Methods for Development of Microsatellite Markers: An Overview

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Abstract

Microsatellite or Simple Sequence Repeat (SSR) markers have evolved to the status of a most versatile and popular genetic marker in a ubiquity of plant systems. Due to their co-dominant, hyper-variable and multiallelic nature, they are the prominent markers of choice for fingerprinting, conservation genetics, plant breeding and phylogenetic studies. Despite its development of a new set of SSR markers for a species remained time consuming and expensive for many years. However, with the recent advancement in genomics, new strategies/protocols are now available for the generation of SSR markers. This review presents an overview on microsatellite markers with a special emphasis on the various strategies used for the development of microsatellite markers.

Keywords: Chloroplast SSR, EST-SSR, Genetic diversity, Genomic SSR, Microsatellite isolation strategies, Simple Sequence Repeats

Introduction

Microsatellites are stretches of DNA consisting of tandemly arranged units of 1-6 bp (Gupta et al., 1996; Thiel et al., 2003), characterised by their co-dominant inheritance, wide genomic distribution, hyper variable and multiallelic nature (Powell et al., 1996; Parida et al., 2009). They are also termed as simple sequences (Tautz et al., 1996), Short Tandem Repeats (STRs) (Edwards et al., 1991) and Simple Sequence Repeats (SSRs) (Jacob et al., 1991). The existence of microsatellites in a wide range of evolutionarily diverse eukaryotic genomes (from yeasts to humans) was first documented by Hamada et al. (1982). Tautz and Renz (1984) further confirmed their abundance in five phylogenetically distinct eukaryotes including human, Drosophila, yeast, sea urchin and Stylonychia. The polymorphic nature of microsatellite was revealed by Litt and Luty (1989) by amplifying (TG)_n microsatellites in the human heart actin gene and detecting allelic variants in unrelated individuals. Weber and May (1989) also reported the successful amplification and polymorphic nature of SSR loci. The genesis of these repeats occur primarily due to slipped-strand mispairing (Levinson and Gutman, 1987) and subsequent errors during DNA replication/ repair/ recombination (Schlotterer and Tautz, 1992; Katti et al., 2001), nucleotide composition of repeat motifs (Katti et al., 2001) or unequal crossing-over between sister chromatids (Innan et al., 1997). However, a minimum size threshold (eight nucleotides or more) is crucial for the dynamic slippage mutation of microsatellites (Messier et al., 1996; Rose and Falush, 1998).

Microsatellites are ubiquitous in the coding and noncoding regions (Tautz and Renz, 1984; Gupta et al., 1996; Toth et al., 2000) with a higher density of simple sequence motifs in the noncoding regions of eukaryotes (Hancock, 1995; Li et al., 2002). In plants, SSRs are much more abundant and preferentially associated within untranslated regions (UTRs) of the transcribed regions (Morgante et al., 2002). The frequency of repeats decrease exponentially with repeat length (Metzgar et al., 2000; Katti et al., 2001) with their informativeness correlating to the repeat length (Lagercrantz et al., 1993). This might be due to the fact that longer microsatellites exhibits higher mutation rates than shorter ones (McConnell et al., 2007).

Among the different types of SSRs, dinucleotide, trinucleotide and tetrancotide repeats are most frequently used in molecular genetic studies (Selkoe and Toonen, 2006). Dinucleotide repeats constitute the majority of microsatellites reported from many species and are less frequent in coding region than in non-coding region (Li et al., 2002). In contrary, trinucleotides are more abundant in the coding regions of the genome (Toth et al., 2000). In plants, monocots are enriched with GC-rich trinucleotide repeats than dicots (Morgante et al., 2002). The relative abundance of trinucleotide repeats in the protein-coding regions of all taxa is attributed to the negative selection against frameshift mutations occurring in coding regions and probably positive selection for specific single amino-acid stretches (Metzgar et al., 2000).
Types of microsatellites

Based on occurrence and source for development

Three types of SSRs:

a. Genomic or nuclear microsatellites (gSSRs) - microsatellites isolated from the nuclear genome (genomic DNA of an organism with or without the construction of genomic DNA library).

b. EST or genic microsatellites (EST-SSRs) - microsatellites developed by data-mining or exploiting EST sequences deposited in public databases.

c. Organellar microsatellites [chloroplast SSRs (cpSSRs) and mitochondrial SSRs (mtSSRs)] - microsatellites developed from the chloroplast or mitochondrial genome of an organism.

