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Molecular Markers in *Oryza* Genomics: Tools for Species Classification and Phylogeny

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Abstract The study explored the utilization of various molecular markers to classify and understand the phylogenetic relationships among *Oryza* species. The genus *Oryza*, which includes the globally significant crop rice (*Oryza sativa*), comprises 22 species with diverse genetic backgrounds. This research evaluates the effectiveness of different molecular markers, including SNPs, AFLPs, ISSRs, and microsatellites, in revealing genetic diversity and phylogenetic relationships. SNP markers, particularly those developed using DArTseq technology, have shown high efficiency in species identification and quality control genotyping. AFLP markers have been instrumental in elucidating the polyphyletic evolution of *Oryza*, indicating multiple independent lineages. ISSR markers have provided insights into the genetic diversity and evolutionary pathways of various *Oryza* genomes. Microsatellite markers, especially those derived from miRNA genes, have proven to be highly polymorphic and useful for genotyping applications. The study underscores the importance of integrating multiple molecular markers to achieve a comprehensive understanding of the genetic diversity and evolutionary history of *Oryza* species, which is crucial for effective germplasm conservation and breeding programs.

Keywords *Oryza* genomics; Molecular markers; Species classification; Phylogeny; Genetic diversity

1 Introduction

The genus *Oryza*, encompassing both wild and cultivated rice species, is of paramount importance in global agriculture. Rice (*Oryza sativa*) serves as the staple food for over half of the world's population, underscoring its critical role in food security (Ganie and Mondal, 2015; Song et al., 2017). The genus includes 24 species with 11 different genome types, providing a vast genetic reservoir that is invaluable for the genetic improvement of rice cultivars. Wild species of *Oryza*, in particular, represent an enormous gene pool that can be harnessed for enhancing disease resistance, stress tolerance, and other agronomic traits in cultivated rice (Stein et al., 2018; Brondani et al., 2003).

Accurate species classification and understanding the phylogenetic relationships within the *Oryza* genus are essential for effective utilization of its genetic resources. Phylogenetic studies reveal the evolutionary pathways and genetic diversity among species, which are crucial for breeding programs and conservation efforts (Joshi et al., 2000). For instance, the identification of species-specific markers and the resolution of phylogenetic relationships help in tracing the lineage-specific emergence and turnover of novel genetic elements, including transposons and potential new coding and noncoding genes (Stein et al., 2018). Such insights are vital for developing strategies to introgress beneficial traits from wild species into cultivated varieties (Tabassum et al., 2022).

Molecular markers are indispensable tools in genomics for species identification, genetic diversity analysis, and phylogenetic studies. Various types of molecular markers, such as chloroplast DNA barcodes, amplified fragment length polymorphisms (AFLP), simple sequence repeats (SSRs), and inter simple sequence repeats (ISSR), have been employed to elucidate genetic relationships and species divergence within the *Oryza* genus (Joshi et al., 2000). For example, chloroplast genomic resources have been developed to provide high-resolution species discrimination and phylogenetic analysis, identifying variable regions that serve as reliable DNA barcodes (Song et al., 2017). Similarly, genome-wide development of novel markers, such as miRNA-based SSRs and InDel markers, has facilitated the genetic analysis and breeding of rice by revealing polymorphisms and genetic

diversity among different *Oryza* species (Stein et al., 2018; Hechanova et al., 2021). These molecular markers not only aid in the classification and phylogenetic studies but also play a crucial role in marker-assisted breeding, enabling the efficient use of wild germplasm for rice improvement (Brondani et al., 2003; Virk et al., 2000).

