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Molecular markers in genetic diversity and hybrid purity testing in rice

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Abstract
Rice is an important food crop in the world. At present the availability of an array of molecular markers allows easy identification of genetic variability present among genotypes as well as species. There are enormous examples of use of molecular markers for hybrid/genetic purity testing in rice crop. The present review highlights applications of molecular markers for genetic diversity analysis as well as genetic/hybrid purity testing in rice.

Keywords: Rice, hybrid, molecular markers, diversity, seed purity

Introduction
Rice belongs to the genus *Oryza* of family Gramineae. The genus *Oryza* includes two cultivated species, namely *O. sativa* (2n=24 AA) grown worldwide and *O.glaberrima* (2n=24 AA) cultivated in a limited area of western Africa. The genus *Oryza* has 22 species (2n=24 or 48) representing AA, BB, CC, BBCC, CCDD, EE, FF, GG and HHJJ genomes (Vaughan, 1989 and Aggarwal *et al.*, 1997) [54, 2] grouped into four main species complexes, Sativa, Officinalis, Ridleyi and Meyeriama (Vaughan, 1989) [54]. The species *O. brachyantha* (FF genome) does not fall into any of these complexes (Aggarwal *et al.*, 1999) [3].

Rice is a well-known holder of two important titles: the most important food crop in the world and a model cereal species. It is a staple food of more than 60% of the world's population. It is primarily a high energy or high calorie food; it contains about 6-7% protein and 2-2.5% fat. Rice occupies a pivotal place in the global food and livelihood security systems. Of the annual world production of 596.485 m tons (paddy) from 155.128 m ha in 1998 -'99 Asia produced 540.621 m tons (90.63%) from 138.563 m ha (89.32%). Average rice yield in Asia was 3.9 T ha^{-1}, compared with 3.8 T ha^{-1} in the world, 6.4 T ha^{-1} in Japan, 6.3 T ha^{-1} in China, 2.9 T ha^{-1} in India, and 10.1 T ha^{-1} in Australia. Per capita consumption of rice in Asia ranges from 132 to 449 g per day. The world population will reach seven billion near the year 2010, eight billion near 2020, where 82% will live in the developing countries. The UN forecasts that the world population will reach 9.4 billion by 2050. It is projected that there will be only 1.2 billion more people in the developed regions of the world, compared to 8.2 billion in the currently less developed countries, with 5.5 billion of them in Asia and 2 billion in Africa. The world must develop the capacity to feed 10 billion within the next 40 to 50 yr, predominantly within Asia and Africa.

Among the rice growing countries, India has the largest growing area of about 44.8 million hectares followed by China and Indonesia with the production of about 131 million tones next to China *i.e.*, about 200 million tones. The projected increase in global population to 9 billion by 2050, require special attention of the rice breeders and agricultural scientists. To meet the global food demands, grain production will need to increase 50% by 2025 (Khush, 1999) [3].

Among all the crops grown in India, rice occupies the largest area (about 44.6 m hectares), which is also the largest in the world among all the rice growing countries. The country had a production of 139.14 million tons of paddy in 2006-07 with a productivity of 3.1 T ha^{-1}. The area under rice increased by 1.5 times, from 30 m hectares in 1950 to about 44 million hectares now; while during the same period the production increased by more than four times i.e. from 22 m tons to 92 m tons of rice (cleaned).

The productivity thus increased three folds i.e. from 0.7 T ha^{-1} to 2.1 T ha^{-1}. This spectacular progress helped India to achieve self-sufficiency in rice production during the early 80s. Among the available and immediately adoptable technologies, hybrid rice technology is one, which is likely to play a very pivotal role in achieving the targeted production increase. Potential of hybrid rice offers an opportunity to increase rice yields and thereby ensure a steady supply of food (Virmani and Kumar, 2004) [56] as per projection made for 2025. Research on hybrid rice in China began in 1964 (Yuan, 1966) [64]. The hybrid rice was developed in different stages in China after the famine condition faced from 1962 to 1964. Hybrid rice helped China to increase rice production by 200 million tons from 1976 to 1991. It has been proved practically on a large scale that hybrid rice has a yield advantage of more than 30% over conventional pure line varieties. Current research in China indicates that improving breeding methodology and achieving high degree of heterosis may enhance the yield potential of rice. Since most of the tropical rice-growing countries in Asia have a high population growth rate and limited land for rice cultivation, there must be an increase in production per unit area per unit time in order to obtain food security. Hybrid rice is one of the time-tested tools for meeting this objective. Rice is the principal food crop, grown across 18 countries feeding more than half of the world's population (Virmani, 1999) [57].

