes have been essentially supplanted by more informative DNA-based molecular markers.

Mitochondrial DNA (mtDNA)

The use of mitochondrial DNA (mtDNA) analysis has grown in recent phylogenetic and population surveys of organisms. Research on vertebrate species has typically demonstrated that mitochondrial DNA experiences a faster rate of sequence divergence accumulation than nuclear DNA (Brown, 1985). Because of its maternal inheritance, lack of recombination, and comparatively fast mutation rate as compared to nuclear DNA, mitochondrial DNA has been a fundamental tool in the study of fish phylogenetics and population genetics (Avise, 1995). Mitochondrial DNA markers, particularly the cytochrome b (cyt b) and cytochrome oxidase subunit I (COI) genes, have been widely used in population genetic and phylogenetic studies of *C. chitala*. Dutta *et al.* (2020) studied the genetic diversity and population structure of the Indian featherback fish, *Chitala chitala* (Hamilton, 1822), by analyzing two complete mitochondrial genes, ATPase 6/8 and cytochrome b. Mandal *et al.* (2012) studied genetic variation in the mitochondrial cytochrome b (cyt b) and D-loop region. Anjansari *et al.* (2021) analyzed and determined the mtDNA sequence of the Cytochrome C Oxidase Subunit I (COI) gene and created phylogenetic trees linking the several species of featherbacks in South Sumatra. Ruzman *et al.* (2024) studied morphology and two mitochondrial markers: cytochrome c oxidase subunit I (COI) and cytochrome b genes to determine and identify *Chitala* species present in Peninsular Malaysia. MtDNA analysis can efficiently focus resource-intensive ecological investigations by providing qualitative signs of population shifts through comparative studies. Thus, there are some quite straightforward applications of mtDNA that may significantly help in the short- and long-term planning of species recovery programs, especially in conjunction with nuclear variation assays (Moritz, 1994).

- **Challenges**: There are also some significant issues with the application of mtDNA in animals, especially fish. The primary drawbacks include the tight demand for fresh or frozen tissue, the requirement for a larger sample size than with most DNA approaches (Park and Moran, 1995), the requirement to sacrifice the animals, and the low level of polymorphism in some populations and species (Ferguson *et al.*, 1995). There are significant drawbacks to using mitochondrial DNA (mtDNA) as a molecular marker. First of all, it solely depicts one evolutionary locus and the maternal lineage (Skibinski, 1994; Magoulas, 1998), which could lead to biased conclusions as it may not fairly reflect the history of the species or the total population. Second, there is a higher rate of allele extinction and faster lineage sorting due to the smaller effective population size of mtDNA compared to nuclear autosomal sequences. Therefore, in order to get more trustworthy evolutionary insights, independent genomic markers must be used in addition to mtDNA (Zhang and Hewitt, 2003; Okumuº & Çiftci, 2003).

Microsatellites (Simple Sequence Repeats or SSRs)

Microsatellites are short, repeating DNA sequences found throughout eukaryotic genomes, commonly used as molecular markers in biology. These sequences, consisting of 1-6 nucleotide repeats, are highly variable, easy to analyze, and accurate, making them ideal for high-resolution population studies. They have significantly contributed to advancements in genetics, molecular biology, biotechnology, and genetic engineering in various organisms (Tripathy, 2018). In *C. chitala*, microsatellites have been a valuable tool for assessing genetic diversity and population differentiation. Mandal *et al.,* 2009, used data from six riverine locations in India-the Satluj, Ganga (Ghagra, Bhagirathi, and Brahmaputra), Mahanadi, and Narmada river systems-to report two microsatellite loci for the first time to determine intraspecific divergence in the natural population of the endangered Indian featherback fish, *Chitala chitala*. Dutta *et al. (2019)* developed a microsatellite marker set for genetic diversity assessment of primitive *Chitala chitala* (Hamilton, 1822) derived through single-molecule real-time sequencing technology for application in population genetics. Punia *et al.,* 2006, identified microsatellite loci that were found to be promising for population genetics studies of *Chitala chitala* and the related species *Notopterus notopterus* (family Notopteridae). Microsatellites have shown that populations of *C. chitala* exhibit moderate to high levels of genetic variation. Research shows that populations with less genetic diversity are found in places impacted by pollution, habitat fragmentation, or overfishing, whereas populations in undisturbed ecosystems have more genetic diversity. The likelihood of genetic bottlenecks is increased by this loss of diversity, underscoring the necessity of focused conservation efforts to maintain genetic health and species survival.

