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Advancements in Molecular Markers: From RFLP to Modern PCR and DNA Barcoding Techniques

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ABSTRACT: Molecular markers are important tools in research, offering a means to identify and measure specific genetic loci within populations. DNA barcoding is a valuable tool for non-expert taxonomists, enhancing the efficiency and accuracy of species identification. These markers, associated with particular genes or traits, facilitate the study of genetic relationships, variety identification, phylogenetic analysis, and gene mapping, including quantitative trait loci (QTLs). They are broadly categorized into two types: hybridization-based and PCR-based markers. Restriction Fragment Length Polymorphism (RFLP) is one of the earliest hybridization-based techniques, detecting DNA sequence variations at restriction enzyme sites. The method involves electrophoresis of DNA fragments, which are then transferred to a membrane and probed with labeled DNA to visualize genetic differences. To enhance specificity and sensitivity, nested PCR can be employed, which involves two rounds of PCR amplification with external and nested primers. PCR-RFLP, an extension of this method, is used for detecting genetic variations such as microindels, single-nucleotide polymorphisms (SNPs), and multi-nucleotide polymorphisms (MNPs). These techniques have been crucial in advancing molecular plant breeding, genomic selection, and genome editing, thereby enriching our understanding of genetic diversity in crops and other organisms.

Keywords: RFLP, PCR, AFLP, Barcoding, Aquaculture.

INTRODUCTION

India's fisheries sector is a cornerstone of its economy, providing employment for millions and contributing significantly to foreign exchange earnings. It is a vital livelihood source of income for many individuals. The sector encompasses both capture fisheries and aquaculture, supporting approximately 07 million people nationwide. India stands as a global leader in fisheries, ranking second in inland fish production and fourth overall. The nation's economic well-being is intricately tied to the health of its fishing industry. With a coastline stretching 8,129 kilometers and marine resources covering 0.5 million square kilometers of continental shelf and 2.02 million square kilometers of exclusive economic zone (EEZ), India has immense fishing potential. Historically, fish captures have increased significantly, driven by key species such as oil sardine, mackerel, Bombay duck, and shrimp. Nonetheless, marine fisheries are now predominantly limited to coastal zones, with production from open waters declining in recent years. Despite reaching a total of 2.99 million tonnes, there remains an untapped potential of around one million tonnes in deeper oceanic regions.

The fishing industry is a crucial income source for approximately two million people in India. Marine fish production grew rapidly during the 1950s and 1970s, but this trend shifted in the 1980s and 1990s, with freshwater fisheries outpacing marine fisheries in both output and growth rate. By the 1990s, marine fish production had plateaued. To sustain marine fish production, it is essential to implement scientifically based management strategies and closely monitor fishing activities. To optimize marine resources and boost production, several strategies have been proposed. These include adopting regulated and diverse fishing practices, targeting underutilized and unconventional resources within the EEZ, identifying potential fishing zones, enhancing fish stocks through sea ranching, deploying fish aggregating devices and artificial reefs, and embracing community-based resource management. Additionally, responsible fishing practices such as closed seasons and mesh size regulations, along with evaluating sustainable exploitation around islands, are crucial. Support for infrastructure development such as deep-sea vessels, onboard facilities, and onshore infrastructure is also necessary. Moreover, there is increasing interest in exploring the marine biodiversity for economic benefits. This includes harvesting genetic material from various microbes, algae, and invertebrates for transgenic applications and extracting bioactive compounds like antibiotics, anesthetics, and anticarcinogens (Mans, 2016).

DNA Barcoding. DNA barcoding is a valuable tool for non-expert taxonomists, enhancing the efficiency and accuracy of species identification in field studies. This

method employs a specific gene as a universal identifier, effectively creating a genetic "barcode" linked to a database of sequence data associated with voucher specimens. The gene typically used for this purpose is a 650-base pair fragment of the cytochrome coxidase I (COI) gene, known as cox1, due to its lower variation within species compared to between species. Although some critics argue that DNA barcoding is not a panacea for the complexities of traditional taxonomy, it offers a rapid and reliable means of confirming species identifications in the field. It also helps identify areas of contention in species classification, signaling where further research is needed. Ward *et al.* (2009) has used DNA barcoding, particularly for marine ecologists studying chondrichthyans (sharks and rays), especially when expert taxonomic support is not available. The Barcode of Life project and the Consortium for the Barcode of Life (CBOL) are key initiatives promoting DNA barcoding. This molecular method, akin to supermarket barcodes, assigns a unique genetic marker to differentiate species. It utilizes PCR-generated amplicons from specific genomic regions to produce sequence data for identification.