Based on the type of repeat sequence

Four types of SSRs (Oliveira et al., 2006):

a. Perfect microsatellite- the repeat sequence is continuous and is not interrupted by any base not belonging to the motif [e.g. AGAGAGAGAG or (AG)n] .

b. Imperfect microsatellite- a pair of bases is present between the repeat motif that does not match the motif sequence [e.g. AGAGAGAGCTAGAG or (AG)nCT(AG)m] .

c. Interrupted microsatellite- a small sequence within the repeated sequence that does not match the motif sequence [e.g. AGAGAGAGCGTAGAG or (AG)nCGTG(AG)m] .

d. Compound/ composite microsatellite- two adjacent distinctive repeats present within the sequence [e.g. AGAGAGAGCTCTCT or (AG)n(CT)n] .

Based on the length of repeat motif

Two types of SSRs (Temnykh et al., 2001):

a. Class I microsatellites- perfect SSRs of \( \geq 20 \) nucleotides in length.

b. Class II microsatellites- perfect SSRs of \( \geq 12 \) nucleotides and \( \leq 20 \) nucleotides in length.

Microsatellites and their influence on molecular functions

The presence of SSRs in the coding regions lead to the appearance of repetitive patterns in the aminoacid sequences (Katti et al., 2001) and thus involve in regulating gene expression or molecular functions. Occurrence of SSRs in the promoter region influences transcriptional activity (Kashi et al., 1997), whereas their presence in non coding regions influences gene regulation, transcription (Martin et al., 2004; Lawson and Zhang, 2006) and recombination events (Bagshaw et al., 2008). The over-representation of CT/GA and CTT/GAA repeats in the 5′-flanks of Arabidopsis thaliana suggest their potential involvement in regulating gene expression (Zhang et al., 2004). The (GA)n repeats in promoters govern the regulation of certain plant genes (Meister et al., 2004) and exhibit protein-binding affinity (Kooiker et al., 2005). The CT/GA repeat variation in the 5′ UTR of the waxy gene is correlated with amylose content in rice (Bao et al., 2002). In maize, presence of (CCG)n in the 5′ UTR of ribosomal protein genes regulate fertilization (Dresselhaus et al., 1999). Poly-stretches of glutamine (Gerber et al., 1994) and proline (Perutz et al., 1994) encoded by rapidly evolving repeats are known to modulate the activity of transcription factors. Similarly, the presence of the trinucleotide repeats like (GAA)n within 5′UTR of ntp303 regulate transcription and translation (Hulzing et al., 2002).

General advantages and disadvantages of microsatellite markers

Though microsatellite markers are considered to be robust, there are also advantages and disadvantages associated with the level of polymorphism and mode of application.

Microsatellites are more variable and informative than RFLP, RAPD (He et al., 2003) and AFLPs (Lee et al., 2004). The technique is PCR-based, thus require only low quantities of template DNA (Kumar et al., 2009; Wolko et al., 2010). The application of lengthy primers and high annealing temperatures during genotyping enhances reproducibility. The ability to use more than one set of optimized SSR markers in a single reaction (multiplexing of markers) significantly reduces the analytical costs involved in genome analysis. They are also useful for parentage analysis and for estimating the degree of relatedness of individuals or groups. Multiallelic microsatellites are considered to be the best marker system for the detection of intervarietal polymorphisms (Stepien et al., 2007). They offer wide applications in the preparation of genome-wide genetic maps and comparative mapping.