2 Molecular Markers in Genomics

2.1 Definition and types of molecular markers

Molecular markers are specific sequences of DNA that can be used to identify a particular location within the genome. These markers are essential tools in genomics for various applications, including genome mapping, gene tagging, and phylogenetic analysis. The primary types of molecular markers include Restriction Fragment Length Polymorphisms (RFLPs), Random Amplified Polymorphic DNAs (RAPDs), Amplified Fragment Length Polymorphisms (AFLPs), Inter Simple Sequence Repeats (ISSRs), Simple Sequence Repeats (SSRs), and Single Nucleotide Polymorphisms (SNPs) (Grover and Sharma, 2016). Each type of marker has unique characteristics and applications. For instance, SSRs, also known as microsatellites, are highly polymorphic and widely used for evaluating genetic diversity and constructing genetic maps (Ni et al., 2002). SNPs, on the other hand, are the most abundant type of genetic variation and are particularly useful for high-throughput genotyping and genome-wide association studies (Gouda et al., 2021).

2.2 Evolution of molecular marker techniques

The development of molecular marker techniques has evolved significantly over the past few decades. Initially, RFLPs were the primary markers used due to their codominant nature and high reproducibility. However, the labor-intensive and time-consuming nature of RFLP analysis led to the development of PCR-based markers such as RAPDs and AFLPs, which allowed for quicker and more efficient genotyping. The advent of microsatellites (SSRs) marked a significant advancement due to their high polymorphism and ease of use in PCR-based assays (Ni et al., 2002). More recently, the focus has shifted towards SNPs and genotyping by sequencing (GBS), which offer high-throughput and ultra-high-throughput capabilities, making them suitable for large-scale genomic studies. The integration of modern transcriptomic and functional markers has further enhanced the resolution and applicability of molecular markers in plant genomics (Grover and Sharma, 2016).

2.3 Advantages of using molecular markers in plant genomics

Molecular markers offer several advantages in plant genomics. They provide a stable, cost-effective, and efficient means of assessing genetic diversity, which is crucial for germplasm conservation and breeding programs (Ni et al., 2002). For example, microsatellite markers have been shown to detect a high degree of polymorphism, making them ideal for evaluating genetic variation among rice cultivars and wild species. Additionally, molecular markers facilitate the identification and introgression of valuable traits from wild species into cultivated varieties, thereby enhancing crop improvement efforts (Brondani et al., 2003; Fang et al., 2011). They also enable precise phylogenetic analysis and species classification, as demonstrated by the use of genome-specific repetitive sequences in the genus *Oryza* to classify unknown species and study genome evolution. Furthermore, the development of diagnostic SNP markers has improved the accuracy of species identification and quality control in breeding programs (Gouda et al., 2021). Overall, molecular markers are indispensable tools in plant genomics, offering numerous benefits for research and practical applications.

3 Commonly Used Molecular Markers in *Oryza* Genomics

3.1 Simple sequence repeats (SSRs)

Simple Sequence Repeats (SSRs), also known as microsatellites, are short, tandemly repeated DNA sequences that are widely distributed throughout the genome. They are highly polymorphic due to variations in the number of repeat units, making them valuable for genetic mapping, population genetics, and phylogenetic studies. In *Oryza sativa*, SSRs have been extensively utilized to enhance the resolution of genetic maps and to study genetic diversity. For instance, a study identified 57.8 Mb of rice DNA sequence to determine the frequency and distribution of SSRs, categorizing them into Class I (hypervariable) and Class II (potentially variable) markers (Figure 1) (Temnykh et al., 2001; Tabassum et al., 2022; Ma et al., 2024). Another research effort developed 200 Class I SSR markers, integrating them into the existing microsatellite map of rice, thus providing links between

genetic, physical, and sequence-based maps. SSRs are also known for their high transferability across species, as demonstrated in a study where SSR loci exhibited broad potential transferability among various angiosperms (Zhong et al., 2022).