Recognizing the potential of hybrid rice to enhance production and productivity, Indian Council of Agricultural Research (ICAR) launched a mission mode project in December 1989, for the development and large-scale adoption of hybrid rice to sustain self-sufficiency in rice production. Hybrid rice project is functioning as national network with 12 centers spread across the country with Directorate of Rice Research, Hyderabad, as coordinating center.

It is estimated that annually more than 4000 tons of hybrid rice seed is produced in the Country, more than 95% of it is produced by the private sector. There has been progressive increase in the quantity of hybrid seed produced over the years but the progress was spectacular during the last four years. The requirement of hybrid seed during 2010 and 2020 is expected to be around 50,000 and 1,00,000 tons, respectively. In India hybrid seed is mainly produced in some parts of southern States during *rabi* (January - April) season because of congenial dry weather condition (Vijay Kumar, 1996) [55]. This hybrid seed is used for sowing to raise the coming *kharif* season crop. Sale price of hybrid seed produced by the private sector ranges from Rs. 120 – 155/- per kg of seed whereas that of public sector ranges from Rs. 75 – 120/- per kg. At present, the hybrid seed production in rice is primarily based on three-line system, which involves a cytoplasmic male sterile (CMS) line or A line, a corresponding iso-nuclear maintainer (B) line and a genetically diverse restorer (R) line. The A line is maintained by crossing it with B line and hybrid seed is produced by crossing A line with R line. This compound system makes it difficult to produce pure hybrid seeds constantly.

Assessment and maintenance of genetic purity of the parental lines and hybrids is crucial for the successful adoption of hybrid rice technology as 1% impurity in hybrid seed brings down the potential yield of hybrid by about 100kg ha^{-1} (Mao *et al.,* 1996) [26]. Cost of seed and its importance make assessment of seed purity, one of the most important quality control components in hybrid seed production.

Therefore, it is very important to manage the seed purity of hybrid varieties and their parental lines. The genetic purity of hybrids and their parents are estimated by Grow Out Test using descriptors. Before sowing, the plants are grown in the field in off-season to confirm the genetic purity based on the expression of morphological traits at plant level *viz.*, fertility, heading date, plant height etc. This test is conceived to verify that the designated cross occurred, the number of self or sib-pollinations between plants of the female parents meets the necessary purity required by law, and the product has an adequate quality (vigor and viability). This entails a lot of cost in terms of locked up capital, time consuming, space demanding and often does not allow the unequivocal identification of genotypes and problem of storage and timely supply of seed.

Methods for the identification of seed purity

The accurate, fast, reliable, and cost-effective methods for identification of seed purity of plant varieties is essential in plant research as well as for protecting plant breeders and farmers rights. Methods of varietal identification are most frequently based on assessment of a range of morphological characteristics or physiological traits expressed by seed, seedlings or mature plants. These methods are very effective for many purposes, although, morphological comparisons may have limitations including subjectivity in the analysis of the character, the influence of environmental or management practices on the character, limited diversity among cultivars with highly similar pedigrees and confining of expression of some diagnostic characters to a particular stage of development, such as flowering or fruit ripening.

The methods like electrophoresis of seed storage proteins, isozymes and molecular markers are most convenient for the analysis on the genetic homogeneity varieties and hybrids. As seed proteins/isoenzymes are products of gene expression these are considered as biochemical markers. The electrophoresis of seed protein/isozymes has been incorporated into the rules of International Seed Testing Association and has been described in Association of Seed Analysts *Cultivar Purity Testing Handbook* (AOSA, 1991) [4]. But these techniques fail to differentiate a number of cultivars in some crops like some vegetable and flower crops. Isozymes and restriction fragment length polymorphisms techniques are useful but have different drawbacks: often an insufficient number of developed isozyme systems are available for a particular species and, on the other hand, RFLPs have the limitation of their cost and complexity for routine commercial testing. Molecular marker is a better approach based on DNA polymorphism among tested genotypes, and thus applicable to biological research. Molecular markers are valuable as genetic markers because, they cover the whole genome with random distribution throughout the genome and, therefore, are much larger in quantity. There is more polymorphism in DNA markers, which are able to reveal the variation and allelism. Require fewer amounts of tissues. Many DNA markers are co-dominant and can differentiate between the homozygous and heterozygous genotypes. Furthermore, DNA markers are 'neutral', and they have no effect on phenotype, no epistatic effect, nondestructive assay, complete penetrance, highly reproducible, high genetic resolution, assay can be automated and independent to environmental stresses, management practices and developmental stages. Therefore, DNA marker is simple, quick and experimentally reproducible. It has been applied widely in the identification, registration of plant variety, and in monitoring of the seed purity and the authenticity with high accuracy, high reliability and low cost.