However, the de novo development of SSR marker is expensive, laborious and time-consuming (Zane et al., 2002; Squirrell et al., 2003; Thiel et al., 2003). Low frequency of SSRs in plants also hinders the large scale isolation of SSRs (Powell et al., 1996). Moreover, SSR markers developed for one species generally exhibit less transferability across same or different taxa which necessitate the development of species specific markers (Roa et al., 2000; Kindiger, 2006). Another important problem associated with microsatellites is the occurrence of null alleles. The potential cause is poor primer annealing caused by nucleotide sequence divergence, inconsistent DNA template quality or low template quantity (Ellegren, 2004) or mutations/indels in the primer binding sites (Pemberton et al., 1995). This leads to complications in the determination of allelic and genotypic frequencies and an underestimation of heterozygosity (Kumar et al., 2009). Homoplasy is another problem when applying microsatellites as a reliable tool for phylogenetic analysis because alleles considered to be identical in state are not necessarily identical by descent (Estoup et al., 2002).

Strategies for microsatellite development

For generating a new set of polymorphic SSR marker for species, microsatellite repeats should be isolated or identified along with sufficient flanking nucleotide sequence information to facilitate primer designing. The
PCR conditions need to be optimized and the primers need to be screened in a set of related and non-related individuals for estimating their polymorphic potential.

The protocol for the "de novo" isolation of microsatellite markers was first described by Rassmann et al. (1991), who identified SSR-containing clones by colony hybridization with SSR probes. However it turned out to be laborious and expensive for species with low frequency of SSRs. The strategies used for the effective isolation of SSR loci were reviewed earlier by Zane et al. (2002) and Kalia et al. (2011). However, with the advancement in genomics, availability of new molecular tools and sequencing platforms for exploring genomic information, several new protocols were developed in the recent years. A general outline on the development of microsatellite markers is summarized (Fig.1) and could be achieved either by:

a) constructing and screening SSR enriched/non enriched genomic libraries or by utilizing the products generated by other molecular markers or by the application of next-generation sequencing systems (gSSRs).

b) exploiting the EST/chloroplast sequences deposited in the public domain (EST-SSRs/ cpSSRs) or sequencing PCR products generated by "consensus/universal chloroplast primers" (cpSSRs).

d) testing the amplification potential of SSR markers developed in other related species (transferability/ cross-species amplification).

Genomic SSR markers

Development of microsatellite markers from SSR-enriched genomic DNA libraries

Briefly, the methods used for isolating SSRs by constructing genomic libraries can be grouped into two categories.

i) Selective hybridization methods

These methods facilitate the selective isolation of microsatellite containing DNA portions of the genome by hybridisation with repeat-specific probes. These protocols generally involve the fragmentation of DNA either by sonication (Karagyozev et al., 1993; Kandpal et al., 1994; Geng et al., 2010) or restriction enzymes (Brown et al., 1995; Chen et al., 1995; Edwards et al., 1996; Prochazka, 1996; Refseth et al., 1997; Fischer and Bachmann, 1998; Hamilton et al., 1999; Glenn and Schable, 2005; Nunome et al., 2006) or nebulisation (Kumpatla et al., 2004; Connell et al., 1998) and its subsequent ligation to a known sequence (linker or adaptors) or directly to a vector. DNA is then denatured and subjected to enrichment by hybridization with:

a) biotinylated oligos followed by capture of biotinylated hybrids (oligo bound DNA fragments) in vectrex-avidin matrix (Kandpal et al., 1994) or

b) oligonucleotides bound to nylon membrane (Karagyozev et al., 1993; Chen et al., 1995; Edwards et al., 1996) or

c) 5′ biotinylated repeat oligos and subsequent capture of biotinylated hybrids by streptavidin coated magnetic beads (Brown et al., 1995; Refseth et al., 1997; Fischer and Bachmann, 1998; Connell et al., 1999; Hamilton et al., 1999; Kumpatla et al., 2004; Dixit et al., 2005; Glenn and Schable, 2005; Nunome et al., 2006; Geng et al., 2010) or

d) biotinylated SSR probe-streptavidin coated magnetic bead complex (Triplex affinity capture protocol; White and Powell, 1997).