· **Challenges:** A key challenge in using microsatellites for fish stock identification is the uncertainty surrounding the correct mutation model, which complicates the selection of an appropriate measure of genetic differentiation. Furthermore, the high mutation rate of microsatellites can result in homoplasy, where unrelated individuals possess the same allele due to convergent evolution, making it more challenging to interpret genetic relationships accurately (Chouhan *et al.,* 2014).

RAPD (Random Amplified Polymorphic DNA)

Random Amplification of Polymorphic DNA is a kind of PCR process in which the amplified DNA segments are chosen at random. Although the primers will bind somewhere in the sequence, it is unclear exactly where, therefore no knowledge of the targeted gene's DNA sequence is necessary. RAPD markers are decamer (10 nucleotide length) DNA fragments that are obtained by PCR amplification of random genomic DNA segments using a single primer of arbitrary nucleotide sequence (Kumar and Gurusubramanian, 2011). Using RAPD markers, Kumar *et al.* (2009) evaluated the population genetics of C. chitala throughout several river basins. Significant genetic differentiation was discovered by the study, and possible conservation units were detected.

Challenges: RAPD has limitations, including poor reproducibility due to sensitivity to experimental conditions, dominance that prevents the differentiation of heterozygous and homozygous loci, and a lack of sequence specificity

Applications of Molecular Markers in Conservation and Management

The rising applications of molecular markers are represented by the frequency of related literature being submitted to different journals. Fisheries encompass a wide range of areas where these markers might be useful tools: species identification; hatchery management; stock improvement; population size and structure; intraspecific relationships; selective breeding; systematic and evolutionary genetics; legal applications; and many more (Maqsood and Ahmad, 2017) (Fig. 2).

Phylogenetic and Evolutionary Studies: Accurate information on evolutionary relationships and resolution of taxonomic uncertainties are provided by the interspecific genetic divergence shown by

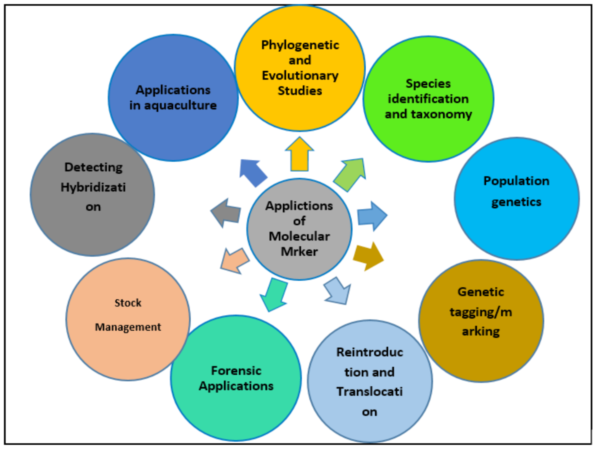

Fig. 1. Application of Molecular Markers

species-specific diagnostic molecular markers. The early life history stage of visually similar species can be distinguished using these markers, both in hatcheries and in natural populations. They can also be used to detect hybrid and introgressed or backcrossed individuals (Chouhan and Rajiv, 2010). Comparative studies involving chitala and related species have helped clarify the species' evolutionary history and biogeographical distribution patterns.

Population Genetics: Molecular marker technology has been widely used to assess genetic diversity and population genetic structure, which has implications for the aquaculture sector of sea cucumbers and the preservation of germplasm resources. For genetic diversity analysis, mitochondrial DNA markers are utilized to measure haplotype diversity (h) and nucleotide diversity (ð), and for population analysis, FST (fixation index) and AMOVA (analysis of molecular variance) are employed.

Species identification and taxonomy: DNA barcoding, which uses short, standardized gene regions (such as the mitochondrial COI gene), has revolutionized species identification, especially for cryptic species and those difficult to distinguish morphologically (Ward *et al.*, 2005). The basic function of DNA-based genetic markers is to identify point mutations, insertions, deletions, or inversions in allelic DNA fragments that distinguish different individuals within the same species (Gebhardt, 2007).

Reintroduction and Translocation Programs: Molecular markers are used to evaluate the genetic suitability of individuals for reintroduction or translocation, ensuring that populations are genetically diverse and can adapt to new environments (Price, 1989). When it comes to studying the genetic variation of closely related species and determining the structure of fish stocks, microsatellites are incredibly potent genetic markers. For the purpose of developing effective conservation strategies for the management of fisheries and aquaculture, microsatellite marker analysis gives crucial information. This can be incorporated into a package for the preservation of genetic variety and the restoration of fish species' native populations, along with other technologies like cry preserved sperm and captive breeding (Abdul-Muneer, 2014).