At present, the main DNA markers are RFLP, RAPD, AFLP and SSR. Among different classes of molecular markers, SSR's are the most suitable for such applications because of ease in handling, reproducibility, multiallelic nature, co-dominant inheritance, relative abundance and genomic wide coverage (Powell *et al.,* 1996) [33]. Molecular marker applications like large-scale genotyping of hybrid rice (Yashitola *et al.,* 2002) [61] and purity assessments demand rapid isolation of high quality genomic DNA in a cost effective manner from a large number of samples. Nine restriction fragment length polymorphism (RFLP) markers on chromosome 10, with Rf-1 gene were converted to PCR-based markers (Komori *et al.,* 2003) [20]. Based on the map information of the Rf-1 gene, it will be possible to introduce a limited chromosomal segment of the Rf-1 region derived from a donor into japonica rice varieties efficiently and effectively by marker-assisted selection (MAS) to facilitate examination of seed purity of hybrid rice varieties and their parental lines

Unambiguous identification of elite crop varieties and hybrids is essential for their protection and prevention of unauthorized commercial use. In India, this is highly relevant especially in rice because the hybrid seed production and marketing of public sector bred hybrids is largely taken up by the private sector. A set of qualitative and quantitative characters known as descriptors are currently in use for variety identification and description. Some of these characters, particularly those showing quantitative inheritance, interact with the environment in which the variety is grown and thus make the process of variety identification subjective. Molecular markers, in contrast, being based on DNA sequence variation, provide an unbiased means of identifying crop varieties. The Biochemical and Molecular Techniques group of the International Union for the Protection of New Varieties of Plants (UPOV) is evaluating different DNA marker parameters prior to its routine use in establishing distinctness, uniformity and stability (DUS) of plant varieties (Bredemeijer *et al.,* 2002; R¨oder *et al.,* 2002 and UPOV-BMT, 2002) [9, 52]. Among the various DNA based markers currently available, genetically mapped sequence tagged micro-satellite sites (STMS) are the markers of choice in rice because of their abundance, co-dominant nature and uniform distribution throughout the genome (McCouch *et al.,* 1997) [10]. Further, the nature of chromosome specificity, greater level of allelic diversity, high power of resolution, operational ease and low cost (Chen *et al.,* 1997 and Garland *et al.,* 1999) [10, 13] make the STMS markers more suitable for fingerprinting.

A random set of these mapped markers providing genome-wide coverage should facilitate an unbiased assay of genetic diversity and thus giving a robust, unambiguous molecular description of rice varieties. The working group on Biochemical and Molecular Techniques of the International Union for the Protection of New Varieties of Plants (UPOV) has in fact identified STMS as the most widely used marker system for plant variety characterization (UPOV, 2002) [56]. It was however, emphasized by this group that prior to the use of molecular markers in DUS testing, it would be essential to evaluate two of the important aspects: (i) correspondence between molecular patterns and phenotypic characteristics, and (ii) the uniformity and stability of the same molecular characteristics as used for distinctness (UPOV, 2002) [54].