The enriched DNA fragments were then amplified, either cloned and sequenced or sequenced directly and searched for the presence of SSR motifs. The efficiency of this approach entirely depends on the specific binding of streptavidin coated beads to the biotin labelled DNA fragments harbouring SSRs.

ii) Primer extension methods

These methods permit selective amplification of microsatellite containing genomic DNA using SSR specific primers (Ostrandr et al., 1992; Pandolfo, 1992; Robic et al., 1994; Paetkau, 1999). This procedure (Ostrandr et al., 1992; Paetkau, 1999) relies on the construction of a primary genomic library in phagemid vector to recover the library as single stranded DNA (ssDNA) which is subjected to primer extension using repeat specific non-biotinylated oligos (Ostrandr et al., 1992) or 5′ biotinylated oligos (Paetkau, 1999). These primer extension steps that selectively generate double stranded products only from vectors containing the desired repeats were transformed into E. coli cells (Ostrandr et al., 1992). The primer extended products generated using 5′ biotinylated oligos (Paetkau, 1999) were selectively captured using streptavidin coated magnetic beads and converted to double stranded DNA (dsDNA) by a second round of primer extension for transformation.

Another protocol based on primer extension (Pandolfo, 1992) involved the ligation of a vectorrete (linker containing a non-complementary region) to restricted YAC (Yeast Artificial Chromosome) DNA. The vectorrete-ligated DNA was amplified (using repeat specific primer along with a universal vector primer) and the products were cloned and sequenced for detecting SSRs. A modified version of this approach termed as the ‘SLiM-PCR’ (Subcloning Ligation Mixture-PCR; Robic et al., 1994) allowed the sequencing of flanking regions of a microsatellite from a cosmid clone using a fluorescent automatic sequencing method without sub-cloning.

Among the different enrichment protocols available, selective hybridization capture is the predominantly used strategy as it allows enrichment and selection prior to cloning thereby providing a faster and easier method to work with multiple samples (Glenn and Schable, 2005). Moreover, it is relatively simple, reproducible and cost effective approach for isolating microsatellites from diverse plant species with higher efficiency (Kalia et al., 2011).

Development of microsatellite markers from non-enriched genomic DNA libraries

In this method, the genomic DNA was restricted, ligated into suitable vectors and transformed to generate a non-enriched genomic DNA library. Clones were then spotted onto gridded nylon filters and screened with
radiolabelled SSR probes or subjected to enrichment with ‘biotin labelled probes-streptavidin capture system’ and sequenced. Cloning of DNA fragments prior to enrichment steps makes it ideal to screen for a wide range of SSR motifs and reduce/avoid redundancy when compared to enrichment protocols. This method was successfully employed for the isolation of SSR markers from few crops like Citrus limon (Golein et al., 2006) and Phaseolus vulgaris (Blair et al., 2009).

Utilization of PCR based molecular markers for generating microsatellites

**SSRs derived from RAPD markers**

This method relies on the fingerprinting and subsequent blotting of the RAPD amplicons to nitrocellulose membrane, followed by the screening of positive clones by digoxigenin labelled probes and its detection by autoradiography (Random amplified hybridization microsatellites [RAHM]; Cifarelli et al., 1995). Some methods utilise labelled SSR probes and chemiluminescent system for the detection of SSRs (Ender et al., 1996) while others facilitate cloning and further screening of intense RAPD bands in duplicate colony PCR (with vector specific and repeat specific probes) for identifying SSR containing clones followed by direct sequencing [PCR isolation of microsatellite arrays (PIMA) approach; Lunt et al., 1999].

