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Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding

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Summary

Inter simple sequence repeat (ISSR)-PCR is a technique, which involves the use of microsatellite sequences as primers in a polymerase chain reaction to generate multilocus markers. It is a simple and quick method that combines most of the advantages of microsatellites (SSRs) and amplified fragment length polymorphism (AFLP) to the universality of random amplified polymorphic DNA (RAPD). ISSR markers are highly polymorphic and are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology. This review provides an overview of the details of the technique and its application in genetics and plant breeding in a wide range of crop plants.

Introduction

DNA markers have proved valuable in crop breeding, especially in studies on genetic diversity and gene mapping. The commonly used polymerase chain reaction (PCR)-based DNA marker systems are random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and more recently simple sequence repeats (SSRs) or microsatellites (Staub et al., 1996; Gupta & Varshney, 2000). The major limitations of these methods are low reproducibility of RAPD, high cost of AFLP and the need to know the flanking sequences to develop species specific primers for SSR polymorphism. ISSR-PCR is a technique that overcomes most of these limitations (Zietkiewicz et al., 1994; Gupta et al., 1994; Wu et al., 1994; Meyer et al., 1993). It is rapidly being used by the research community in various fields of plant improvement (Godwin et al., 1997). The technique is useful in areas of genetic diversity, phylogenetic studies, gene tagging, genome mapping and evolutionary biology in a wide range of crop species. In this method SSRs are used as primers to amplify mainly the inter-SSR regions. SSRs or microsatellites are short tandem repeats (STRs) or variable number of tandem repeats (VNTRs) of 1–4 bases of DNA ubiquitously present in eukaryote genomes (Tautz & Renz, 1984). They are dispersed throughout the genome and vary in the number of repeat units. The details of the technique and its major applications are discussed in this review.

The technique

Inter simple sequence repeat (ISSR) technique is a PCR based method, which involves amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. The technique uses microsatellites, usually 16–25 bp long, as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter- SSR sequences of different sizes. The microsatellite repeats used as primers can be di-nucleotide, tri-nucleotide, tetra-nucleotide or penta-nucleotide. The primers used can be either unanchored (Gupta et al., 1994; Meyer et al., 1993; Wu et al., 1994) or more usually anchored at 3' or 5' end with 1 to 4 degenerate bases extended into the flanking sequences (Zietkiewicz et al., 1994) (Figure 1). The technique combines most of the benefits of

