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[Review]

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Inter Simple Sequence Repeat (ISSR) Polymorphism and Its Application in Mulberry Genome Analysis

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Molecular markers have increasingly been used in plant genetic analysis, due to their obvious advantages over conventional phenotypic markers, as they are highly polymorphic, more in number, stable across different developmental stages, neutral to selection and least influenced by environmental factors. Among the PCR based marker techniques, ISSR is one of the simplest and widely used techniques, which involves amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. Though ISSR markers are dominant like RAPD, they are more stable and reproducible. Because of these properties ISSR markers have recently been found using extensively for finger printing, pohylogenetic analysis, population structure analysis, varietal/line identification, genetic mapping, marker-assisted selection, etc. In mulberry (*Morus* spp.), ISSR markers were used for analyzing phylogenetic relationship among cultivated varieties, between tropical and temperate mulberry, for solving the vexed problem of identifying taxonomic positions of genotypes, for identifying markers associated with leaf yield attributing characters. As ISSR markers are one of the cheapest and easiest marker systems with high efficiency in generating polymorphism among closely related varieties, they would play a major role in mulberry genome analysis in the future.

Key words: Molecular markers, PCR, Primers, Genetic diversity, Phylogenetic relationship

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Introduction

Recent advances in molecular biology have introduced a new generation of markers to facilitate dissection of plant genomes more easily, efficiently and speedily. These DNA based markers are versatile tools having a number of advantages over the conventional phenotypic markers. DNA markers are known to be present more in numbers, stable across the developmental stages, least influenced by environmental factors, devoid of the pleaotripic and epistatic effects. Depending on the techniques used, theses markers can be broadly classified as hybridization based markers and polymerase chain reaction (PCR) based markers. In hybridization based marker systems like restriction fragment polymorphism (RFLP), the DNA profiles are visualized by hybridizing the restriction enzyme-digested DNA blotted onto a solid membrane with radio labeled probe. In PCR based marker system, *in vitro* amplification of a particular DNA sequences is carried out with the help of specifically or arbitrarily chosen primers and a thermostable enzyme, called *Taq* polymerase. The amplified fragments are separated electrophorectically on polyacrylamide gels or agarose gels and the banding patterns are detected by either staining or by autoradiography. Some of the important PCR based marker systems are random amplified polymorphic DNA (RAPD), amplified fragment polymorphism (AFLP), inter simple sequence repeats (ISSR), simple sequence repeats (SSR), expressed sequence tag (EST). Each of this marker system has its own merits and demerits (Table 1). Markers like RFLP, SSR and EST are co-dominant in nature, thus, can detect genetic variability at allelic level. However, the development and utilization of these marker systems are costly, laborious and time taking. RAPD, ISSR and AFLP, on the other hand, are dominant marker systems but are less costly and easier to be developed and used. Thus, while selecting a suitable marker system, a number of factors like availability of equipments, time and expertise along with

Table 1. Merits and demerits of different marker systems

Marker	PCR-based	Polymorphism	Nature	Developmental cost	Running cost
RFLP	No	Medium	Co-dominant	High	High
RAPD	Yes	High	Dominant	Low	Low
SSR	Yes	High	Co-dominant	High	Medium
ISSR	Yes	High	Dominant	Low	Low
AFLP	Yes	High	Dominant	Medium	Medium
Isozyme	No	Low	Co-dominant	High	Medium
STS	Yes	High	Dominant	High	Medium
EST	Yes	High	Co-dominant	High	Medium
SCAR	Yes	High	Co-dominant	High	Medium
CAPS	Yes	High	Co-dominant	High	Medium
SNP	Yes	Very High	Co-dominant	High	Medium

the nature of the crop, are to be taken into account.