**SSRs derived from ISSR/SSR amplicons**

These methods are based on the fact that ISSR primers bind specifically to SSRs and facilitate the amplification of genomic DNA between two distinctly placed SSRs, providing an opportunity to design primers (based on the flanking regions) by cloning and sequencing of ISSR amplicons. The ‘dual suppression method’ (Lian et al., 2001; Lian and Hogetsu, 2002) involved cloning and sequencing of ISSR amplicons and initiating a nested PCR (with primers ‘IP1’- designed from the region flanking the microsatellite sequence and ‘IP2’- sequence between IP1 and the microsatellite sequence). Adaptor-ligated, restricted DNA libraries were then constructed using restricted DNA fragments ligated to a blunt ended suppression PCR adaptors (a 48mer and a complementary 8mer capped with amino residue). Two adapter specific primers (AP1 and AP2), containing portions of the 48mer were designed and used for nested PCR amplification in two steps-first reaction with IP1 & AP1 and second reaction using the PCR product as template and IP2 & AP2 as primer. The single bands generated in the PCR were cloned and sequenced for designing a specific primer (IP3). A combination of either IP1 or IP2 primer along with the IP3 primer constituted the new SSR marker.

Another improved protocol described by Lian et al. (2006) differed from the earlier protocol in that a compound SSR primer and adapter primers were used for constructing genomic library. A specific primer (IP1) designed from the nucleotide sequence flanking the SSR and the initial compound SSR primer were used as a compound SSR marker.

Korpelainen et al. (2007) utilized genome screening with ISSR primers to obtain nucleotide sequence information flanking one side of the microsatellite followed by a restriction-ligation technique with a specific adaptor to facilitate sequence walking (in order to identify nucleotide sequences flanking the other side of the microsatellites). Wu et al. (2008) described an IC-SSR (intercompound microsatellite) method in which DNA was amplified with a mixture of SSR primers and the products that showed multibands were cloned and sequenced. The primer designed from the flanking sequence and the SSR primer with shorter inner repeat constituted a compound SSR marker.

**SSRs developed using AFLP**

AFLP markers along with enrichment steps (Hakki and Akkaya, 2000; Zane et al., 2002) or a combination of randomly amplified microsatellite primer and a selective primer capable of amplifying restricted fragments containing SSR motifs (Van Eijk et al., 2001) were used for generating SSR markers.

Hakki and Akkaya (2000) utilised selectively amplified AFLP bands along with an enrichment step (using biotinylated target repeat oligonucleotide and streptavidin coated magnetic beads) to generate SSR markers. The enriched AFLP fragments were re-amplified using same set of selective AFLP primer combinations, size selected, reamplified, and relatively long fragments (containing both restriction sites) were directly sequenced using site selective primers to reveal the nucleotide sequences flanking SSRs.

In the FIASCO (Fast Isolation by AFLP of Sequences Containing repeats) protocol, the AFLP bands were hybridized with biotinylated probes and subjected to selective capture using streptavidin-coated beads, followed by cloning and sequencing of enriched DNA fragments (Zane et al., 2002) to generate SSR markers.

Van Eijk et al. (2001) developed Microsatellite-AFLP (M-AFLP) technique that utilised the combination of a RAMP (Randomly Amplified Microsatellite Polymorphism) primer that binds to the microsatellite repeat at the transition point (between the repeat and flanking sequence) and a selective AFLP primer to amplify restriction fragments containing SSR motif sequences. The Amplified Fragment-Length Microsatellite (AFLM) approach (Douhan and Rizzo, 2003) utilised the selective amplification of the genomes with linker-adaptor-PCR followed by enrichment of microsatellite motifs with 5’ biotinylated oligonucleotides and recovery using streptavidin coated magnetic beads. The recovered fragments were re-amplified, cloned and sequenced to reveal flanking nucleotide sequence information.