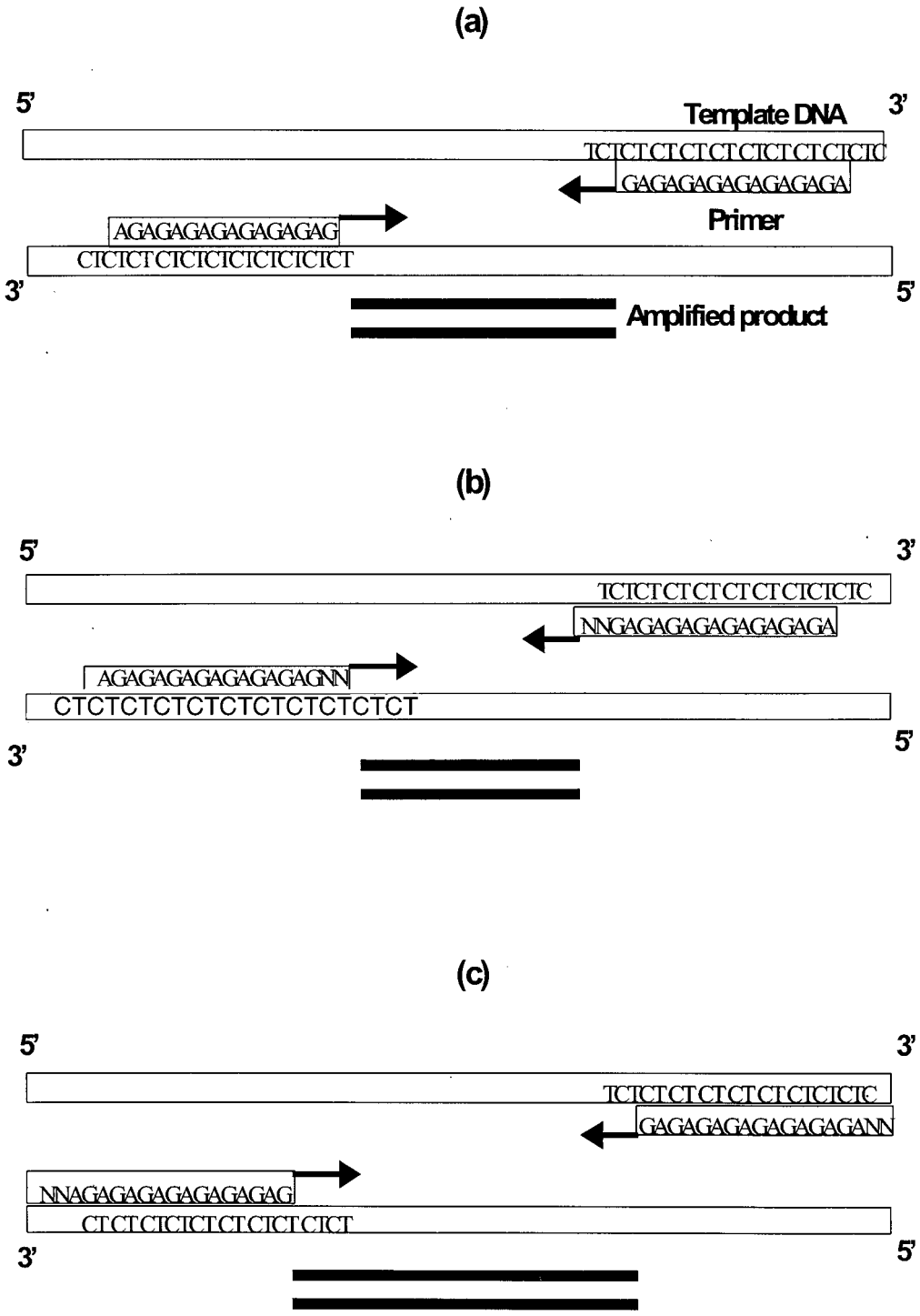

Figure 1. ISSR-PCR: A schematic representation of a single primer $(AG)_8$, unanchored (a), 3'-anchored (b) and 5'-anchored (c) targeting a $(TC)_n$ repeat used to amplify inter simple sequence repeat region flanked by two inversely oriented $(TC)_n$ sequences. (a) Unanchored $(AG)_n$ primer can anneal anywhere in the $(TC)_n$ repeat region on the template DNA leading to slippage and ultimately smear formation (b) $(AG)_n$ primer anchored with 2 nucleotides (NN) at the 3' end anneals at specific regions on the template DNA and produces clear bands (c) $(AG)_n$ primer anchored with 2 nucleotides (NN) at the 5' end anneals at specific regions and amplifies part of the repeat region also leading to larger bands.

AFLP and microsatellite analysis with the universality of RAPD. ISSRs have high reproducibility possibly due to the use of longer primers (16–25 mers) as compared to RAPD primers (10- mers) which permits the subsequent use of high annealing temperature (45–60 °C) leading to higher stringency. The studies on reproducibility show that it is only the faintest bands that are not reproducible. About 92–95% of the scored fragments could be repeated across DNA samples of the same cultivar and across separate PCR runs when detected using polyacrylamide (Fang & Roose, 1997; Moreno et al., 1998). 10 ng template DNA yielded the same amplification products as did 25 or 50 ng per 20μl PCR reaction. The annealing temperature depends on the GC content of the primer used and usually ranges from 45 to 65 °C.