Inter simple sequence repeat (ISSR) markers

Inter simple sequence repeat (ISSR) technique is a PCR based technique, reported by Ztetkiewicz *et al.* (1994), which involves amplification of DNA segments between two identical microsatellite repeat regions 'oriented in opposite direction using primers designed from microsatellite core regions. The technique uses microsatellite primers, usually 16 – 25 bp long, of di-nucleotide, tri-nucleotide, tetra-nucleotide or penta-nucleotide repeats to target multiple genomic loci. The primers can be either unanchored (Meyer *et al.*, 1993; Gupta *et al.*, 1994; Wu *et al.*, 1994) or more usually anchored at 3' or 5' end with 1 to 4 degenerate bases extended into the flanking sequences (Zetkiewicz *et al.*, 1994). ISSR primers generate polymorphism whenever one genome misses the sequence repeat or has a deletion or insertion or translocation that modifies the distance between the repeats. Usually di-nucleotide repeats anchored either at 3' or 5' end reveal high polymorphism (Nagaoka and Ogihara, 1997; Blair *et al.*, 1999; Joshi *et al.*, 2000). The primers anchored at 3' end give clearer banding pattern as compared to those anchored at 5' end (Tsumura *et al.*, 1996; Nagaoka and Ogihara, 1997; Blair *et al.*, 1999). In general, primer; with (AG) (GA), (CT), (TC), (AC), (CA) repeats show higher polymorphism than those with (AT) repeats as the primers with (AT) repeats tend to be self-annealed. ISSR markers are generally considered as dominant markers following Mendelian inheritance (Tsumura *et al.*, 1996; Ratnaparkhe *et al.*, 1998; Wang *et al.*, 1998). However, there are incidences where they segregated as co-dominant markers and helped to distinguish homozygotes from heterozygotes (Wu *et al.*, 1994; Akagi *et al.*, 1996; Wang *et al.*, 1998; Sankar and Moore, 2001).

ISSR markers have many advantages over other marker systems. ISSR technique is simple, quick and less costly like the RAPD technique. ISSR markers have high reproducibility than RAPD primers due to the longer primer length. The studies on reproducibility showed that about 92 – 95% of the scored fragments could be repeated across DNA samples of the same cultivars and across separate PCR runs (Fang and Roose, 1997; Moreno *et al.*, 1998). Development of ISSR markers does not need prior knowledge of the genome to be analyzed; hence, it can be used universally for plant genome analysis. ISSR markers provide more polymorphism (Fang *et al.*, 1997; Wolfe *et al.*, 1998) as compared to isozymes, because of the lack of mutational constraints in the inter simple sequence repeats as they are largely part of the noncoding regions of the genome, while isozymes are from the coding regions of the genome (Wolfe *et al.*, 1998). Similarly, mitochondrial and chloroplast DNA markers are haploid, maternally inherited, lack recombination and show gametic disequilibrium. Further, the rate of evolution of ISSR, mitochondrial and chloroplast markers was reported to be quite different as the ISSR markers evolve faster than the other two, despite the fact that mitochondrial genome shows extensive rearrangement of its structural organization (Wolfe *et al.*, 1987; Palmer and Herbon 1988; Provan *et al.*, 2001). It has also been observed that genomic regions containing microsatellites are evolving and mutating more rapidly than other areas due to slip-strand mispairing during replication, with the slippage rate dependent upon the length of the repeat (Burgess *et al.*, 2001). The slow rate of evolution exhibited by the mitochondrial gene made it less useful in genome analysis at family and subfamily levels but has proved to be very useful in inferring ancient phylogenetic relationships, and time and mode of diversifications (Hwang *et al.*, 1999; Damgaard and Sperling, 2001).

Owing to the above mentioned advantages, ISSR markers have extensively been used for characterization of germplasm (Wolff *et al.*, 1995; Charters and Wilkinson,

2000), to estimate the extent of genetic diversity at inter- and intra-specific level in a wide range of crop species (Salimath *et al.*, 1995; Nagaoka and Ogihara, 1997; Prevost *et al.*, 1998; Joshi *et al.*, 2000; Ajibade *et al.*, 2000), to identify DNA markers closely linked to important agronomic traits (Ratnaparkhe *et al.*, 1998; Hussain *et al.*, 2000; Levin *et al.*, 2000), to determine the distribution of microsatellites in the genome (Gupta *et al.*, 1994; Nagaoka and Ogihara, 1997; Blair *et al.*, 1999; Pasakinskiene *et al.*, 2000), to test the hypothesis of speciation (Wolfe *et al.*, 1998) and to study the history of colonization of plant communities, to distinguish closely related cultivars and varieties (Prevost *et al.*, 1999) and also to detect somaclonal variations in plants (Albani and Wilkinson, 1998). ISSR markers have also been used for screening for duplicate accessions in germplasm collections (Virk *et al.*, 2000). Thus, ISSR markers have many applications in plant genome analysis.

