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REVIEW ARTICLE

Trends in Biotechnology and Plant Sciences

A Comprehensive Analysis of Molecular Markers in Molecular Research

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Abstract

Recent developments in cellular and molecular genetics have opened up new genetic opportunities for advancement in the field of modern science, including the development of molecular markers. Molecular markers have been used as significant tools in agriculture and biomedical research for genetic relationship studies between individuals and hybrids, phylogenetic relationships among species, gene mapping, and tracking quantitative trait loci. During the past three decades, several molecular marker approaches have been developed and exploited in a variety of techniques throughout the world. However, only a handful of these techniques, namely RAFLPs, RAPDs, AFLPs, ISSRs, and SSRs, have gained widespread implementation. These markers can be broadly categorized into hybridization-based and PCR-based markers. The main focus is on analyzing how molecular markers have improved our perception of genetic variety and structure, enabling researchers to clarify complex evolutionary linkages and population dynamics. Furthermore, this review also covers the merits and demerits of molecular marker technology, highlighting the significance of integrating multiple marker types with progressive analytical techniques to provide robust and trustworthy results. As molecular marker technology continues to advance, its importance in evolving science and shaping numerous research disciplines will grow, paving the way for a more focused and individualized approach to addressing global challenges.

KEYWORDS
Hybridization-based markers, Genetic diversity, polymerase chain reaction, Plant breeding, Molecular markers, Polymorphism

1 | INTRODUCTION

Modern genetic studies depend mainly on molecular markers, also known as genetic markers, which enables researchers to uncover DNA-level polymorphisms and identify particular genes within the genome. These markers have been shown to be crucial in the search for genetic advancement and host genetic resistance in many species (Grover & Sharma, 2016; Zafar et al., 2024). Molecular markers offer a variety of advantages by offering an integrated strategy for their use, particularly in the developing world. Molecular markers have emerged as powerful and precise tools that complement traditional breeding methods, playing a pivotal role in expediting breeding programs, enhancing accuracy, and reducing labor and costs (Adhikari et al., 2017; Zafar et al., 2023). The advent of polymerase chain reaction (PCR) and molecular markers in the early 1980s was a turning point in molecular biology. and their continuous evolution through advanced tools and equipment led to the ideas of genomics, bioinformatics, and proteomics by the mid-1990s. The emergence of genomics owes much to advances in marker technology, resulting in a diverse array of genetic markers such as ISSRs, SSRs, RFLPs, RAPDs, AFLPs, microsatellites, and SNPs (Table 1). These markers have vast potential across various scientific endeavors, particularly in the study of agricultural and biomedical sciences (McGuire et al., 2020). In this review, we discuss the applications, advantages, and limitations of different kinds of genetic markers. Genetic markers categorize based on morphological traits such as DNA markers and molecular markers, relying on visually assessable features. Molecular markers involve DNA assays, revolutionizing our understanding of biological sciences. It is important to note that genetic markers are distinct from normal genes as they lack direct biological effects.

Instead, they are identifiable DNA sequences located at specific positions within the genome, adhering to the standard laws of inheritance across generations (Callahan et al., 2017). On the other hand, In the 1950s, the discovery of DNA markers visible through electrophoresis of proteins brought about a revolution in the field. Differences in DNA sequences were manifested as bands of varying sizes, which could be observed, recorded, and analyzed using different methods (Jamali et al., 2019). Given the variety of molecular techniques and methodologies available, careful consideration is essential in selecting the most appropriate marker types for specific research purposes. The selection of an appropriate molecular marker depends on several critical characteristics, such as genomic abundance, polymorphism level, locus specificity, reproducibility, and technical requirements (Amiteye, 2021; Ali et al., 2023). In summary, this review aims to explore the significance of molecular markers in genetic studies, their potential applications in different research areas, and the diverse array of marker types, each with its own set of advantages and limitations. By understanding the intricacies of genetic markers, we can pave the way for new and exciting advancements in the field of genetics and genomics.