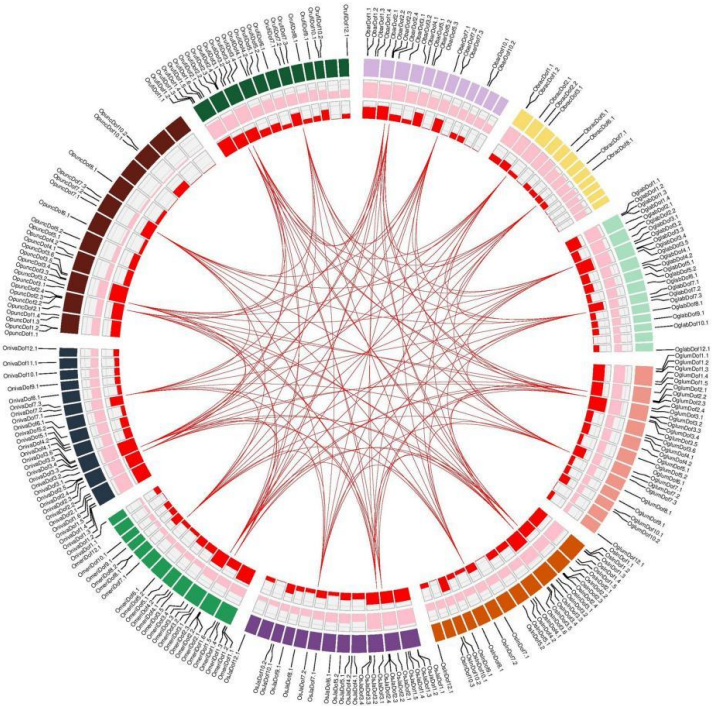

Figure 1 Genomic landscape of Dof genes among ten *Oryza* genomes (Adopted from Tabassum et al., 2022)

Image caption: Circular diagram from outside to inside are gene names and locations on individual species-specific colored chromosomal bands, density of high confidence protein-encoding genes (count/Mb; min=65, max=133), density of Dof genes (count/Mb; min=0.0, max=0.17) and links indicating duplicated genes among ten rice species (Adopted from Tabassum et al., 2022)

3.2 Single nucleotide polymorphisms (SNPs)

Single nucleotide polymorphisms (SNPs) are the most abundant type of genetic variation in genomes. They involve a single base pair change and are highly stable, making them ideal for high-resolution genetic mapping and association studies. SNPs have been used in *Oryza* genomics to identify genetic variations associated with important agronomic traits (Duhan et al., 2023). The high density and widespread distribution of SNPs across the rice genome allow for detailed genetic analysis and the development of SNP-based markers for marker-assisted selection. Although the provided data does not include specific studies on SNPs in *Oryza*, their general utility in plant genomics is well-documented, and they are often used in conjunction with other markers like SSRs to provide a comprehensive understanding of genetic diversity and structure.

3.5 Comparative analysis of these markers in the context of *Oryza*

When comparing SSRs and SNPs in the context of *Oryza* genomics, several key differences and complementary strengths emerge. SSRs are highly polymorphic and multiallelic, which makes them particularly useful for studies requiring high levels of genetic diversity detection, such as population genetics and phylogenetic studies. They are also relatively easy to develop and analyze, with a high degree of reproducibility and codominant inheritance (Kalia et al., 2011). However, SSRs can be less abundant than SNPs and may require more effort to develop species-specific markers.

On the other hand, SNPs are more abundant and evenly distributed across the genome, providing higher resolution for genetic mapping and association studies. They are also more stable than SSRs, which can be advantageous for certain types of genetic analysis. The development of high-throughput SNP genotyping technologies has further enhanced their utility in large-scale genetic studies (Kumar et al., 2020).

In *Oryza*, the integration of both SSR and SNP markers can provide a more comprehensive understanding of genetic variation. For example, SSRs can be used to quickly assess genetic diversity and structure, while SNPs can provide detailed insights into specific genetic loci associated with important traits (Temnykh et al., 2000). The complementary use of these markers allows researchers to leverage the strengths of each type, leading to more robust and informative genetic analyses.