ISSRs segregate mostly as dominant markers following simple Mendelian inheritance (Gupta et al., 1994; Tsumura et al., 1996; Ratnaparkhe et al., 1998; Wang et al., 1998). However, they have also been shown to segregate as co-dominant markers in some cases thus enabling distinction between homozygotes and heterozygotes (Wu et al., 1994; Akagi et al., 1996; Wang et al., 1998; Sankar & Moore, 2001).

Source of variability / polymorphism

The evolutionary rate of change within microsatellites is considerably higher than most other types of DNA, so the likelihood of polymorphism in these sequences is greater. The source of variability in the ISSRs can be attributed to any one of the following reasons or any combination of these.

(a) Template DNA

Slippage of DNA polymerase during DNA replication and failure to repair mismatches is considered as a mechanism for creation and hypervariability of SSRs (Levinson & Gutman, 1987). Mutations at the priming site i.e. SSR could prevent amplification of a fragment, as also in RAPD markers and thus give a presence/absence polymorphism. An insertion/deletion event within the SSR region or the amplified region would result in the absence of a product or length polymorphism, depending on the amplifiability of the resulting fragment size. Variability in number of nucleotides within a microsatellite repeat would result in length polymorphisms when using a 5'-anchored primer.

(b) Nature of primer used

The extent of polymorphism also varies with the nature (unachored, 3'-anchored, or 5'-anchored) and sequence of the repeats (motif) in the primer employed. When unanchored i.e only the SSRs are used as primers, the primer tends to slip within the repeat units during amplification leading to smears instead of clear bands (Figure 1a). Extending the primer (anchoring) with 1 to 4 degenerate nucleotides at the 3' end (Figure 1b) or 5' end (Figure 1c) assures annealing only to the ends of a microsatellite in template DNA thus obviating internal priming and smear formation. Secondly, the anchor allows only a subset of the microsatellites to serve as priming sites. When 5' anchored primers are used, the amplified products include the microsatellite sequences and their length variations across a genome and therefore give more number of bands and a higher degree of polymorphism. Usually di-nucleotide repeats, anchored either at 3' or 5' end reveal high polymorphism (Blair et al., 1999; Joshi et al., 2000; Nagaoka & Ogihara, 1997). The primers anchored at 3' end (Figure 1b) give clearer banding pattern as compared to those anchored at 5' end (Tsumura et al., 1996; Blair et al., 1999; Nagaoka & Ogihara, 1997). Since the primer is a SSR motif the frequency and distribution of the microsatellite repeat motifs in different species also influence the generation of bands. There is a difference of abundance of SSRs between nuclear and organelle DNA sequences. Taking di- and tri-nucleotides together, one SSR was found every 33Kb in nuclear DNA compared to every 423-Kb of organelle DNA sequence (Wang et al., 1994). In general, primers with (AG), (GA), (CT), (TC), (AC), (CA) repeats show higher polymorphism than primers with other di-, tri- or tetra-nucleotide repeats. (AT) repeats are the most abundant di-nucleotides in plants but the primers based on (AT) would self- anneal and not amplify. Tri- and tetra-nucleotides are less frequent and their use in ISSRs is lesser than the di-nucleotides. The (AG) and (GA) based primers have been shown to amplify clear bands in rice (Blair et al., 1999; Joshi et al., 2000; Reddy et al., 2000; Sarla et al., 2000), trifoliate orange (Fang et al., 1997), Douglas fir and sugi (Tsumura et al., 1996) and chickpea (Ratnaparkhe et al., 1998), whereas primers based on (AC) di-nucleotide repeats were found more useful in wheat (Nagaoka & Ogihara, 1997; Kojima et al., 1998) and potato (McGregor et al., 2000). Resolving power Rp is an index developed to compare the value of different primers in terms

of the informative bands obtained in a given set of germplasm (Prevost & Wilkinson, 1999).