PCR-based Markers

The revolutionary breakthrough that significantly shaped the landscape of markers was the introduction of polymerase chain reaction (PCR). This technique sparked a transformative shift by allowing DNA replication to occur in controlled laboratory settings, mirroring the natural DNA replication process (Amom & Nongdam, 2017). The polymerase chain reaction has become a cornerstone of genetic research and evolution. The utilization of PCR-based methods has become widespread, particularly in the investigation of genetic diversity among various plant, animal and human species. These markers have emerged as powerful tools for identifying genetic variations and probing into the diversity and genetic relationships present in different populations. The spectrum of these markers encompasses several key types, each with its own unique attributes and applications. Significant among them are the RAPD, SSR, ISSR, and AFLP markers (Al-Khayri et al., 2022). These markers leverage the potency of PCR technology to unravel genetic relationships, providing researchers with valuable insights into polymorphisms, diversity patterns, and genetic connections among different species. In essence, the advent of PCR-based markers has been a pivotal turning point in genetic research. It has streamlined the investigation of genetic diversity in populations, offering an efficient, cost-effective, and non-radioactive approach to unraveling the intricacies of genetic makeup and relationships among different cultivars (Ramesh et al., 2020).

Inter- Simple Sequence Repeat (ISSR) Markers

These markers, pioneered by Zietkiewicz and colleagues, have emerged as valuable tools in the fields of genetic research and molecular biology due to their ability to reveal polymorphisms at the DNA level without requiring prior sequence information. They function by amplifying polymorphic regions between microsatellites using relative short primer sequences (Zietkiewicz et al., 1994). Notably, ISSR primers possess greater length ranging from15 to 35 nucleotides, enabling the use of higher annealing temperature, which enhance stringency. It is important to note that specific annealing temperatures can be influenced by GC content of primer.

Following PCR amplification, the resulting products, which typically range from 200 to 2000 base pairs in length, is separated using either agarose or polyacrylamide gel electrophoresis (Nilkanta et al., 2017).

Applications

ISSR markers have found diverse applications across different areas of science. They have made significant

Table 1: Characteristics of some widely used molecular markers (Raza et al., 2019)

	RFLP	RAPD	SSR	ISSR	AFLP
Abundance	High	High	Medium	High	High
Reproducibility	High	Low	Medium	High	High
Degree of polymorphism	Medium	Medium	Medium	High	Medium
Amount of DNA required	10 µg–50	100 ng–1	120 ng–50	10-100 ng	100 ng–1
Quantity of DNA required	High	Low	Low	Low	Medium
Inheritance	Co-dominant	Dominant	Co-dominant	Dominant	Dominant/co-dominant
Locus specificity	Yes	No	No	No	No
Type of probes/primers	Low copy DNA or cDNA clone	Usually, 10 bp random nucleotides	Specific sequence	No Specific sequence	Specific sequence
Major application	Physical mapping	Gene tagging	Genetic diversity	Genetic diversity	Gene tagging

contributions to understanding genetic diversity, population structure, and evolutionary relationships, which have aided in uncovering cryptic species, identifying distinct population, and unraveling the effect of habitat fragmentation on biodiversity (Etminan et al., 2016). In genetics and genomics, these markers are employed for mapping and characterization of gene of interest. ISSRs play a crucial role in assessing genetic diversity, structure, and gene flow among populations in population genetics studies. In phylogenetic and biodiversity research, ISSR markers assist in elucidating evolutionary relationships, and gene divergence also identifies unique genetic profiles when analyzing species conservation status (Hossain et al., 2021). ISSRs have also advanced plant breeding programs by assisting in development of new cultivars and the preservation of genetic resources through germplasm characterization, hybrid identification, and marker-assisted selection (Bagmohammadi et al., 2014).

Advantages

1. **No prior sequence information:** One of the major advantages of ISSRs is their capacity to generate polymorphic data without requiring prior knowledge of DNA sequences.
2. **Cost-effective:** ISSR markers involve simple PCR reactions with short primers, making them a cost-effective option for many research projects.
3. **High polymorphism:** ISSR markers exhibit high levels of polymorphism, making them effective for distinguishing between individuals and populations.
4. **Rapid analysis:** ISSR analysis is relatively quick, allowing researchers to analyze a large number of samples in a short period of time.
5. **Minimal DNA requirements:** ISSR markers can be amplified from small amounts of DNA, enabling studies on limited or degraded DNA samples (Haroon et al., 2023).