**Other PCR based methods for SSR development**

Other methods utilised SAM (Selectively Amplified Microsatellite) for the selective amplification of restricted fragments with anchored primers, sequence tags, biotinylated probes and hybrid capture using streptavidin coated magnetic beads (Hayden and Sharp, 2001) or the use of multi locus marker system called RAMs (Random Amplified Microsatellites) to convert the amplified SSR bands to a marker, amplifying a single locus through a series of steps (Choy et al., 2005). The
Microsatellite Amplified Library’ (MAL) approach (Acquadro et al., 2005) utilised a two step primer extension protocol that allowed the construction of enriched SSR libraries based on PCR and avoided the necessity of hybridisation, enrichment steps and conversion of SSRs into sequence tagged STMS markers. Based on the cross-hybridisation of SSR sequences between distantly related organisms, Nolte et al. (2005) described a new protocol for the direct cloning of SSRs using an enrichment strategy. Wu et al. (2009) utilised a new genome walking method with a random tailed primer and multiple primer extensions (using Phu DNA polymerase) for isolating SSRs.

The ‘Sequential Reverse Genome Walking’ (SRGW) strategy (Joy et al., 2011) primarily involved the generation of a genomic walking (GW) library, which was enriched in two consecutive primary and nested secondary PCR steps (using SSR oligos as reverse primers in combination with two adaptor specific primers). The PCR products were cloned and sequenced. Based on the flanking sequence identified from one end of the microsatellite motif, two sets of flanking primers (F1 and nested secondary F2 primer) were designed. A ‘sequential reverse walk’ was then initiated with the rest of GW libraries using the flanking primers (F1 and F2) and the adaptor specific primers (AP1 and AP2). The secondary nested PCR products were cloned and sequenced to generate SSR markers.

The ‘Recombinant microsatellite amplification’ method (Wu et al., 2012) permitted rapid and large scale isolation of microsatellites by normalising adapter-ligated restricted DNA using a suppression PCR. This was followed by the selective amplification of SSR containing sequences using anchored SSR primer and a suppressor primer. The amplified products were restricted, ligated, re-amplified using anchored primer, cloned and sequenced. From the sequenced clone, a primer targeting the SSR motif was designed while for designing the suppressor primer. The amplified products were restricted, ligated, and the adaptor specific primers (AP1 and AP2). The secondary nested PCR products were cloned and sequenced to generate SSR markers.

Using high throughput sequencing/ Next generation sequencing technology

High throughput sequencing technology along with bioinformatics tools provide a superior alternative to the conventional methods used for developing SSR markers (Abdelkrim et al., 2009; Santana et al., 2009). With the advent of next generation sequencing (NGS) platforms, large volumes of sequencing data are being generated that could be screened with the aid of bioinformatics tools for identifying microsatellite repeats. This avoids the construction of microsatellite-enriched DNA libraries and provides a rapid approach for the large-scale generation of microsatellite loci. The recent availability of massively parallel sequencing (MPS) facilitated the sequencing of microsatellite-enriched genomic libraries in multiplex pools, thereby reducing sample preparation and sequencing costs (Jennings et al., 2011). Current advances in NGS technology and reduction in sequencing costs will further enable easier, cheaper and rapid identification of microsatellite markers in future. The pyrosequencing technique has been applied for the generation of microsatellite markers from Comarum palustre (Sonne et al., 2012), Vaccinium macrocarpon (Zhu et al., 2012), Linum usitatissimum etc. (Kale et al., 2012).

Using these various approaches, a large number of genomic SSR markers have been developed in several economically important crops. However, comparing the efficiency of SSR isolation protocols is generally difficult due to the difference in search criteria used for identifying SSRs and other variance factors existing among different laboratories and researchers (Techen et al., 2010). High redundancy, lack of SSRs in majority of sequenced clones and varying enrichment efficiency (observed when the same protocol is applied to members of same or different genus/ species) are some of the inherent problems associated with the improved protocols. Other factors that influence SSR frequencies include variation in sampling regions of the genome used for SSR detection (coding vs. non coding), library preparation and limits set for SSR detection using probe hybridization methods (Iniguez-Luy et al., 2008). High proportions of DNA fragments lacking microsatellite repeats in enrichment protocols is mainly attributed to the high level of non-specific binding of streptavidin-coated magnetic beads to the DNA (St. John and Quinn, 2008). However, the variation in enrichment efficiency between different species arise mainly due to the quality of genomic DNA used for library construction, difference in genome size and complexity, variation in the frequency of microsatellite repeats in the genome and attrition problems during isolation.