(c) Detection method

The level of polymorphism detected has been shown to vary with the detection method used. Polyacrylamide gel electrophoresis (PAGE) in combination with radioactivity (labelled nucleotide in PCR reaction) was shown to be most sensitive, followed by PAGE with silver staining and then agarose-ethidium bromide system of detection. Markedly higher number of bands were resolved per primer when polyacrylamide was used compared to agarose (Moreno et al., 1998). In a study on trifoliate orange germplasm, silver staining using high quality chemicals could detect all the bands detected by autoradiography (Fang et al., 1997). However, high levels of polymorphism have been detected even when products of ISSR amplification are resolved on agarose gels without radiolabelling (Tsumura et al., 1996; Arcade et al., 2000; Kojima et al., 1998; Wolff & Morgan-Richards, 1998; Sankar & Moore, 2001) Thus, the need for radioactivity can be avoided when many samples have to be screened as in germplasm characterization.

ISSR-PCR is a simple, quick, and efficient technique. It has high reproducibility. The use of radioactivity is not essential.The primers are not proprietary (as in SSR-PCR) and can be synthesized by anyone. Variations in primer length, motif and anchor are possible. The primers are long (16–25 bp) resulting in higher stringency. The amplified products (ISSR markers) are usually 200–2000 bp long and amenable to detection by both agarose and polyacrylamide gel electrophoresis. In the literature this technique and its variations have been referred to by different names (Table 1).

Application

The potential for integrating ISSR-PCR into programs of plant improvement is enormous (Table 2). The major areas of the application of ISSR-PCR in different crops are discussed below.

Genomic fingerprinting

DNA fingerprinting is an important tool for characterization of germplasm and establishment of the identity of varieties/hybrids/parental sources etc. in plant breeding and germplasm management. Di-nucleotide based ISSR primers anchored at 5' or 3' end have been used in fingerprinting studies with high reproducibility for maintenance of cocoa collection (Charters & Wilkinson, 2000). ISSRs showed sufficient polymorphism to distinguish between various cultivars of chrysanthemum (Wolff et al., 1995). Microspore derived plants could be distinguished from those derived from somatic tissues in anther culture of flax at an early seedling stage (Chen et al., 1998).

Genetic diversity and phylogenetic analysis

ISSRs have been successfully used to estimate the extent of genetic diversity at inter- and intra-specific level in a wide range of crop species which include rice (Joshi et al., 2000), wheat (Nagaoka & Ogihara, 1997), fingermillet (Salimath et al., 1995), *Vigna* (Ajibade et al., 2000), sweet potato (Huang & Sun, 2000) and *Plantago* (Wolff & Morgan-Richards, 1998). Superiority of ISSR-PCR over other marker techniques has been brought out in such investigations by various workers. Anchored SSR primers for instance, have been found to be more useful and reproducible than isozymes, RFLPs and RAPDs in the diversity analysis of trifoliate orange germplasm (Fang et al., 1997). ISSRs were more useful for the analysis of diversity in the genus *Eleusine* in terms of quality and quantity of data output as compared to RFLP and RAPD (Salimath et al., 1995). Significantly, the efficiency of the technique was evident in characterization even at the varietal level of a species. For instance, three 5' anchored primers together could distinguish 20 cultivars of *Brassica napus* (Charters et al., 1996). ISSR is the marker of choice for assessment of genetic diversity in cocoa (Charters & Wilkinson, 2000), gymnosperms such as Douglas fir and sugi (Tsumura et al., 1996) and even fungi (Hantula et al., 1996). In a study on white lupin it has been demonstrated that among 10 primers used any two were sufficient to distinguish all the 37 accessions studied (Gilbert et al., 1999). Similarly, 4 primers were sufficient to distinguish 34 cultivars of potato (Prevost & Wilkinson, 1999) and 3 primers could distinguish 16 genotypes of redcurrant (Lanham & Brennan, 1998). The use of such highly informative primers lowers the cost, time and labour for diversity analysis.