Limitations:

1. **Dominance of bands:** ISSR markers produce dominant bands, which may not accurately reflect heterozygosity within individuals.
2. **Inconsistency:** ISSR results can be influenced by factors like DNA quality, primer sequences, and PCR conditions, potentially leading to inconsistencies (Baloch et al., 2015).
3. **Lack of sequence information:** While ISSRs provide valuable genetic information, they do not offer sequence data, limiting their utility in certain applications.
4. **Limited transferability:** ISSR primers designed for one species may not always work optimally in other species due to variations in microsatellite sequences.

ISSR markers underscore their pivotal role in genetic research and molecular biology. These markers have proven invaluable in a wide range of applications, contributing to our understanding of genetics, biodiversity, and evolution (Hadipour et al., 2020). Despite their limitations, they continue to be a popular choice for researchers seeking cost-effective and informative markers for their research. As technology advances, ISSR markers remain an essential tool in genetics' toolkit.

Amplified Fragment Length Polymorphism (AFLP)

It is a widely used molecular technique in genetic and genomics research for detecting genetic variations among individuals or populations. AFLP merges the advantages of polymerase chain reaction and restriction fragment length polymorphism (RFLP), allowing researchers to assess a huge number of genetic markers simultaneously. An important aspect of AFLP is its flexibility in accommodating a large range of DNA quality (Leipold et al., 2020). Notably, both intact high-quality DNA and partially degraded DNA can be employed for AFLP analysis although it is imperative that DNA samples are devoid of residual restriction enzymes and PCR inhibitors. The process initiates with genomic DNA digestion by a combination of restriction enzymes, naturally combining an infrequent cutter (e.g., Eco RI or PstI) with a frequent cutter (e.g., MseI or TaqI). To deliver a recognized restriction sites are ligated to both ends of subsequent fragments (Hou et al., 2021).

Principle of AFLP

AFLP comprises several steps, including DNA digestion, adapter ligation, pre-amplification, selective amplification, and fragment analysis. Primarily, digestion of genomic DNA with restriction enzymes generates a pool of DNA fragments. Ligation of adapters to the ends of these fragments enables PCR amplification. The AFLP amplification phase is a multi-step PCR strategy. Pre-amplification with primers targeting the adapter sequences is followed by discriminatory amplification using primer pairs with selective nucleotides. This results in the amplification of specific fragments centered on the existence or non-existence of restriction sites, generating a complex banding pattern for analysis (Vos et al., 1995).

Applications of AFLP

These are markers broadly used to access genetic diversity within and between populations of different species. This knowledge is important for conservation biology, evolutionary studies, and understanding population patterns. AFLP is utilized in plant breeding to identify genetic markers associated with desirable traits

this allows for the selection of plants with modified characteristics such as diseases resistance, yield, and stress tolerance (Al-Hadeithi & Jasim, 2021). They also aid in analyzing microbial diversity and community structure within different environments, including soil, water, and human microbiota. It helps in understanding microbial interactions and their responses to habitat modifications (Kumar et al., 2016). AFLP data is crucial to interfering with evolutionary relationships among species, assisting in construction of phylogenetic trees, and unraveling the evolutionary history of organisms. It also aids in detecting specific pathogens accountable for disease outbreaks and recognizing genetic variations associated with diseases in humans, animals, and plants (Evanno et al., 2005).

Advantages:

1. **High throughput:** AFLP can instantaneously investigate abundant genetic markers, making it a prevailing tool for large-scale genetic studies.
2. **No prior genomic information requisite:** Contrasting to some other techniques, AFLP does not require prior genomic sequence knowledge, permitting its utilization in non-model organisms.
3. **Dominant markers:** AFLP produces dominant markers, making it appropriate for populations with mixed genotypes and polyploid species.
4. **Scalability:** the techniques can be adapted for diverse levels of genetic resolution (Johnson et al., 2007), depending on the research objectives.