4 Applications of Molecular Markers for Species Classification

4.1 Case studies of molecular markers used for classifying *Oryza* species

Molecular markers have been extensively utilized to classify and differentiate various *Oryza* species. For instance, a study compared the effectiveness of AFLP, isozymes, ISSR, and RAPD markers in revealing genetic diversity and discriminating between infraspecific groups of *Oryza sativa* germplasm. The study found that isozymes and AFLPs were most effective in classifying the germplasm into three major groups, although there were differences in the precise classifications generated by ISSR markers (Virk et al., 2000). Another study focused on the use of RAPD and SSR markers to assess genetic diversity among 40 cultivated varieties and five wild relatives of *Oryza sativa*. The study concluded that SSR markers provided a more definitive separation of clusters of genotypes, indicating a higher level of efficiency for accurate determination of relationships between accessions (Ravi et al., 2003). Additionally, diagnostic SNP markers developed using DArTseq technology have been validated for quality control genotyping in a collection of four rice species, demonstrating their utility in species classification (Figure 2) (Hechanova et al., 2021; Gouda et al., 2021).

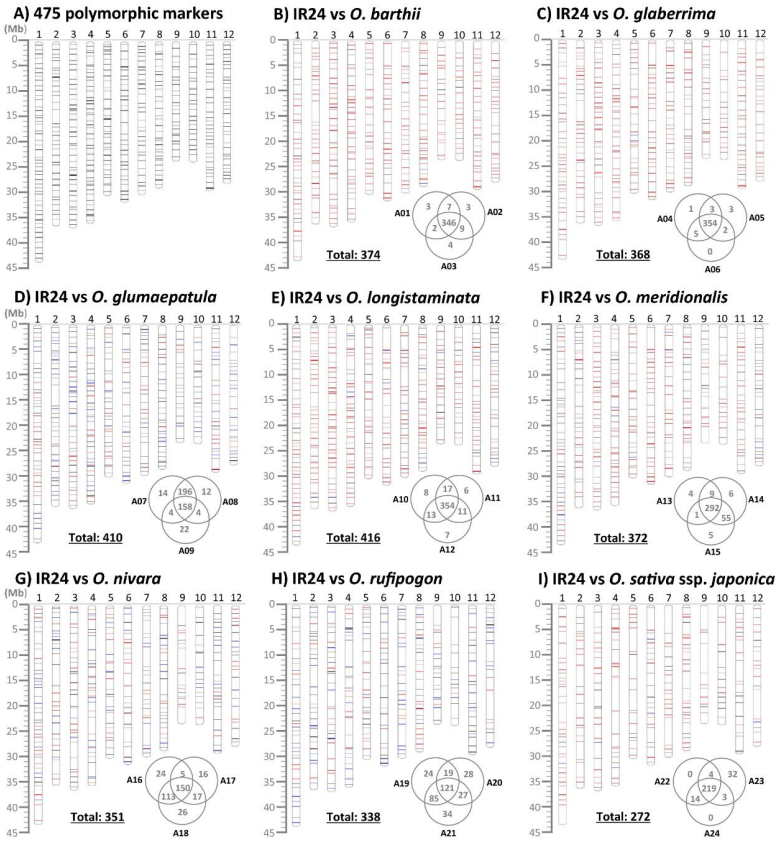

Figure 2 Physical locations of the polymorphic markers (Adopted from Hechanova et al., 2021)

Image caption: Physical locations of the polymorphic markers. The position of each marker was mapped on the rice reference genome (Os-Nipponbare-Reference-IRGSP-1.0) with a horizontal bar. (A) All available 475 polymorphic markers between *O. sativa* and the other AA-genome species. The selected polymorphic markers showing polymorphism between IR24 and *O. barthii* (B), *O. glaberrima* (C), *O. glumaepatula* (D), *O. longistaminata* (E), *O. meridionalis* (F), *O. nivara* (G), *O. rufipogon* (H), and *O. sativa subsp. japonica* (I), respectively. Within a species, the common polymorphic markers among three accessions and between two accessions are highlighted by red and blue bars, respectively, and the accession-specific polymorphic markers are depicted with a black bar. (Adopted from Hechanova et al., 2021)