EST-SSR markers

Large scale EST sequencing projects for gene discovery programmes have generated and deposited a wealth of EST sequences in databases (Rudd 2003). With the availability of SSR mining tools like TROLL (Castelo et al., 2002), MISA (Thiel et al., 2003), SciRoKo (Kofler et al., 2007), Msatcommander (Faircloth, 2008), QDD (Maglez et al., 2010) etc., it has now become a fast approach to search for microsatellite repeats in the EST sequences/ databases and exploit the possibility of converting it into polymorphic SSR markers.

EST-SSR markers have both advantages and disadvantages. The generation of SSR markers from EST resources is relatively fast and inexpensive (Thiel et al., 2003; Gupta et al., 2003) and could be achieved rapidly using bioinformatics softwares (Varshney et al., 2005). EST-SSRs reveal variation in the expressed regions of the genome, thereby detecting perfect marker-trait associations (Gupta et al., 2003). They exhibit high transferability across a much broader taxonomic range (Gupta et al., 2003) and null alleles are less problematic (Leigh et al., 2003; Rungis et al., 2004) than those derived from untranslated regions (Rungis et al., 2004; Pashley et al., 2006).

However, the generation of EST-SSR markers is limited to the availability of EST sequences and hence
restricted to economically and widely exploited crops (Varshney et al., 2005; Pashley et al., 2006), whose sequences are deposited or shared in the public domain. The relatively low abundance of SSRs within the transcribed region (Hancock, 1995; Katti et al., 2001) is also a limiting factor for the large scale development of genic SSR markers. Moreover, EST-SSR markers exhibit less polymorphism and are less efficient in distinguishing closely related individuals (Cho et al., 2000; Gupta et al., 2003; Chabane et al., 2005) because of greater DNA sequence conservation in transcribed or coding regions (Rungis et al., 2004; Varshney et al., 2005).

Chloroplast SSR markers

Microsatellites occurring in the chloroplast genome of higher plants (cpSSRs; Powell et al., 1995, 1996) are usually composed of mononucleotide (A and T) repeats rather than di-, tri- or tetra-nucleotide repeats (Bryan et al., 1999).

The identification and development of cpSSRs is achieved principally by utilizing the nucleotide sequence information retrieved from public databases (Weising and Gardner, 1999; Chung and Staub, 2003) and also by sequencing PCR products generated by "consensus/universal primers", capable of amplifying cpDNA regions in several species. The de novo sequencing of noncoding chloroplast DNA regions is recommended to be the most efficient way for identifying large number of chloroplast microsatellites (Ebert and Peakall, 2009).

Taberlet et al. (1991) reported the first set of 'universal' PCR primers for analyzing intra-specific variation in the chloroplast nucleotide sequence across plant genera/species. Later on, several universal primers were reported for amplifying chloroplast regions in various crops (Demesure et al., 1995; Dumolin-Lapegue et al., 1997; Weising and Gardner, 1999; Ebert and Peakall, 2009). Sequencing of amplicons generated by these universal cpDNA-PCR primers provide basic information about mononucleotide tracts and flanking sequences in the amplicons. Since the flanking regions of cpSSR loci are highly conserved, 'universal cpSSR primers' (Weising and Gardner, 1999; Chung and Staub, 2003; Ebert and Peakall, 2009) that enable the amplification of cpSSRs across species/taxa have been identified. Most of the molecular studies involving chloroplast microsatellites now rely on testing these universal cpSSRs in the target species.