Molecular markers in genetic diversity analysis of Rice

With the application of RAPD markers using 17 random oligonucleotides (Operon Technologies, USA) each of three hybrids of rice and their parents were distinguished, following a combination of selected primers for the purpose of Plant Variety Protection and for testing the genetic purity (Santhy *et al.,* 2003) [42]. Singh *et al*. (2003) [43] had determined the genetic diversity among 21 rainfed lowland rice genotypes. The genotypes included both landraces and improved cultivars, and were evaluated for important morphological characters and 47 RAPD markers. A RAPD assay for 47 random primers revealed a total of 275 alleles, 91% of which were polymorphic. The number of alleles detected by primers ranged from 1 to 17, with an average of 5.8, whereas gene frequencies ranged from 0.14 to 0.96, except for AA09, AC02, AB17, HI46, and F19. Gene diversity was high (0.53 to 0.85). Genetic diversity among 51 Sali rice accessions from Assam was characterized based on 72 RAPD markers (Dakshina and Sarma, 2004). 11 polymorphic primers showed a high degree of molecular variation with the range of polymorphic bands from 33 to 100%,. The Jaccard's similarity coefficient (0.515) indicated high level of diversity. The genetic diversity studied among bulk samples of 14 populations and individual samples of within two populations (Chengjun *et al.,* 2004) [24], using RAPD and ISSR amplification markers revealed that 44% natural populations of *O. granulata* in Yunnan had become extinct during the last 30 years whereas, of the 37 remaining wild populations, 28 (76%) was on the verge of extinction and nine (24%) were unaffected. The percentage of polymorphic bands (PPB) was 59% for RAPDs and 64% for ISSRs among the 14 populations. AFLP, ISSR and SSR marker systems generated higher levels of polymorphism among the 18 rice genotypes (traditional Basmati, cross-bred Basmati and non-Basmati) with distinguishability among all the cultivars (Saini *et al.,* 2004) [41]. The minimum number of assay-units per system required to distinguish all the cultivars was one for AFLP, two for ISSR and five for SSR. A total of 171 (110 polymorphic), 240 (188 polymorphic) and 160 (159 polymorphic) bands were detected using five primer combinations of AFLP, 25 ISSR primers and 30 well distributed, mapped SSR markers, respectively. Out of 16 ISSR primers tested to distinguish rice hybrids and their parents, 10 showed amplification among all the samples (Joshi *et al.,* 2004) irrespective of male or female parent. Six primers showed male parental lines useful in determining the true/selfed seeds. Twelve RAPD markers showed polymorphism among 54 cultivars with 78% polymorphic bands of 500-4000 bp nucleotide length (Vaesi *et al.,* 2005) [53]. Seven pairs of ISSR primers generated clear, reproducible 34 banding patterns with 53.0%, polymorphic bands were used to detect the genetic diversity of allelopathic potential in 57 rice accessions introduced from more than 10 countries (Lin *et al.,* 2005) [23]. Both SSR (0.51) and ISSR (0.46) primers showed almost similar values for polymorphic information content (PIC) in 3 landraces and 2 elite cultivars of rice (Prasad *et al.,* 2005) [29]. Maximum PIC value was observed with trinucleotide ISSR primers (0.67) followed by dinucleotide ISSR primers. RAPD primers could be successfully used with ISSR primers for the detection of new genomic loci and applied in a new way for genomic mapping, fingerprinting, and gene tagging as the primers of both elicit different genomic information in the same polymerase chain reaction (ChunJiang *et al.,* 2005) [30]. Molecular characterization of 32 Indian rice varieties of different agro-climatic zones resulted in mean heterozygosity value of 0.622, 0.819 and 0.890 over polymorphic loci and marker index value of 1.00, 6.75 and 4.16, respectively for RAPD, ISSR and STMS primers (Khandelwal *et al.,* 2005) [5]. The three

marker systems resulted in 201 polymorphic bands (94.36%) out of a total of 213 bands. The probability of a chance identical match between two varieties was very low (2.08x10-10) in combined molecular marker analysis as compared to individual marker system (RAPD, 7.5x10-4; ISSR, 1.5x10-3 and STMS, 3.9x10-6). Evaluation of genetic diversity among 103 aerobic rice accessions using 80 random primers showed polymorphism for 14 with reproducibility (Zargar *et al.*, (2007). A total of 81 RAPD and 201 ISSR markers were generated with 92.5 and 98% polymorphism respectively among 24 rice genotypes from Assam (Nipon-Bhuyan *et al.*, 2007) [30]. The average polymorphism information content (PIC) was 0.741 and 0.888for with 0.480 and 0.257 Jaccard's similarity coefficient for RAPD and ISSR markers, respectively. The nature of somaclonal variation at the nucleotide sequence level was studied in rice by Ngezahayo *et al.* (2007) [31] with the help of two molecular marker systems *i.e.*, RAPD and ISSR followed by sequencing of selected bands that represented genomic variations, and pairwise sequence analysis taking advantage of the whole genome sequence of rice. The genetic polymorphisms and identities of rice evaluated by RAPD showed that 25 decamer-primers could generate a total of 208 RAPD fragments, of which 186 or 89.4% were polymorphic (Rabbani *et al.*, 2008) [35]. The number of amplification products produced by each primer varied from 4 to 16 with an average of 8.3 bands primer-1. The size of amplified fragments was ranged from 200 to 4000 bp. The average similarity index in 63 deep-water rice accessions based on RAPD and ISSR was 0.325%, indicating a high level of genetic diversity among the accessions (Dakshina and Sarma, 2008) [12].