4.2 Comparative analysis of marker efficiency for classification purposes

Different molecular markers vary in their efficiency for species classification. For example, SSR markers have been shown to be more efficient than RAPD markers in accurately determining relationships between closely related accessions of *Oryza sativa* (Ravi et al., 2003). Similarly, a study comparing the transferability of microsatellite and sequence-tagged site (STS) markers across 16 *Oryza* species found that microsatellite markers had a higher level of polymorphism and were more effective in detecting genetic diversity compared to STS markers (Brondani et al., 2003). Another study highlighted the utility of sequence-tagged microsatellite sites (STMS) markers, which were found to be highly reproducible and effective in identifying polymorphisms among different *Oryza* species (Dalai et al., 2021). These findings suggest that while all marker systems have their strengths, SSR and STMS markers may offer higher efficiency for species classification in *Oryza*.

4.3 Limitations and challenges in species classification using molecular markers

Despite their utility, molecular markers also present certain limitations and challenges in species classification. One major challenge is the partial agreement in relationships between individual accessions when different markers are used, as observed in a study comparing AFLP, isozymes, ISSR, and RAPD markers (Virk et al., 2000). Additionally, the transferability of markers across different species can be variable, with some markers showing reduced levels of genetic diversity detection, as seen with STS markers (Brondani et al., 2003). Another limitation is the potential for misidentification due to morphological similarities among closely related species, which can complicate the validation of molecular markers. Furthermore, the development and validation of new markers, such as SNPs, require extensive testing to ensure their reliability and cost-effectiveness for routine use (Gouda et al., 2021). These challenges highlight the need for careful selection and validation of molecular markers to ensure accurate species classification in *Oryza* genomics.

5 Case Studies

5.1 A Specific case of using ssr markers to construct a phylogenetic tree of the genus *Oryza*

Simple sequence repeats (SSRs) have been extensively used in constructing phylogenetic trees due to their high polymorphism and codominant inheritance. In the genus *Oryza*, SSR markers have been particularly effective. For instance, a study on the rice genome (*Oryza sativa* L.) identified a high frequency of SSRs, with Class I SSRs occurring every 16 kb in continuous genomic sequences and Class II SSRs every 1.9 kb. This high density of SSRs allows for detailed genetic mapping and phylogenetic analysis. The study developed 200 Class I SSR markers, which were integrated into the existing microsatellite map of rice, providing a robust framework for phylogenetic studies (Temnykh et al., 2001). This integration facilitates the construction of a detailed phylogenetic tree, revealing the genetic relationships within the genus *Oryza*.

5.2 Case studies on phylogeny reconstruction using different markers

Phylogenetic reconstruction in plants has utilized various molecular markers beyond SSRs. For example, a study on *Cryptomeria japonica* developed EST-SSR markers from expressed sequence tags, which are useful for genome analysis due to their abundance and polymorphism (Ueno et al., 2012). These markers were found to be less polymorphic than genomic SSRs but still valuable for phylogenetic studies. Another study on *Cucurbita pepo* used SSR markers to construct a genetic linkage map, demonstrating high inter-species transferability and polymorphism (Gong et al., 2008). This map included 178 SSRs and provided insights into the genetic relationships within the genus *Cucurbita*. Additionally, a study on *Picea abies* highlighted the use of both codominant and dominant SSR markers for population studies and phylogenetic analysis, despite the challenges posed by null alleles and dominant markers (Yazdani et al., 2003). These examples illustrate the versatility of different molecular markers in phylogenetic reconstruction across various plant species.