Various marker techniques have been used in phylogenetic investigations based on relative similarity. Inspite of their higher efficiency and reproducibility ISSR markers have as yet not been used extensively. It has however been found effective in

Table 1. Synonyms of the ISSR-PCR technique and its variants

S. No	Terms used	Reference
1	MP-PCR, Microsatellite primed PCR (refers to unanchored primer)	Meyer et al. (1993)
2	SSR-anchored PCR, Inter-SSR amplification	Zietkiewicz et al. (1994)
3	SPAR (single primer amplification reaction)	Gupta et al. (1994)
4	RAMPs (random amplified microsatellite polymorphisms)	Wu et al. (1994)
5	RAMs (randomly amplified microsatellites)	Hantula et al. (1996)
6	AMP-PCR (anchored microsatellite primed PCR)	Weising et al. (1998)
7	ASSR (anchored simple sequence repeats)	Wang et al. (1998)

resolving problems relating to the phylogeny of Asian cultivated rice *Oryza sativa* (Joshi et al., 2000), wheat (Nagaoka & Ogihara, 1997), finger millet (Salimath et al., 1995), *Vigna* (Ajibade et al., 2000) and *Diplotaxis* species (Martin & Sanchez-Yelamo, 2000). There is immense scope to use this powerful technique in resolving species/inter-species status in many a genus and in deciding the distinctness of different genera within a family. Significantly, genome/species specific ISSR markers have been reported in four genera *Oryza* (Joshi et al., 2000), *Lolium* and *Festuca* (Pasakinskiene et al., 2000) and *Diplotaxis* (Martin & Sanchez-Yelamo, 2000) which are useful in delineating species.

Genome mapping

ISSR markers are unmapped but can be used to saturate RFLP and SSR linkage maps. The RFLP map of barley was saturated with 60 ISSRs (referred as RAMPs in the study) which mapped to all chromosomes (Becker & Heun, 1995). Many of these markers are mapped in between clustered RFLPs, flanking RFLP clusters, at the tips of chromosomes and more importantly in areas of low RFLP marker density. In *Einkorn* wheats, however, the nine ISSR markers mapped at or close to the RFLP marker positions (Kojima et al., 1998). ISSRs have also been used along with AFLP and RAPD markers in the mapping of Japanese and European larch genomes (Arcade et al., 2000). The genetic linkage map of *Citrus* was further saturated using 75 ISSR markers, which were dispersed among all the linkage groups (Sankar & Moore, 2001). Also it was shown that the level of segregation distortion of ISSRs is lower compared to RAPDs. In soybean, 58 ISSR markers were mapped onto 18 RAPD/RFLP linkage groups (Wang et al., 1998). CA polymorphisms had a biased distribution and GA polymorphisms were randomly dispersed.

Gene tagging and marker assisted selection

DNA markers closely linked to important agronomic traits greatly contribute to practical crop improvement programs. In rice, an ISSR marker generated by primer $(AG)_8$YC was converted to a sequence tagged site (STS) marker to identify the fertility restoration gene, Rf-1 (Akagi et al., 1996*)*. This co-dominant marker can be used in management of genetic purity of hybrid seed. In chickpea, ISSR markers UBC 855_{500} generated by primer $(AG)_8$YT and UBC 825_{1200} using primer $(AG)_8$T were linked to the gene conferring resistance to race 4 of *Fusarium* wilt (Ratnaparkhe et al., 1998). Markers closer to a given gene are generated by altering 5' or 3' anchors. Recently, ISSR-PCR was used in identifying two allelic dominant DNA markers, one linked in coupling and the other in repulsion phase to a major locus *Fgr*, which modulates fructose to glucose ratio in tomatoes (Levin et al., 2000). These PCR products were obtained from two ISSR-PCR reactions using $(TC)_8$CC and $(TC)_8$CG as primers. Another trait of value in hybrid seed production viz., temperature-sensitive genic male sterility has been tagged with an ISSR marker UBC 855_{1060} in rice (Hussain et al., 2000).