Microsatellites in genetic diversity analysis and hybrid purity testing of Rice

With the advent of DNA marker technology, several types of DNA markers like RAPD, ISSR, RFLP, etc now available, but SSR markers are widely used in comparison to other types of markers because they are reproducible, co-dominant, species specific and highly polymorphic. Microsatellites (Litt and Luty, 1989) [25] are tandemly arranged repeats of short DNA motifs (1-6 bp in length) that frequently exhibit variation in the number of repeats at a locus. Microsatellite (or simple sequence repeats-SSR), (Akkaya *et al.* 1992) [1] markers have been proposed for gene mapping in species. RAPD and ISSR were widely used markers initially for genotyping and purity *e.g.*, *Brassica*, tomato, chili, cereals, papaya etc. After the development of SSR marker, become the first choice for genotyping and genetic purity as highly reproducible and transferability nature of the marker. SSR markers have been effectively used in rice, sorghum, chillies, tomato and citrus etc. Canada Grain Commission has developed a catalogues of DNA fingerprints of all the registered 67 two-rowed varieties of barley and identified 12 sequence tagged-site (STS) markers for discriminate all the two-rowed barley. Similarly, for olive varieties, a set of 12 SSR markers can discriminate 27 olive varieties grown in Istria. Studies in rice indicate that microsatellite markers are highly polymorphic (Wu and Tanlsley, 1993, Zhao and Kochert, 1993 and Yang *et al*., 1994) [65, 67]. Several hundred microsatellite markers and a genetic map consisting of approximately 2,740 SSR markers have been genetically mapped in rice, *i.e.*, about one SSR marker every 157 kb (Chen *et al.*, 1997, Temnykh *et al.*, 2000 & 2001 and McCouch *et al.*, 2002) [10, 28]. Ninety-four newly microsatellite markers which were integrated into existing RFLP framework maps of four rice populations, including two doubled haploid, a recombinant inbred, and an interspecific backcross population had developed by Chen *et al.* (1997) [10]. This simple sequence repeats (SSR) were predominantly poly (GA) motifs, targeted because of their abundance in rice. Mapping of the 94 newly developed markers as well as 27 previously reported microsatellites provided genome wide coverage of the 12 chromosomes, with an average distance of 1 SSLP (simple sequence repeat polymorphism) per 16-20 cM.

Microsatellite markers (312) of different SSR motifs were relatively uniformly distributed over rice genome with an average density of one SSLP per 6 cM, regardless of whether they were derived from genomic clones or cDNA sequence (Temnykh *et al.*, 2000) [54]. Genetic polymorphisms of 10 microsatellite DNA loci among 238 accessions of landraces and cultivars that represent a significant portion of the distribution range for both *Indica* and *Japonica* groups of cultivated rice was studied by Yang *et al.* (1994) [66]. In all, 93 alleles were identified with ten markers. The linkage map was constructed containing 108 SSR markers distributed throughout the whole 12 chromosomes with average marker interval of 16.26 cM (Yang *et al.*, 2005) [55]. Molecular markers are potent tools to identify genetic relatedness effectively and efficiently (Kresovich *et al.*, 1992) [34]. SSR's (Temnykh *et al.*, 2001 and Temnykh *et al.*, 2000) [5, 4] markers have been used to analyze diversity and to locate genes and QTLs on rice chromosomes using both intra and inter specific crosses (Bao *et al.*, 2000 and Moncada *et al.*, 2001) [1, 5, 4]. SSRs are increasingly useful for integrating the genetic, physical, and sequence based maps of rice and they simultaneously provide information to link phenotypic and genotypic variation efficiently.