5.3 Insights gained from phylogenetic studies in *Oryza*

Phylogenetic studies in the genus *Oryza* have provided significant insights into the genetic diversity and evolutionary relationships among species. The use of SSR markers has been particularly informative. For instance, the high degree of allelic variation revealed by SSR markers in rice has been attributed to replication slippage and unequal crossing-over during meiosis, which contribute to the genetic diversity observed within the genus (Kalia

et al., 2011). Moreover, the integration of SSR markers into genetic maps has facilitated the identification of genetic linkages and the construction of detailed phylogenetic trees (Martina et al., 2022). These studies have also highlighted the importance of SSR markers in marker-assisted selection and breeding programs, as they provide valuable information on genetic relationships and trait inheritance. Overall, the use of SSR markers in phylogenetic studies has enhanced our understanding of the genetic architecture and evolutionary history of the genus *Oryza*.

6 Advancements in Molecular Marker Technologies

6.1 Next-generation sequencing (NGS) and its impact on marker discovery

The advent of next-generation sequencing (NGS) has revolutionized the field of genomics, providing unprecedented capabilities for the discovery and genotyping of genetic markers. NGS technologies enable the rapid sequencing of millions of DNA fragments simultaneously, which has significantly reduced the cost and time required for genome-wide studies (Davey et al., 2011; Satam et al., 2023). This high-throughput approach is particularly beneficial for both model and non-model organisms, facilitating the discovery of genetic markers even in species with no existing genomic data. Techniques such as restriction-site-associated DNA sequencing (RAD-seq) and reduced-representation libraries (RRLs) have been developed to reduce the complexity of target genomes, making marker discovery more efficient and cost-effective. Moreover, NGS has expanded the scope of genomics research, enabling studies on rare genetic diseases, cancer genomics, microbiome analysis, and population genetics.

6.2 High-throughput genotyping platforms

High-throughput genotyping platforms have emerged as powerful tools for large-scale genetic analysis, allowing researchers to genotype thousands of samples simultaneously. These platforms leverage the advancements in NGS technologies to provide detailed information on genetic variations across populations (Dijk et al., 2014). For instance, targeted multiplex NGS techniques enable the simultaneous resequencing of multiple genomic regions from numerous individuals, enhancing the efficiency of population genomic studies (Hancock-Hanser et al., 2013). Such platforms are particularly useful for non-model organisms, where traditional genotyping methods may be less effective (Cross et al., 2016). The integration of high-throughput genotyping with NGS has also facilitated the development of novel applications in clinical diagnostics, agrigenomics, and forensic science, further broadening the impact of these technologies (Pabinger et al., 2013).

6.3 Integration of multi-omics data for enhanced phylogenetic and classification accuracy

The integration of multi-omics data, including genomics, transcriptomics, proteomics, and metabolomics, has significantly enhanced the accuracy of phylogenetic and species classification studies. By combining data from multiple molecular levels, researchers can obtain a more comprehensive understanding of the evolutionary relationships and genetic diversity within and between species (Kumar and Kocour, 2017). NGS technologies play a crucial role in this integration, providing the high-throughput sequencing capabilities needed to generate large-scale multi-omics datasets. For example, the use of both mitochondrial and nuclear DNA sequencing has improved the resolution of phylogeographic studies, allowing for more precise identification of genetic structure and evolutionary history (Hancock-Hanser et al., 2013). Additionally, the application of NGS in systematics and population genetics has demonstrated the potential of multi-omics approaches to uncover novel insights into the genetic and biological significance of various species (Cross et al., 2016).

7 Future Directions in Molecular Marker Research for *Oryza*

7.1 Potential of CRISPR-based and other emerging molecular tools

The advent of CRISPR/Cas9 technology has revolutionized genome editing, offering unprecedented precision and efficiency in genetic manipulation. This technology holds immense potential for advancing molecular marker research in *Oryza* genomics. CRISPR/Cas9 allows for targeted modifications at specific genomic loci, facilitating the study of gene function and the development of new molecular markers (Figure 3) (Zhang et al., 2014; Adli, 2018). Recent advancements in CRISPR technology, such as the development of high-fidelity variants and base editors, further enhance its applicability by reducing off-target effects and enabling precise nucleotide changes.