Limitations:

1. **Labor-intensive:** AFLP comprises manifold steps, which can be time-consuming, and necessitate cautious optimization.
2. **Difficulties in fragment interpretation:** Complex banding patterns can be challenging to interpret precisely, leading to possible complications in recognizing true genetic alteration (Roldán-Ruiz et al., 2000).
3. **Limited sequence information:** AFLP generated anonymous markers, providing no sequence knowledge for identified fragments.
4. **Cost:** The cost of reagents and equipment mandatory for AFLP analysis can be moderately high, mainly for large-scale projects.

As molecular techniques continue to progress, AFLP remains a valuable tool for providing insights for identifying genetic variations associated with genetic variations in agriculture and biomedical research (Tavasoli et al., 2011). However, researchers should carefully consider labor-intensiveness, potential challenges in fragment interpretation, and associated costs before choosing as a method for their research.

Random Amplified Polymorphic DNA (RAPD) Analysis

Random amplified polymorphic DNA (RAPD) analysis is an extensively utilized molecular technique in genetic research permits for the valuation of genetic diversity, population structure, and evolutionary relationships among organisms. Random Amplified Polymorphic DNA (RAPD), as introduced by Williams et al*.* (1990), is a reformed sort of polymerase chain reaction (PCR) that employs short 10-base primers of arbitrary sequence with a minimum 60% GC content. The core of RPAD lies in the statement that PCR amplification is anticipated to happen only when the primer site is present twice in opposite orientations within a span of around 2000 bases (Dobhal & Kumar, 2021).

Methodology

The RAPD procedure comprises a simple experimental protocol, which adds to its popularity. Researchers use short single-stranded DNA primers of arbitrary sequences that anneal to multiple sites within the genomic DNA of interest. The primers are naturally 10-12 nucleotides long and designed to target repetitive and variable regions in the genome. Amplified by means of PCR, resulting in the generation of multiple DNA fragments of varying lengths, which were separated by agarose gel electrophoresis. The subsequent banding patterns were then used to infer genetic relationships and variations among individuals or populations (Saxena & Bisen, 2017).

Applications

RAPD analysis comprehensively casts-off to estimate the genetic diversity within and among populations. By compromising the DNA banding patterns of diverse individuals or groups, researchers can estimate the degree of genetic variation, which is vital for considerate population-changing aspects, management approaches, and adaption to eco-friendly modifications. Taxonomy and phylogenetic analysis have contributed to the explanation of evolutionary affiliations and groupings of organisms (Vilatersana et al., 2005). By compromising RAPD profiles among species or genera, researchers can infer genetic relatedness and construct phylogenetic trees, shedding light on the evolutionary history and origins of various taxa. The ability to assess population structure and gene flow is valuable in studying the genetic differentiation of populations. RAPD provides insights into migration patterns, gene flow barriers, and the impact of geographical isolation on genetic diversity (Thakur et al., 2014). It has been employed in plant breeding programs to identify desirable genetic traits and markers associated with specific characteristics. This technique aids in selecting

individuals with preferred traits, leading to the development of improved crop varieties.

Advantages

1. **Cost and time proficiency:** RAPD analysis requires an insignificant initial investment and is relatively quick compared to other molecular techniques.
2. **Minimal DNA requirement:** Small amounts of DNA are sufficient for RAPD analysis, making it suitable for working with limited or degraded samples (Mahasi et al., 2009).
3. **No prior sequence information:** RAD does not require previous knowledge of the target DNA sequence, making it applicable to non-model organisms.

Limitations

1. **Lack of reproducibility:** RAPD can be sensitive to experimental conditions, leading to variability in results between different laboratories or runs.
2. **Dominance of dominant markers:** RAPD markers are often treated as dominant, which limits the information gained about heterozygosity and genetic diversity within populations.
3. **Limited transferability:** The arbitrary nature of RAPD primers can lead to difficulties in comparing results across studies or laboratories (Khan et al., 2009).

Despite its limitations, advancements in other molecular techniques like microsatellite markers and next-generation sequencing have expanded researchers' insights for studying genetic variation with greater precision and accuracy (Dobhal et al., 2019). As such, RAPD analysis continues to be used alongside these newer techniques, serving as a complementary method in various research activities.