Application of ISSR techniques in mulberry

Mulberry (*Morus* spp.), the most important tree crop in sericulture industry, is highly a heterozygous tree with long juvenile period. Hence, genetic analysis using the conventional methods is very difficult, as developing pure lines or near isogonic lines is very laborious and time taking. In order to circumvent the problems associated with the genetic analysis of mulberry, molecular techniques have recently been adopted. Since no prior knowledge on the genome of mulberry was available, application of RAPD was the easiest way to begin with. Accordingly, Lichun *et al.* (1996), Lou *et al.* (1998), Zhang *et al.* (1998), Bhattacharya and Ranade (2001) and recently Chatterjee *et al.* (2004) used RAPD markers to study genetic diversity among different genotypes. Considering the disadvantages associated with RAPD markers, particularly on the reproducibility point, Vijayan and Chatterjee (2003) tested the suitability of ISSR primers in mulberry by using the primers obtained from University of British Columbia, Canada (set # 9). From this study it was found that (AG), (TG) and (AC) repeat primers generated excellent band profiles. Primers synthesized from (AT) repeats failed to amplify even at a low annealing temperature. The important tri and tetra nucleotide repeat primers generated excellent amplification are those from (ACC), (ATG), (AGC), (GAA), (GATA) and (CCCT). The penta-nucleotide repeats (GGAGA) and (GGGGT) also gave good amplification. Another important point noticed was the ineffectiveness of the use of formamide to enhance the clarity of the bands by reducing the faint bands and streaks associated with the PCR products of certain primers. The PCR mixture and amplification cycles followed by Vijayan and Chatterjee (2003) were as follows; The 20 µl PCR reaction mixture used for the ISSR amplification contained 2 µl of the 10× PCR buffer provided by the manufacturer (Genetaq, Genetix, Singapore; it contained 100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin, 0.5% Tween-20 and 0.05% NP-40), 0.2 mM dNTPs, 2 mM $MgCl_2$, 1.0 mM primer, 20 ng genomic DNA, and 1 unit of *Taq* polymerase enzyme. The PCR cycles followed were as follows; an initial denaturation of the genomic DNA at 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 2 min and a final extension at 72°C for 5 min. The PCR products were then resolved on 1.5% agarose gel and subsequent stained with ethidium bromide (0.5 µg/ml) and visualized the band profiles under UV illumination.

Genetic diversity and phylogenetic analysis

The central Sericultural Germplasm Resources Center, Hosur, Tamil Nadu has collected more than 750 mulberry accessions from different parts of India. For proper conservation and utilization, these materials have to be properly assessed for their genetic inter relationship. Attempts have, thus, been made with ISSR primers to generate molecular profiles for each mulberry accessions and also to estimate the genetic diversity present among these accessions. Vijayan and Chatterjee (2003) estimated genetic diversity among 11 locally popular cultivars. Later, Vijayan *et al.* (2004a) worked out the genetic diversity among sixteen genotypes of *M. serrata* present in Uttaranchal (29° 22' – 30° 45' N latitude and 75° 52' – 80° 12' E longitude) and Himachal Pradesh (30° 30' – 30° 54' N latitude and 77° 06' – 77°40' E longitude). Using 58 ISSR markers developed with 10 ISSR primers, Srivastava *et al.* (2004) worked out the genetic diversity among mulberry genotypes belonged to a single species. In another attempt, with ISSR markers Vijayan *et al.* (2004b) distinguished newly evolved mulberry varieties from traditional land cultivars and established their phylogenetic relationships. Recently, Vijayan *et al.* (2005a) worked out the genetic diversity among 34 mulberry accessions. Using different statistical methods, it has been shown that how the genetic materials might have migrated in the past so that genetic admixture between genetic pools of the eastern India and the southern India. These studies clearly revealed the usefulness of ISSR markers in the genetic diversity analysis of mulberry.

Genetic relationship between tropical and temperate mulberry

Since, morphologically the temperate mulberry doest not differ much from the tropical mulberry, though in certain