Additionally, the use of CRISPR/Cas systems in plants, including rice, has demonstrated significant improvements in yield, stress tolerance, and biofortification, underscoring its potential for crop improvement. Future research should focus on optimizing CRISPR delivery systems and exploring its integration with other genomic tools to maximize its utility in *Oryza* genomics.

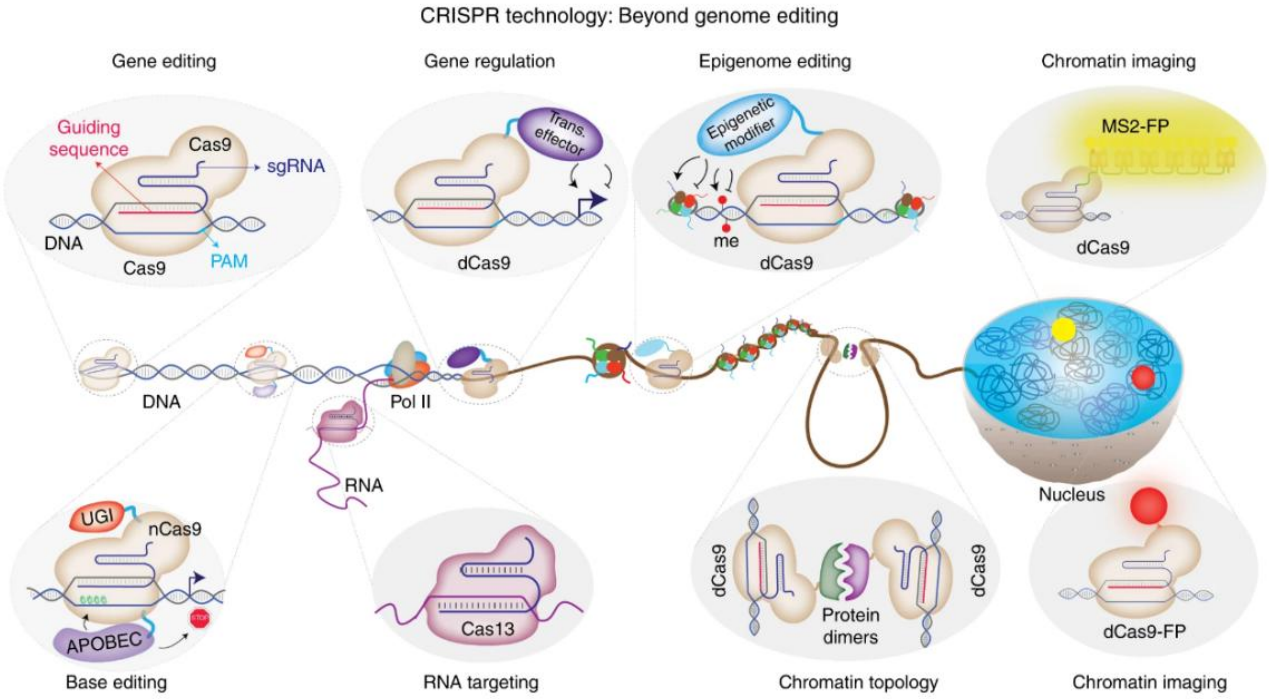

Figure 3 Major application areas of CRISPR-Cas-based technologies beyond genome editing (Adopted from Adli, 2018)

Image caption: While WT Cas9 enables genome editing through its guidable DNA cleavage activity, catalytically impaired Cas9 enzymes have been repurposed to achieve targeted gene regulation, epigenome editing, chromatin imaging, and chromatin topology manipulations. Furthermore, the catalytically impaired nickase Cas9 enzyme has been used as a platform for base editing without double strand breaks. In addition to DNA-targeting Cas proteins, novel RNA-targeting CRISPR/Cas systems have been described as well (Adopted from Adli, 2018)