SSR (Simple Sequence Repeat) Markers

Simple sequence repeats (SSRs), also known as microsatellites, are short DNA sequences consisting of tandem repeats of 1-6 nucleotide motifs. They are abundant and extensively distributed throughout the genomes of various organisms. The unique characteristics of SSRs, including high polymorphism, codominant inheritance, and multi-allelic nature, have made them valuable genetic markers for a wide range of applications. The concept of SSR markers emerged in the late 1980s with the pioneering work of Litt and Luty (1989), who demonstrated the potential for the amplification of SRR loci, leading to their widespread adoption across species. The initial focus was on developing SRR markers in humans, but their utility quickly expanded to include plants, animals, and microbes (Ali et al., 2023).

Applications

SRR markers are widely used to assess genetic diversity within and between populations. The high level of polymorphism allows researchers to accurately distinguish between individuals and populations, making SSRs valuable tools in conservation genetics, crop improvement, and understanding the genetic basis of traits. The markers have been pivotal in constructing genetic maps and identifying quantitative trait loci (QTLs) associated with various phenotypic traits. These markers aid in elucidating the genetic basis of complex traits and facilitating marker-assisted selection in breeding programs (Hasan et al., 2021). By analyzing the variation in SSR alleles across populations, researchers can infer historical migration patterns, population linkage, and evolutionary relationships. SSR markers provide insights into population dynamics, speciation events, and adaptive evolution. They are employed in forensic science for human identification, paternity testing, and criminal investigations (Kliuiev, 2023). Similarly, in agriculture, these markers can establish parentage and verify the authenticity of plant varieties. SSR markers aid in characterizing germplasm collection, ensuring the preservation of genetic diversity in crops, and protecting endangered species. These markers contribute to the efficient management and utilization of genetic resources (Li et al., 2022).

Advantages

1. **High polymorphism:** SSR markers exhibit high levels of polymorphism due to their repetitive nature, making them ideal for distinguishing between closely related individuals or populations (Ambreen et al., 2018).
2. **Co-dominant inheritance:** Unlike dominant markers, SRRs are co-dominant, allowing heterozygotes to be accurately genotyped, which is crucial for various genetic analyses.
3. **Wide genomic distribution:** SSRs are dispersed throughout the genome, providing numerous marker options for mapping and genotyping studies.
4. **PCR-based amplification:** The use of PCR to amplify SSR loci makes genotyping relatively straightforward and efficient (Phougat et al., 2018).

Limitations

1. **Genomic complexity:** The repetitive nature of SSRs can lead to difficulties in isolating and characterizing these sequences accurately, especially in complex genomes.
2. **Allele size variation:** Interpreting allele size from electrophoresis gel can be challenging due to

variations in gels conditions and equipment, leading to issues in data consistency.
3. **Transferability across species:** While SSR markers are highly polymorphic within a species, their transferability across different species can be limited due to differences in genome structure and repeat motifs (Ramu et al., 2013).
4. **Cost and throughput:** SSR marker development and genotyping can be time-consuming and costly, particularly when compared to high-throughput sequencing-based methods.

Recent Developments

1. **Next-generation sequencing (NGS):** NGS technologies have revolutionized the field of marker development. SSR markers can now identified and characterized using NGS data, enabling the rapid discovery of SSRS in non-model species (Reis et al., 2022).
2. **Database resources:** Numerous SSR marker databases and online resources have been established (Martina et al., 2022), providing researchers with curated SSR datasets and primer information, facilitating marker selection and utilization.
3. **In silico analysis:** Bioinformatics tools allow for in silico identification and characterization of SSR markers from genomic data, reducing the need for traditional wet lab techniques (Patil et al., 2020).
4. **SNP-SSR integration:** Integration of SSR markers with single nucleotide polymorphism (SNP) markers enhances the resolution and informativeness of genetic analyses (Alsamman et al., 2020).

Simple Sequence Repeats (SSRs) have proven to be versatile genetic markers with a broad range of applications in genetics and genomics. Their high polymorphism, codominant inheritance, and genome-wide distribution make them valuable tool for understanding genetic diversity, mapping traits, studying populations, and supporting various breeding and conservation efforts (Adjebeng-Danquah et al., 2020). Advances in sequencing technologies and bioinformatics have further expanded the utility of SSR markers, ensuring their continued relevance in modern genetic research.