Genetic tagging/marking: There are times when we must mark individual fish for a variety of reasons, such as monitoring migration or movement, calculating population size, or assessing the contributions of individual stocks to mixed fisheries. There is no way to use physical tags for generations because they are not heritable. By identifying a rare allele in an individual or populations and tracking them over several generations, genetic marking can reveal information about the number of stocked individuals that contribute to the growth of the targeted population, the impact of hatchery programs on harvest, and the identification of migrants from source populations (Sukumaran and Gopalakrishnan, 2019).

Forensic Applications in Wildlife Conservation: Molecular markers are used in forensic science to combat wildlife crime, including poaching and illegal trade of endangered species. Population biology and species conservation have entered a new phase thanks to recent developments in molecular technology (Haig *et al.,* 1998). Molecular markers may be utilized for identification and certification in samples such as dead or stranded fish, preserved or canned fish meat, and fish fillets where morphological identification is not feasible. Scientific approaches are employed by forensics to make conclusions about historical occurrences and their usage in the certification of fisheries goods, and the identification of illicit fish and fisheries product trading is growing. Utilizing molecular techniques, it is also possible to monitor intentional or unintentional releases into natural waters.

Stock Management and Breeding Programs: Molecular markers are vital for identifying genetically unique stocks and guaranteeing genetic variety in breeding programs, which are critical for sustainable fisheries and aquaculture. In captive breeding, molecular methods are used to monitor genetic variety and minimize inbreeding, which can lead to lowered growth rates, lower reproductive success, and higher susceptibility to illnesses. In hatcheries, genetic markers aid in the selection of different broodstock to maximize genetic variability in offspring. This is particularly crucial for species like chitala, where excessive breeding by a select few breeders can result in inbreeding and the loss of critical genetic features.

Detecting Hybridization and Inbreeding: The genetic integrity of endangered species may be threatened by interspecies hybridization, which is detected using molecular markers (Vilà *et al*. 2003). In tiny, isolated populations, molecular markers aid in the monitoring of genetic drift and inbreeding. This is especially important for threatened species, as lower genetic variety can cause depression from inbreeding. In small populations, SNP and microsatellite markers are very useful for determining inbreeding coefficients and evaluating genetic drift. Early detection of inbreeding enables the implementation of management strategies, such as the introduction of genetically diverse individuals into the population.

Applications in aquaculture: Primarily used in aquaculture, molecular markers can be used for a wide range of tasks such as genetic identification and discrimination of hatchery stocks, detection of inbreeding events, assigning progeny to parents via genetic tags, identification of quantitative trait loci, marker-assisted selection for selective breeding trials, and evaluation of the impact of gynogenesis and polyploidy induction. Molecular markers also enable the assessment of genetic variability both within and between stocks. In figuring out the potential parents' involvement in mass spawning occurrences, they are also helpful. Moreover, it is feasible to map entire genomes and identify quantitative trait loci (QTLs), or the sites of quantitative traits of significant commercial value.

Challenges and Future Directions

Limited Genetic Resources: A major obstacle in the genetic research of *C. chitala* is the scarcity of genomic resources and species-specific molecular markers. Although microsatellites and SNPs have advanced, more thorough genetic research would be possible with the addition of genomic resources like transcriptome data and whole-genome sequencing.
Integrating Genomic and Environmental Data: To gain a better understanding of how *C. chitala* populations react to environmental stressors, future research should combine genomic data with ecological and environmental parameters. A more comprehensive understanding of the species' capacity for adaptation in shifting environments would be possible with such an approach.
Conservation Applications: For *C. chitala*, the use of increasingly sophisticated molecular tools in conservation genetics will be essential. Further research connecting molecular marker data with field conservation initiatives is required. Restocking initiatives, habitat restoration, and protected area designations based on genetic data may all be part of this.

CONCLUSION

Molecular markers have proven pivotal to understanding the inheritable diversity, population structure, and evolutionary history of *Chitala chitala*. While mitochondrial DNA markers are still helpful for literal and phylogenetic population studies, microsatellites are particularly helpful for sensitive inheritable study. Despite the progress, further has to be done to make genomic resources and integrate inheritable information into conservation operation plans if this important species is to endure over the long term. Molecular markers will be a pivotal tool for the success of *C. chitala* conservation efforts and for populations to endure ongoing environmental and human pressures.

Conflict of Interest- None

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