ISSRs have also been used to generate species specific, gene specific and trait specific markers. While delineating the phylogenetic relationship among different species of the genus *Oryza,* 87 putative genome/species specific markers were identified (Joshi et al., 2000). The 582 bp inter-SSR *Festuca* specific sequence and 1350 bp *F. arundinacea* specific sequence have potential as markers to confirm presence of closely linked *Festuca* genes (Pasakinskiene et al., 2000). Likewise, race specific markers have been de-

Table 2. Applications of ISSR-PCR technique

S. No	Application	Reference
1	**Genomic fingerprinting**	
	Cocoa germplasm	Charters & Wilkinson, 2000
	Potato cultivars	Prevost & Wilkinson, 1999
	Chrysanthemum cultivars	Wolff et al., 1995
2	**Genetic diversity and phylogenetic analysis**	
	Rice cultivars	Virk et al., 2000
	Oryza granulata	Qian et al., 2001
	Wheat (*Triticum* sp.)	Nagaoka & Ogihara, 1997
	Barley (*Hordeum vulgare*)	Sanchez et al., 1996
	Maize inbred lines (*Zea mays*)	Kantety et al., 1995
	Fingermillet (*Eleusine* sp)	Salimath et al., 1995
	Sorghum (Chinese) (*Sorghum bicolor*)	Yang et al., 1996
	White lupin germplasm (*Lupinus albus*)	Gilbert et al., 1999
	Vigna sp	Ajibade et al., 2000
	Pea germplasm (*Pisum sativum*)	Lu et al., 1996
	Soybean (*Glycine max*)	Wang et al., 1998
	Oilseed rape cultivars (*Brassica napus*)	Charters et al., 1996
	Sweet potato, wild relatives (*Ipomoea* sp)	Huang & Sun, 2000
	Potato cultivars (*Solanum tuberosum*)	McGregor et al., 2000
	Redcurrant germplasm (*Ribes* sp)	Lanham & Brennan, 1998
	Grapevine germplasm (*Vitis vinifera*)	Moreno et al., 1998
	Citrus cultivars (*Citrus* sp)	Fang & Roose, 1997
	Trifoliate orange germplasm (*Poncirus trifoliata*)	Fang et al., 1997
	Plantago major subspecies	Wolff & Morgan- Richards, 1998
	Gymnosperms, Douglas fir and sugi	Tsumura et al., 1996
3	**Genome mapping**	
	Saturating RFLP linkage map in barley	Becker & Heun, 1995
	Construction of a genetic linkage map in Einkorn wheat	Kojima et al., 1998
	Genetic mapping of Japanese and European types of larch	Arcade et al., 2000
	Saturating genetic linkage map in citrus	Sankar & Moore, 2001
	Saturating RFLP/RAPD linkage map in soybean	Wang et al., 1998
4	**Determining SSR motif frequency**	
	Recovery of microsatellite sequences in the mustard genome	Varghese et al., 2000
	Distribution pattern of microsatellites across eukaryotic genomes	Gupta et al., 1994
	Analysis of microsatellite frequency in rice cultivars	Blair et al., 1999
5	**Gene tagging and use in marker assisted selection**	
	Rf-1 gene for fertility restoration in rice	Akagi et al., 1996
	Gene for resistance to *Fusarium* wilt Race 4 in chickpea	Ratnaparkhe et al., 1998
	Temperature sensitive genic male sterility in rice	Hussain et al., 2000
	Fgr gene for modulating fructose to glucose ratio in tomato	Levin et al., 2000
	Genome/species specific markers in *Lolium* and *Festuca*	Pasakinskiene et al., 2000
	Putative genome/species specific markers in *Oryza.*	Joshi et al., 2000
	Race specific markers in fungi	Hantula et al., 1996
6	**Evolutionary biology**	
	Diplotaxis species	Martin & Sanchez-Yelamo, 2000
	Diploid hybrid speciation in *Penstemon*	Wolfe et al., 1998

veloped in various fungi groups using ISSRs (Hantula et al., 1996).