Hybridization-based Markers

The class of genetic markers that depend on the complementary base pairing between probe and target DNA sequence. These markers play a critical role in molecular biology and genetics by enabling detection and analysis of specific DNA sequences. They have been instrumental in many applications from genome mapping to disease diagnosis. Hybridization-based markers work on the principle of complementary base pairing between DNA strands. A probe which is a single-stranded DNA or RNA sequence is designed to correspond to a precise target DNA sequence. When probe and target DNA come into contact, they bind together through hydrogen bonding between complementary nucleotides (A-T and G-C base pairs). This binding forms a stable duplex structure that can be detected and analyzed (Younis et al., 2020).

Restriction Fragment Length Polymorphism (RFLP) Markers

These markers are a category of genetic markers generally utilized in molecular biology and genetics to study genetic variation, map genomics, and identify specific alleles. They were among the first molecular markers developed and played a vital role in progressing our understanding of genetic variation in various species. RFLP markers were first introduced in the late 1970s and early 1980s by Alec Jeffreys as an innovation procedure that empowered the isolation and investigation of genetic variation at the DNA level (Roewer, 2013). The procedure is related to the differential fragmentation of DNA sequences due to variation in recognition sites of restriction enzymes. These enzymes cleave DNA at specific nucleotide sequences, resulting in fragments of varying sizes that can be separated and analyzed using gel electrophoresis.

Principles of RFLP Analysis

These markers are constructed on the basis of polymorphisms that happen in DNA sequences. Polymorphism can result from single nucleotide changes (SNPs), insertions, deletions, and rearrangements. To analyze these variations, genomic DNA is digested with restriction enzymes to generate DNA fragments. The resulting fragments are then separated on an agarose gel based on size using gel electrophoresis (Singh & Singh, 2015). The separated fragments are visualized using techniques such as southern lotting, where the DNA fragments are transferred to a membrane and hybridized with labeled probes complementary to the target DNA sequence.

Applications

RFLP markers played a pivotal role in the construction of genetic linkage maps. By analyzing the inheritance patterns of RFLP markers in family pedigrees, researchers determine the relative positions of genes on chromosomes. They have been extensively utilized to analyze genetic variation and population dynamics within and among species. This knowledge is important for conservation efforts, understanding evolutionary processes, and tracing migration patterns (Liu, 2007).

These markers were employed in forensic DNA analysis and paternity testing due to their polymorphic nature, which permits the distinction of individuals based on their unique DNA profiles (Evett et al., 1993). RFLP markers have been used to assist in selective breeding programs by identifying individuals with desirable traits and tracking the inheritance of these traits across generations.

Advantages

1. **High polymorphism:** RFLP markers can detect a wide range of genetic variation, including both subtle and large changes, making them suitable for various applications and research.
2. **Stale over generations:** unlike some other types of markers, RFLP tends to be relatively stale across generations, allowing for long-term studies (Angelopoulou et al., 2023).

Limitations

1. **Labor-intensive:** the process of generating RFLP data is labor-intensive and time-consuming, involving multiple steps such as DNA extraction, restriction enzyme digestion, gel electrophoresis, blotting and hybridization.
2. **Low throughput:** RFLP analysis is limited in its ability to process a large number of samples simultaneously, which is a drawback when dealing with extensive datasets.
3. **Dependence on probes:** the reliance on specific probes for hybridization limits the versatility of RFLP markers compared to more modern techniques like PCR-based markers (Hashim & Al-Shuhaib, 2019).

Restriction fragment length polymorphism (RFLP) markers have played a fundamental role in molecular genetics and significantly contributed to our understanding of genetic diversity, evolution, and inheritance (Kumar et al., 2022). While newer marker technologies have since emerged with higher throughput and efficiency, the historical significance and continued application of RFLP markers in various fields demonstrated their enduring impact on genetic research.

Conclusion

In conclusion, molecular markers have emerged as requisite tools across a range of disciplines, revolutionizing genetic understanding and applications. From agriculture to medicine, these markers enable precise genetic characterization, accelerating progress in personalized medicine, crop improvement, and conservative efforts. Despite challenges, advances in genotyping technologies and data analysis continue to expand their potential. As technology continue to advance, molecular markers have the potential to make ongoing transformative contributions to scientific discovery and practical solutions.

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