For seed commercialization of hybrid rice, 160 microsatellite markers were used for PCR amplification of rice seedling DNAs of Gangyou-22, which is a major hybrid rice in China, and its parents, Gang46A(CMS line) and CDR22(restorer line). A microsatellite marker, RM168, was screened out for its ability to produce polymorphic bands specific to each of the two parents but different from other 22 restorer lines and 9 cultivars. Li *et al*. (2000) [68]. Computational and experimental analysis of microsatellites in rice for frequency, length variation, transposon associations, and genetic marker potential had performed by Temnykh *et al.* (2001) [59]. A set of 200 class ISSR markers was developed and integrated into the existing microsatellite map of rice, providing immediate links between the genetic, physical, and sequence based maps. Their contribution brought the number of microsatellite marker that has been rigorously evaluated for amplification, map position, and allelic diversity in *Oryza* spp. to a total of 500. A total of 2414 new di, try and tetra-nucleotide non-redundant SSR primer pairs, representing 2240 unique marker loci, which had been developed experimentally validated for rice. Duplicate primer pairs are reported for 7% (174) of the loci. Using electronic PCR (e-PCR) to align primer pairs against 3284 publicly sequenced rice BAC and PAC clone containing at least one genetically mapped marker and could be mapped by proxy (McCouch *et al.*, 2002) [28].

Twenty-five fluorescent-labelled SSR markers were used to genotype 242 accessions of the Brazilian Rice Core Collection. Based on the Polymorphism Information Content (PIC) values of each marker, groups with 5, 10, 15 and 20 markers were formed. The average PIC values varied from 0.67 to 0.89 and the number of alleles sampled per group, from 70 to 377 (Borba *et al.*, 2005) [7]. A total of 58 DNA

simple sequence repeat (SSR) markers were selected to detect polymorphisms among 28 parental lines of commercial F< sub>1</ sub> hybrid rice in China. Fourteen of the markers were assayed using agarose gel electrophoresis and non-denaturing PAGE (Shi-Yong Feng *et al.,* 2005) [44]. Ten specific primers were screened and selected to identify subspecies between *indica* and *japonica* by He-HaoHua *et al.* (2006) [16] and to classify some sterile lines and their restorers of hybrid rice with SSR markers. Rajendra Kumar *et al.* (2006) [38] reported abundant simple sequence repeats (SSRs) across genomes. However, the significance of SSRs in organellar genomes of rice has not been completely understood.

Microsatellites are abundant across prokaryotic and eukaryotic genomes. However, comparative analysis of microsatellites in the organellar genomes of plants and their utility in understanding phylogeny has not been reported. The purpose of this study was to understand the organization of microsatellites in the coding and non-coding regions of organellar genomes of major cereals *viz*., rice, wheat, maize and sorghum (Rajendra Kumar *et al.,* 2008) [37]. A genetic linkage framework map was constructed by Zhang *et al.* (2006) [59] with the application of new markers from the F_2 population of 90 lines derived from a single cross between *Japonica* and *Indica* rice varieties. Comparison of coding region SSR markers and non-coding region SSR markers on 60 genotypes of rice using 17 pairs of coding region SSR markers and 12 pairs of non-coding region SSR markers exhibited an average of 3.59 polymorphic loci with an average polymorphism information content (PIC) value of 0.447 (Li-YuGe *et al.,* 2007) [23]. Simple sequences repeat assays with 37 primer pairs were performed, on 37 maintainer and 44 restorer lines of the wild abortive (WA) CMS system of different origins (IRRI, Philippines and China) used in the tropical rice breeding programme (Xu *et al.,* 1999) [60]. The seed purity of hybrid rice is estimated conventionally by Grow out Test (GOT), based on assessment of morphological and floral characteristics in plants grown to maturity. To replace the GOT with DNA based assays, cytoplasmic male sterile (CMS), restorer, and hybrid lines were screened by means of microsatellite and sequence tagged site (STS) polymorphisms (Yashitola *et al.,* 2002) [61] on 6-days old seedlings. It could be used for detection of off-types in hybrid seed lots. SSR markers were successfully used in discriminating the DNA profiles and seed quality of the rice hybrid (Gao-FangYuan, 2002) [69]. Six rice hybrid combinations (HCs) and their corresponding parents were used to study their polymorphic patterns (PP) by using microsatellite markers. Number of primers displayed polymorphism on 20, 21, 22, 25, 32 or 37 pairs. The purity grades of seeds (adulterated with the seeds of their parents) were identified by 2 pairs of polymorphic primers (Zhan, 2002). He advocated microsatellites for identifying the purity of hybrid seeds because of rapid, simple and low cost method on DNA extraction with highly accurate, reliable, highly polymorphic, high stability and easy operation. Rice hybrids and their parental lines may be Identified following a combination of selected RAPD markers *i.e.,* oligonucleotides (Operon Technologies, USA) for the purpose of Plant Variety Protection and testing the genetic purity of A line and hybrid seed lots (Santhy *et al.,* 2003) [42]. Twenty-six simple sequence repeat (SSR) primers from 12 rice chromosomes were able to distinguish all restorer lines and a majority of the sterile lines of nine major hybrid rice combinations and their parents (Peng *et al.,* 2003) [32]. The bands of hybrid rice were complementary with their parents, which were adapted to examine the purity of hybrid rice seeds. The primer RM17 was selected for SSR identification with 100 single seeds.