DNA barcoding was introduced to the scientific community in 2003 through Paul Hebert's research group at the University of Guelph. Their groundbreaking paper, "Biological Identifications through DNA Barcodes," proposed using a short, standardized DNA segment for species identification and exploration. This approach provides a quick and cost-effective method for identifying eukaryotic organisms, revolutionizing taxonomy, especially for well-studied groups like fishes. It also enhances understanding of ecological dynamics, biodiversity, and fish taxonomy across various regions. To support this approach, a global initiative was launched to develop and coordinate the Barcode of Life Database, aiming to compile species-specific markers for all known living organisms. DNA barcoding relies on the principle that genetic variations between species are greater than those within a species, enabling effective species differentiation through nucleotide sequences. The standardized approach uses a 650-nucleotide segment from the 5′ region of the cytochrome c oxidase subunit I (COI) gene, serving as a universal barcode for identifying and categorizing life forms on Earth (Costa and Antunes 2012). Overfishing is a major factor contributing to the decline and local extinction of ray and shark populations worldwide.

The accumulation of DNA data for ray species, including 1,255 COI sequences cataloged on GenBank, indicates a substantial genetic database. This suggests that there is now sufficient information to use DNA barcoding for taxonomic identification of batoids. The Barcode of Life Data Systems (BOLD) provides an accessible platform for the operation, analysis, and dissemination of DNA barcodes. Understanding the taxonomy and systematics of fish species is crucial for the sustainable management of genetic resources. DNA barcoding significantly enhances species identification through a standardized and validated DNA-based approach (Bhattacharya *et al.,* 2016). The use of the COI gene for fish species identification has spurred global initiatives such as FISH-BOL (www.fishbol.org) (Ward *et al.,* 2009). Polymerase Chain Reaction (PCR) technique Introduced in 1983, has revolutionized molecular biology by enabling the selective amplification of specific nucleic acid segments. Over time, PCR has evolved into several advanced forms, including end-point PCR, digital PCR (dPCR), and quantitative PCR with real-time fluorescence detection (qPCR). Despite these advancements, the fundamental principle remains consistent across all variations: the enzymatic replication of targeted nucleic acid sequences using short oligonucleotides known as primers. Primers bind to single DNA strands and initiate the amplification process by guiding DNA polymerase to synthesize complementary strands, creating double-stranded DNA. This DNA is then subjected to high temperatures that denature it into single strands. The process of amplification is cyclical and exponential, enabling the production of substantial amounts of DNA from a minimal starting quantity. Depending on the PCR method used, the amplified products can be detected and analyzed in various ways. In end-point PCR, the amplicons are typically identified by size using electrophoresis on an agarose gel, though this detection can be performed post-quantitative PCR (qPCR) as well. qPCR and dPCR enhance sensitivity by detecting and quantifying target DNA through fluorescent signals. Fluorescent dyes such as SYBR Green I or Eva Green, which intercalate with the DNA, emit signals that are measured to monitor the amplification process.

Quantitative PCR generates an amplification curve that reflects the increase in fluorescence over time, indicating the quantity of the target DNA. However, the use of intercalating dyes may affect specificity, necessitating additional techniques like High-Resolution Melting (HRM) analysis for accurate species identification. PCR is foundational to many molecular biology techniques, including Amplified Fragment Length Polymorphism (AFLP) and various sequencing methods, establishing it as a benchmark for DNA-based analyses (Cermakova *et al.,* 2023). Recent studies have applied PCR to address issues such as red snapper mislabeling through phylogenetic mitochondrial DNA analysis and sequencing. For example, Pepe et al. (2005) used PCR products from a conserved region of the cytochrome b gene to differentiate between fish from the Gadidae and Merlucciidae families in processed products. Additionally, sequencing of nuclear gene segments, such as those encoding growth hormone, p-53, alpha-actin, and 5S ribosomal DNA, has been employed to distinguish between fish species. In a 2021 study, the Loop-Mediated Isothermal Amplification (LAMP) technique was utilized for identifying Atlantic salmon (*Salmo salar*). The Salmonidae family encompasses a diverse group of fish known as salmonids, which includes several species of significant economic value to the fishing industry. These species consist of wild-caught *Oncorhynchus kisutch*, *O. tshawytscha*, *O. nerka*, *O. keta*, and *O. gorbuscha*, alongside the farmed

Salmo salar and *O. Mykiss* (Pereira *et al.,* 2011).

Molecular Marker Techniques. Molecular markers are specific genetic loci that can be easily identified and measured within a population. They are often associated with particular genes or traits and have become indispensable tools in genetic research. These markers are crucial for studying genetic relationships, identifying varieties, conducting phylogenetic analyses, and mapping genes or quantitative trait loci (QTLs). Advances in molecular plant breeding, genetics, genomic selection, and genome editing have significantly enhanced our understanding of these markers, offering valuable insights into crop variety diversity. Molecular markers are primarily categorized into two groups: hybridization-based and PCR-based.

