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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 1989), 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 cardiac 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 tetranucleotide 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. AGAGAGAGAGAG or $(AG)_6$]

b. Imperfect microsatellite- a pair of bases is present between the repeat motif that does not match the motif sequence [e.g. AGAGAGAGAG<u>CT</u>AGAGAG or $(AG)_5CT(AG)_3$].

c. Interrupted microsatellite- a small sequence within the repeated sequence that does not match the motif sequence [e.g. AGAGAGAG<u>CGTG</u>AGAGAGAG or $(AG)_4CGTG(AG)_4$].

d. Compound/ composite microsatellite- two adjacent distinctive repeats present within the sequence [e.g. AGAGAGAGAGTCTCTCTC or $(AG)_5(TC)_4$].

Based on the length of repeat motif

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

a. Class I microsatellites- perfect SSRs of ≥20 nucleotides in length.

b. Class II microsatellites- perfect SSRs of ≥12 nucleotides and ≤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 (Hulzink *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. Multiallellic 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 (Karagyozov *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 (Karagyozov *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.,* 1998; 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 (Ostrander *et al.,* 1992; Pandolfo, 1992; Robic *et al.,* 1994; Paetkau, 1999). This procedure (Ostrander *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 (Ostrander *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 (Ostrander *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 vectorette (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 digoxygenin 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 multilocus 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 reverse primer, the whole SSR locus was isolated by genome walking.

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 new 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* (Somme *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 (Meglécz *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 economical 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

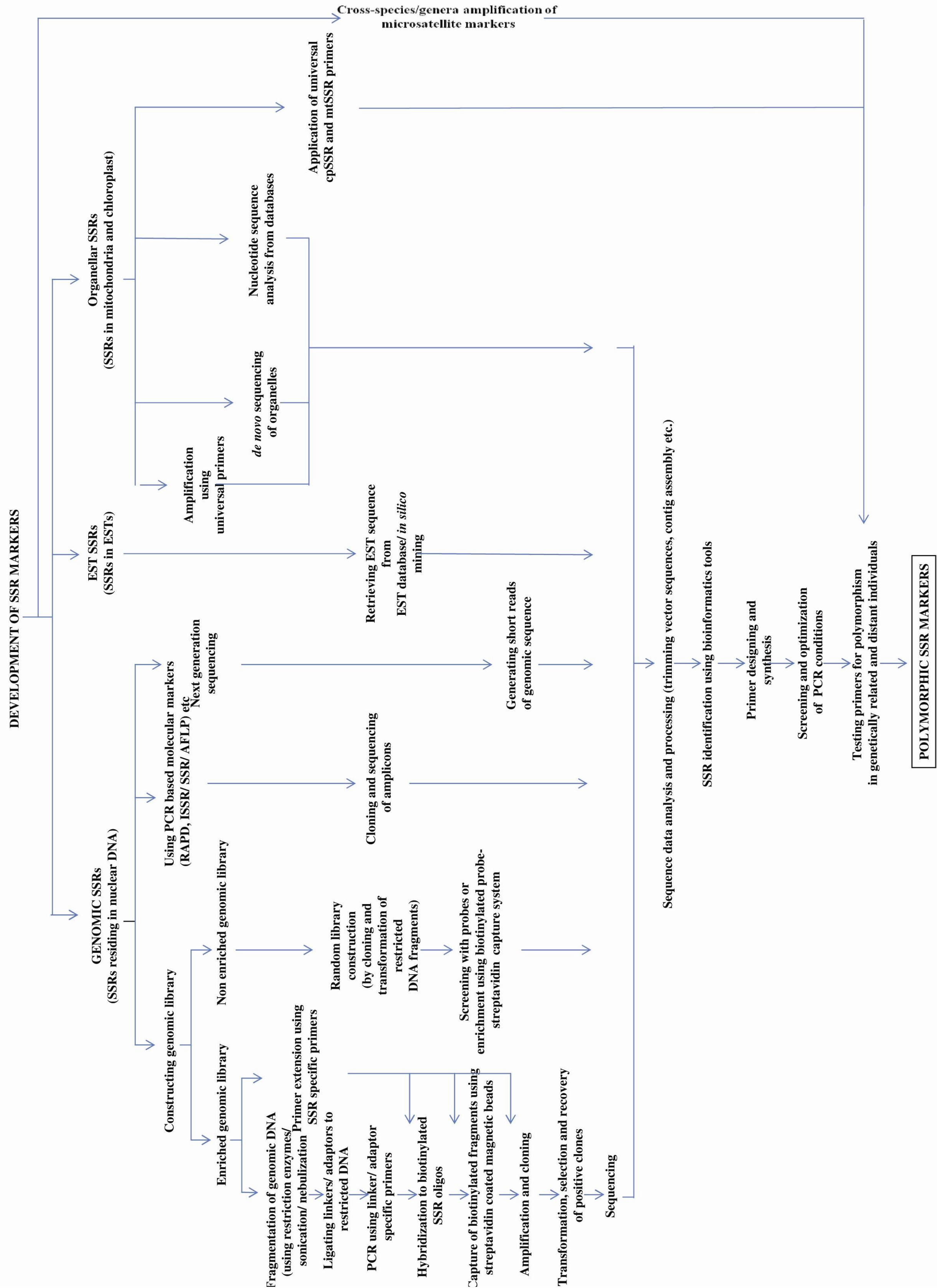
The high cost and labour involved in developing microsatellite repeats is a serious factor that restricts the wide-spread application of SSR markers in different plants. Hence the development of SSR markers is often focussed only to economically important crops. However, flanking sequences are reported 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 makers, 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

			Methodology								Advantages and Disadvantages			
SSR type	Strategy	Method	Restriction digestion	Adapter/ linker ligation	PCR amplification	Enrichment using probes/ SSR primers	Cloning	Sequencing	Primer designing	Testing of primers	Metholodology used for generation	Cost involved	Time taken for development	Labour involved
Genomic SSR	Enrichment	Selective hybridization	✓	✓	✓	✓	✓	✓	✓	✓	Complex	High	More	High
		Primer extension			✓	✓	✓	✓	✓	✓	Complex	High	More	High
	Non-enrichment		✓	✓	✓		✓	✓	✓	✓	Complex	High	More	High
	PCR based molecular markers	RAPD markers			✓		✓	✓	✓	✓	Moderate	Moderate	Medium	Medium
		ISSR/SSR amplicons			✓		✓	✓	✓	✓	Moderate	Moderate	Medium	Medium
		AFLP	✓	✓	✓	✓	✓	✓	✓	✓	Complex	High	More	High
		Other methods (SLiM PCR, Recombinant microsatellite amplification etc)	✓	✓	✓		✓	✓	✓	✓	Complex	High	Moderate	High
	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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