Based on analysis of mitochondrial DNA sequences of CMS and fertile rice, the orf61-atp6-orf79 region of the rice mitochondrial genome revealed two regions polymorphic among IR58025A (CMS), its B line (IR58025B), and hybrid (Mestizo). The a6P5 and MdF1 primers designed to amplify these regions produced 300-bp and 500-bp bands, respectively, that discriminated the B line from the CMS line and the hybrid. Successful detection of B line off-types in mixtures was demonstrated using the bulked DNA assay. This technique is starting to be used by the Philippine National Seed Quality Control Services for seed purity testing in the hybrid commercialization program. (Sundaram *et al.* 2008) [38]. Out of 10 sequence tagged microsatellite sites (STMS) markers nine STMS markers were found polymorphic across the 11 hybrids with unique fingerprint. A set of four markers (RM 206, RM 216, RM 258 and RM 263) differentiated all the hybrids and parental lines from each other (Nandkumar *et al.,* 2004) [21].

Three dominant polymerase chain reactions (PCR)-based markers (M1, M2 and M3) were developed to detect mutual contamination in seed batches of CMS lines, maintainer lines, restorer lines and hybrids (Komori *et al.,* 2004) [20]. By the strategic use of these markers, japonica hybrids and their parental lines could be efficiently distinguished from each other. The cytoplasmic male sterile (CMS) lines that are utilized for developing the popular “three-line” hybrids often get contaminated with their isonuclear maintainer lines during CMS line multiplication. Use of such CMS lines in hybrid seed production results in the production of genetically impure hybrid seed. In a polymerase reaction (PCR) using total genomic DNA as a template, oligo- nucleotide primers based on this unique DNA sequence could amplify a fragment from CMS lines of rice and their hybrids but not from their cognate maintainer lines (Yashitola *et al.,* 2004) [62].

Micro-satellite or simple sequence repeat (SSR) marker analysis was carried out to assess allelic diversity and prepare a DNA fingerprint database of 24 rice genotypes, including three premium traditional Basmati, 9 cross-bred Basmati, a local scented selection, eight *indica* and three *japonica* rice cultivars. A total of 229 alleles were detected at the 50 SSR loci and 49 alleles were present in only one of the 24 cultivars. The size difference between the smallest and largest allele varied from 1 (RM333) to 82 (RM206). A number of SSRs have been identified, which can be used to differentiate the traditional Basmati cultivars and between traditional Basmati and other crossbred Basmati or long grain, non-Basmati rice cultivars (Priyanka *et al.,* 2004) [34]. Five super hybrid rice combinations and their parental lines were tested by means of 144 SSR primer pairs distributed on 12 rice chromosomes. Out of which 47 detected polymorphism whereas primers, RM337 and RM154 produced polymorphic patterns in four or more of the tested experimental materials respectively that could distinguish among most of the tested genotypes (XIN Ye-yun, 2005) [69]. SSR has become an ideal molecular marker for purity identification of hybrid rice seeds, especially for the identification of closely-related varieties because it is abundant in number, high in polymorphism, co-dominant in genes, simple in experimental operation, reliable in results and easy in exchange of primer sequence (Li-ZhaoHua, 2006) [16]. Sixty-five out of 66 varieties of rice, including 5 photoperiod/thermo-sensitive genetic male sterilie lines, 7 cytoplasmic male sterile lines, 53