7.2 Opportunities for integrating molecular markers with other genomic technologies

Integrating molecular markers with other genomic technologies presents a promising avenue for advancing *Oryza* genomics. The combination of CRISPR/Cas9 with high-throughput sequencing technologies can facilitate the identification and validation of novel molecular markers (Wang et al., 2017). Moreover, the integration of CRISPR-based epigenome editing tools can provide insights into the regulatory mechanisms governing gene expression, thereby aiding in the discovery of epigenetic markers (Nakamura et al., 2021). The use of CRISPR in conjunction with transcriptomics and proteomics can also enhance our understanding of gene function and interaction networks, leading to the identification of functional markers associated with important agronomic traits (Adli, 2018; Manghwar et al., 2020). By leveraging these integrated approaches, researchers can develop more comprehensive marker-assisted selection strategies, ultimately accelerating the breeding of improved rice varieties.

7.3 Challenges and prospects for future research in *Oryza* genomics

Despite the significant advancements in molecular marker research, several challenges remain in the field of *Oryza* genomics. One major challenge is the efficient and precise delivery of CRISPR/Cas9 components into plant cells, which is crucial for achieving high editing efficiencies and minimizing off-target effects (Leisen et al., 2020). Additionally, the complexity of the rice genome, with its high degree of genetic diversity and polyploidy, poses challenges for marker development and validation (Wang et al., 2017). Future research should focus on addressing these challenges by developing more efficient delivery methods, such as nanoparticle-based systems, and by employing advanced bioinformatics tools for accurate marker identification and validation (Manghwar et al.,

2020). Furthermore, the ethical and regulatory considerations surrounding the use of genome editing technologies in agriculture must be carefully addressed to ensure their safe and responsible application (Li et al., 2021). By overcoming these challenges, the prospects for future research in *Oryza* genomics are promising, with the potential to significantly enhance rice breeding and production.

8 Concluding Remarks

Molecular markers have proven to be invaluable tools in the classification and phylogenetic analysis of *Oryza* species. Techniques such as AFLP, ISSR, RAPD, and SSR have been extensively utilized to unravel the genetic relationships and evolutionary pathways within the genus *Oryza*. AFLP markers, for instance, have demonstrated the polyphyletic nature of *Oryza* evolution, revealing multiple independent lineages diverging from a common ancestor. Similarly, ISSR markers have provided insights into the genetic diversity and phylogenetic relationships among various *Oryza* species, highlighting the distinctiveness of species like *Oryza brachyantha* and *Oryza australiensis*. The use of multiple marker systems, including RAPDs, ISSRs, and SSRs, has further elucidated the genomic differentiation between wild and cultivated species, as well as between diploid and tetraploid genomes within the genus. These molecular markers not only facilitate the accurate classification of *Oryza* species but also enhance our understanding of their evolutionary history and genetic diversity, which is crucial for conservation and breeding programs.

The application of molecular markers in *Oryza* research holds promising potential for further advancements in species classification, phylogenetic studies, and genetic resource management. The development of new marker systems, such as INDEL markers, offers rapid and reliable discrimination of genome types, which can significantly improve the identification and conservation of wild *Oryza* species. Additionally, the integration of high-throughput sequencing technologies and phylogenomics approaches can provide more comprehensive and accurate phylogenetic reconstructions, as demonstrated by the use of nuclear genes and intergenic regions to resolve the phylogeny of AA-genome species. The continued exploration of genetic diversity through molecular markers will also aid in the discovery of novel alleles and haplotypes, which are essential for crop improvement and disease resistance. As the field progresses, the combination of traditional molecular markers with advanced genomic tools will undoubtedly enhance our ability to study and utilize the genetic wealth of the *Oryza* genus, ultimately contributing to sustainable agriculture and food security.

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Conflict of Interest Disclosure

The author affirms that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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