Hybridization-Based Markers. One of the earliest hybridization-based markers is Restriction Fragment Length Polymorphism (RFLP). RFLP detects variations in DNA sequences at specific restriction enzyme sites. Differences in these sites lead to distinct patterns of DNA fragments, which are separated by electrophoresis based on length, size, or molecular weight and then transferred onto a membrane using the Southern blot technique. By hybridizing labeled DNA probes with these fragments, the DNA profiles become visible. RFLP can reveal the presence or absence of restriction sites, thereby identifying genetic variations. To improve specificity and sensitivity, nested PCR can be used. This technique involves two sequential PCRs with external and nested primers, enhancing amplification efficiency even at low DNA concentrations (Zhang *et al.,* 2006; Pardo *et al.,* 2018). PCR-RFLP is a widely used method for detecting species-specific genetic variations and includes various changes such as microindels, single-nucleotide polymorphisms (SNPs), and multi-nucleotide polymorphisms (MNPs) (Cermakova *et al.,* 2023).

PCR-Based Markers. Among PCR-based markers, Amplified Fragment Length Polymorphism (AFLP) stands out for its sensitivity in detecting genetic variations. AFLP uses a combination of primers to amplify fragments from across the entire genome without prior knowledge of the organism's genome. The fragments are separated by electrophoresis on a polyacrylamide gel, and the resulting patterns can be visualized via autoradiography or with fluorescently labeled primers using automated sequencers. AFLP allows simultaneous analysis of multiple loci but does not pinpoint specific DNA regions, making it less precise compared to other methods. It is also resource-intensive and requires high-quality DNA, which can lead to variability in fragment intensity and uncertain band origins (Zhang *et al.,* 2014).

Random Amplified Polymorphic DNA (RAPD) is another PCR-based technique that amplifies random DNA segments using a single, short primer typically around 10 nucleotides long. This primer anneals at random sites on the DNA, producing species-specific band profiles or DNA fingerprints. RAPD is cost-effective, requires minimal DNA, and allows rapid analysis. It is suitable for differentiating species without prior genetic information. However, challenges include incomplete fingerprints due to DNA degradation and the potential for misleading results if PCR fragments of similar lengths are produced by different species (Gil *et al*., 2001).

Other Techniques. Genotyping-by-Sequencing (GBS) represents an advanced method that leverages next-generation sequencing (NGS) to discover and genotype SNPs in various crop genomes and populations. This technique enhances the capacity to identify and analyze genetic variations comprehensively. Loop-Mediated Isothermal Amplification (LAMP) is a rapid and highly accurate gene amplification technique that operates at a constant temperature, eliminating the need for complex laboratory equipment. LAMP uses two or three pairs of primers (outer F3, B3; inner FIP, BIP; loop primers) for amplification. While designing primers for LAMP is complex, the method streamlines routine analysis and achieves high specificity. The amplification process results in amplicons of varying lengths, and loop primers can accelerate exponential amplification, reducing analysis time by approximately half (Cermakova *et al*., 2023). LAMP typically employs Bst polymerase due to its higher tolerance to inhibitors, though other polymerases like Z-Taq or BcaBEST may be used depending on the DNA quantity and requirements (Notomi *et al.,* 2000). The amplification products can be detected on agarose gels or through dyes that change color or emit fluorescence, such as calcein, ethidium bromide, SYBR Green, and hydroxynaphthol blue. So, molecular marking techniques have transformed genetic analysis, providing tools for detailed study and application across various fields, including fish species identification. Each technique offers unique advantages and is suited to different aspects of genetic research and practical applications.

CONCLUSION

Molecular marking techniques have fundamentally transformed genetic research and applications across diverse fields. These methods facilitate precise identification and measurement of genetic loci, enabling detailed studies of genetic relationships, variety identification, phylogenetic analysis, and gene mapping, including quantitative trait loci (QTLs). The evolution of molecular markers from hybridization-based techniques like Restriction Fragment Length Polymorphism (RFLP) to advanced PCR-based methods and beyond illustrates the dynamic advancements in this field. Hybridization-based markers such as RFLP have laid the groundwork for detecting genetic variations through restriction enzyme sites, while PCR-based markers, including Amplified Fragment Length Polymorphism (AFLP) and Random Amplified Polymorphic DNA (RAPD), offer sensitivity and cost-effectiveness for analyzing genetic diversity. Although techniques like RAPD are valuable for rapid analysis and mapping population genetics, they are often complemented by more precise methods in current research. The development of Genotyping-by-Sequencing (GBS) and Loop-Mediated Isothermal Amplification (LAMP) highlights the shift towards

high-throughput and efficient approaches. GBS leverages next-generation sequencing to provide comprehensive genetic insights, while LAMP offers a rapid, user-friendly alternative for gene amplification without the need for complex equipment. These advancements have not only enhanced our understanding of genetic diversity in crops and other organisms but have also significantly contributed to practical applications in areas such as plant breeding, aquaculture, and species identification. By integrating these molecular marking techniques, researchers can achieve more accurate, efficient, and detailed genetic analyses, fostering advancements in both scientific research and applied genetic technologies.

Conflict of Interest. None.

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