Determining SSR motif frequency

ISSR analysis provides insights into the organization (clustered or not), frequency and levels of polymorphism of different simple sequence repeats in a genome. In rice and wheat, di-nucleotide simple sequence repeats used as primers gave the maximum number of bands and are, therefore, more common than any SSRs with larger units (Blair et al., 1999; Nagaoka & Ogihara, 1997). Poly(GA) based 3'-anchored primers produced 5 times as many bands as those with poly(GT) motif indicating low frequency or lack of clustering of (GT) motif (Blair et al., 1999). Using ISSRs it has been shown that tetra-nucleotide repeats were abundant across eukaryotic genomes (Gupta et al., 1994) and that tetramers of tetra-nucleotides AGAC and GACA are scattered within the genome of grasses (Pasakinskiene et al., 2000). It has been demonstrated in *Brassica* that enhanced recovery of microsatellite markers is possible using ISSR primers (Varghese et al., 2000).

Studies on natural populations/ speciation

The hypervariable nuclear ISSR markers have proved useful in testing hypotheses of speciation, introgression and systematics (Wolfe et al., 1998). The hybrid origin of *Penstemon clevelandi* was clearly brought out by the use of just 8 ISSR markers. Population of *P. clevelandi* has been found to have an additive profile of bands of the two proposed progenitor species viz. *P. centranthifolius* and *P. spectabilis.* On the other hand the population of *P. spectabilis* lacked the additive profile of bands of its proposed putative parents. The hybrid origin of *P. spectabilis* was thus negated and its origin was attributed instead to introgression of genes and not the genome of a related species. The utility of the technique has been demonstrated in a wide range of applications in molecular ecology in plant families which include Asteraceae, Brassicaceae, Hippocastanaceae, Orchidaceae, Poaceae, Scrophulariaceae and Violaceae (http://www.biosci.ohio-state.edu/ ∼awolfe/issri.issr.html). Variation within and between populations can be compared using dispersed multilocus markers such as ISSR. It was shown that the amount of variation between *O. granulata* populations from different regions (49.2%) was higher than that between populations within a region (38%) or within a population (12%) using ISSR markers (Qian et al., 2001).

Perspectives

As the need to protect proprietary germplasm is likely to increase in the future, ISSRs will have an important role in securing plant variety rights by virtue of its unique efficiency in distinguishing even closely related germplasm. To date, more polymorphism has been detected with the use of ISSRs than with any other assay procedure (Gupta et al., 1994; Salimath et al., 1995; Virk et al., 2000). In many of the studies for determining the extent of polymorphism or comparing marker systems only one family of SSRs, eg. tri-nucleotides or tetra-nucleotides had been used as primers. Such repeats are infrequent as compared to di-nucleotides and their use may not help arrive at precise classification. As more data on the occurrence and distribution of SSR motifs becomes available, it should be possible to use primers that give more accurate span of the whole genome. Also, different combinations of the motif, anchor and length of primers can be used. Strategies to detect additonal polymorphism could include use of ISSRs in combination with RAPD (Joshi et al., 2000; Becker & Heun, 1995; Wu et al., 1994) or AFLP primers in the same reaction or restriction digestion of ISSR products (Becker & Heun, 1995). Unlimited combinations of motif and length of both primers and use of different restriction enzymes are thus possible. Well chosen primers can provide reasonably accurate fingerprinting and thereby quick estimate of genetic diversity especially in large sized accessions to identify core sets and the pattern of geographical distribution.

The technique is not without limitations. For instance, there is the possibility as in RAPD, that fragments with the same mobility originate from non-homologous regions, which can contribute to some distortion in the estimates of genetic similarities (Sanchez et al., 1996). The molecular nature of the polymorphisms can be known only if the fragments extracted from the gel are sequenced. ISSR markers linked to the traits of agronomic importance have been sequenced and used as STS markers in marker aided selection. An attractive possibility is thus the use of ISSRs as probes for in-situ hybridization for physical mapping of homologous chromosome sites (Pasakinskiene et al., 2000). Another advantage in the use of ISSR markers lies in their linkage to SSR loci.

Although microsatellites themselves are probably non-functional and selectively neutral, they are known to be linked to coding regions, so that ISSRs are likely to mark gene rich regions (Kojima et al., 1998).

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