Chloroplast SSR markers also have advantages and disadvantages. The haploid nature and high copy number of the chloroplast genome facilitate easy working of these markers using PCR based methods (Bryan et al., 1999). However, the mutation rate is lower than nuclear SSRs (Provan et al., 2001) and the level of polymorphism is variable across loci and species, with some loci found to be monomorphic in all species (Navascues and Emerson, 2005). The short length and limited number of alleles generated by cpSSRs further necessitate the confirmation of allele size by sequencing (Weising and Gardner, 1999).

Cross-amplification/transferability of microsatellite markers

The high cost and labour involved in developing microsatellite repeats is a serious factor that restricts the wide-spread application of SSR markers in different plants. Hence the development of SSR markers is often focussed only to economically important crops. However, flanking sequences are reported to exhibit slower mutation rate than SSR region (Holmen et al., 2009), permitting their sequence conservation across species or genera. This homology allows the amplification of primers designed for one species to other members of the same species or genera (cross-species/cross-genera amplification or transferability). Transferability offers potential for the low cost generation of microsatellite markers for related or distant species.

In general, the strategy is applicable to species belonging to the same genus or recently separated genera. However, successful cross-species amplification is inversely related to the evolutionary distance between two species (Primmer et al., 1996; Steinkellner et al., 1997), conservation of flanking sequences and maintenance of long arrays to generate sufficient polymorphisms (FitzSimmons et al., 1995). Among the class of SSR markers, EST-SSR markers shows greater cross-species transferability than genomic SSRs (Varshney et al., 2005), as they reside in the more conserved regions (genic) of the genome. Transferability studies are ample within genus (Roa et al., 2000; Takayama et al., 2008) and across genus (Raji et al., 2009; Datta et al., 2010).

Conclusion

It is evident that microsatellite markers are one of the most widely exploited molecular markers in various research areas, including the assessment of genetic diversity, gene mapping and marker assisted selection. Each type of SSR markers has its own advantages and disadvantages. Though the development of genomic SSR is cumbersome, it is of wide application in genetic diversity analysis and population genetics, due to its robustness and high polymorphism. EST-derived markers have a prominent role when the study is concerned with the identification of functional polymorphisms in key genes. Though several new isolation strategies have been described (Tab. 1), careful attempts need to be made to choose an appropriate strategy by considering factors like operation cost, rapid generation, high efficiency and species transferability. Among the methods available till date, when concerned with the short time and rapid mode of generation, NGS offers wide possibilities for the large scale generation of microsatellite markers.
Fig.1. Strategies for the development of microsatellite markers
### Tab. 1. Comparison of different methods used for the development of microsatellite markers

| SSR type | Strategy | Method | Restriction digestion | Adapter/linker ligation | PCR amplification | Methodology | Sequencing | Primer designing | Testing of primers | Methodology used for generation | Advantages and Disadvantages |
|----------|----------|--------|------------------------|--------------------------|------------------|-------------|------------|------------------|------------------|-------------------------------|-----------------------------|
|          |          |        |                        |                          |                  | Enrichment using probes/SSR primers | Cloning |             |                  |                  |                              |                            |
|          |          |        |                        |                          |                  |                          |            |                  |                  |                              |                            |
| Enrichment | Selective hybridization | Primer extension | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | Complex | High | More | High |
| Non-enrichment | RAPD markers | ISSR/SSR amplicons | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | Complex | High | More | High |
| PCR based molecular markers | Other methods (SLiM PCR, Recombinant microsatellite amplification etc) | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | Complex | High | More | High |
| Genomic SSR | High throughput/Next generation sequencing | | | | | | ✓ | ✓ | ✓ | Easy | Medium | Less | Less |
| EST-SSR | Data Mining | | ✓ | ✓ | | | Easy | Less | Less | Less |
| cpSSR | Data mining/Testing of universal primers | | ✓ | ✓ | | | Easy | Less | Less | Less |
| Genomic SSR/EST-SSR/cpSSR | Transferability/Cross Species-amplification | | | | | ✓ | Easy | Very less | Less | Less |

* - optional
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