restorers and one wild rice variety, were distinguished using 36 SSR (simple sequence repeats) primer pairs which disperse on 12 chromosomes (He-Hao Hua, 2006). Ten specific primers were screened and selected to distinguish subspecies *indica* and *japonica*. A total of 208 simple sequence repeat (SSR) markers were used to identify 42 parental lines of hybrid rice. Genetic polymorphism was detected using 123 primers. Genetic diversity was higher in chromosome 9 and 10, and lower in chromosome 12 (Xiao-Xiao Yu, 2006) [59]. Twelve out of 10 primers showed polymorphism between parental lines. The seed purity of Dyou 527 was confirmed using the SSR primers RM337, RM244 and RM346. The lines Dyou 527 and Dyou 68 were distinguished using the SSR primers for Dyou 527. With the objective of identifying SSR markers that can distinguish parental lines of rice hybrids; Sundaram *et al.*, (2008) [38], characterized 10 each of cytoplasmic male sterile (CMS) and restorer (R) lines along with 10 popular Indian rice varieties using a set of 48 hyper polymorphic SSRs distributed uniformly across the rice genome. All the SSR markers were polymorphic. Twenty-seven SSR markers showed amplification of an allele, which was very specific and unique to a particular parental line and not amplified in any other rice genotype tested. Through multiplex PCR, SSR marker combinations that were unique to a particular parental line or hybrid were also identified. With a set of 10 SSR markers, all the public bred Indian rice hybrids along with their parental lines could be clearly distinguished.

Studies were conducted to evaluate the genetic diversity of major commercial rice cultivars in China. A total of 63 conventional rice cultivars and parental lines of hybrid rice crosses were collected in China (Ying-Jie Zheng, 2007) [63] and assayed using a set of 24 microsatellite markers (SSRs) distributed on the 12 rice chromosomes (2 markers in each chromosome). By using the 24 SSR markers, a total of 135 alleles were detected from all the tested cultivars (5.6 alleles per marker). Genetic diversity among the conventional *indica* cultivars was higher compared to the conventional *japonica* cultivars. Based on these results, the trends in the genetic relationship of the different rice cultivars in China constructed by the use of SSR markers were almost identical or more accurate than results based on pedigree analysis.

Simple sequence repeat (SSR) allele polymorphism of 6 hybrids and their parents was studied by analysing 40 SSR loci distributing on 12 chromosomes in rice (Shi-Kuan Yu, 2005) showed that SSR locus RM224 could discriminate F_1 and their parents in all the 6 hybrids. A total of 58 DNA simple sequence repeat (SSR) markers were selected to detect polymorphisms among 28 parental lines of commercial F_1 hybrid rice in China. Fourteen of the markers were assayed using agarose gel electrophoresis and non-denaturing PAGE. Each of the rice lines was distinguished with significant variations in the frequency of polymorphism among the markers. Similar results were generated by agarose gel electrophoresis and non-denaturing PAGE. Higher resolution by non-denaturing PAGE advocated feasibility for identification of cultivar. On average, the probability of distinguishing the rice lines by detection with 12 SSR markers was higher than 99.9%, but markers of higher variation and/or a higher number of markers may be required for cultivars with high genetic similarity (Shi-Yong Feng, 2005) [44].

Molecular marker applications like large-scale genotyping, hybrid rice (Yashitola *et al.*, 2002) [61] and Basmati (Bligh 2000) [8] purity assessments demand rapid isolation of high quality genomic DNA in a cost effective manner from a large numbers of samples. An ideal DNA isolation method should require only a small amount of tissue, involve simple procedures, use a minimal number and amounts of chemicals, should be rapid and yield reasonably good quality as well as quantity of DNA. Rajendrakumar *et al.* (2007) [36] had standardized a modified Cetyl trimethyl ammonium bromide (CTAB) based procedure for isolation of high quality and quantity of DNA from single rice grain, seed and leaf tissue for deployment in hybrid seed and Basmati grain purity assessments and also for rapid genotyping in marker-assisted breeding programmes. Diverse data sets including morphology (Bar-Hen *et al.*, 1995) [5], isozymes (Hamrick and Godt, 1997) [15] and storage protein profiles (Smith *et al.*, 1987) [46] have been used to assess genetic diversity among parental lines. The utility of DNA markers has been suggested for precise and reliable characterization and discrimination of genotypes (Karkousis *et al.*, 2003) [18]. Among different classes of molecular markers, SSRs are the most suitable for such applications because of the ease in handling, reproducibility, multiallelic nature, codominant inheritance, relative abundance and genome-wide coverage (Powell *et al.*, 1996) [33]. Determining genetic diversity among rice genotypes and purity of the hybrids are important. It is an essential requirement to analyze purity of seed before it is sold commercially for cultivation in the field for more productivity as well as production. There are chances of contamination of the hybrid seed production plot through various means. Physical mixtures during the subsequent handling of the harvested materials (especially during threshing, drying, cleaning, grading, bagging) also facilitate contamination. So, molecular markers provide a platform to test hybrid purity of rice and among all markers microsatellites have been found to be superior due to their co dominance character.

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