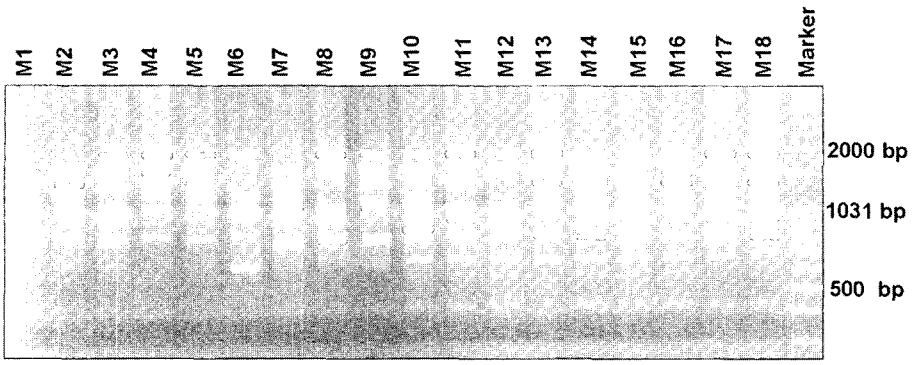

Fig. 1. An example of the molecular profile generated with GGGGT- repeat primer (UBC-881); M1, Okinawa; M2, Kenmochi; M3, Kokuso-13; M4, Seijuurou; M5, Ichibei; M6, Wasemidori; M7, Mizusawagua; M8, Kairyou Nezumi Gaeshi; M9, Goshoerami; M10, Surat; M11, Almora local; M12, Sujanpur-5; M13, MR-2; M14, Punjab local; M15, Dhidiawhite; M16, Bushmalda; M17, Assamjati; M18, Tippufort (Vijayan, 2004).

cases the temperate differ in sprouting and rooting from the tropical, it is difficult to distinguish the tropical mulberry genotypes from the temperate ones. Since many of the temperate mulberry genotypes like Goshoerami, Kairyou Nezumi Gaeshi have many good agronomical traits, utilization of these materials for the improvement of tropical mulberry, it is necessary to understand the genetic relationship between these two groups. ISSR markers have been employed for this purpose (Vijayan, 2004) and it has been found that using ISSR markers it is possible to distinguish tropical mulberry from the temperate ones (Fig. 1). A few ISSR markers specific for temperate mulberry have also been identified (Fig. 2).

Genetic relationship of Himalayan mulberry with others

Plants of *M. serrata* Roxb. are commonly known as

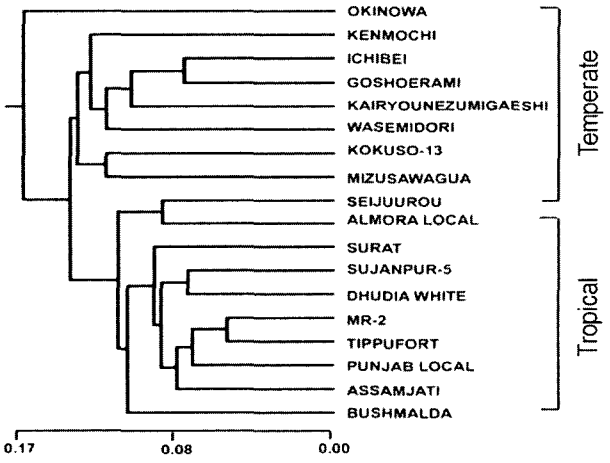

Fig. 2. Dendrogram showing clustering of temperate and topical mulberry as distinct clusters (Vijayan, 2004).

"Himalayan mulberry" because their natural distribution is mostly confined to the northwestern parts of the Himalayas (Ravindran *et al.*, 1997). Plants of this species harbor a number of agronomically desirable traits like resistance to biotic and abiotic stresses (Ravindran *et al.*, 1997; Tikader and Dandin, 2001), which are of much use for improving the mulberry varieties cultivated for silkworm rearing. Since, the hybridization studies in mulberry (Das and Krishnaswami, 1965; Dwivedi *et al.*, 1989; Tikader and Rao, 2002) showed no reproductive barrier among different species, widening of the narrowing genetic base of the cultivated varieties through inter-specific hybridization is quite possible. Since genetic interrelationships among parents are a pre-requisite for any breeding program, ISSR technique has been employed to understand the genetic relationship of *M. serrata* to other Indian mulberry species *viz., M. laevigata*, *M. India* and *M. alba* (Vijayan *et al.*, 2005b) The study reveled *M. serrata* is genetically far distant from *M. indica* and *M. alba* but closer to *M. laevigata* (Fig. 3).

As a supplementary tool to the taxonomist

Attempts have also been made to use ISSR markers along with RAPD markers as supplementary tools to confirm the correct taxonomic identity of genotypes. Using 15 ISSR primers the genetic relationships among nineteen genotypes of five mulberry species *viz., M. latifolia*, *M. bombycis*, *M. alba*, *M. laevigata* and *M. indica* were tested (Vijayan *et al.*, 2004c). The study could group all genotypes of *M. laevigata* into one cluster and genotypes from other four species were grouped together into another cluster. Population structure analysis was also resorted to elaborate the result, which revealed that *M. laevigata* can be considered as a separate species of mulberry while the other four species may be grouped

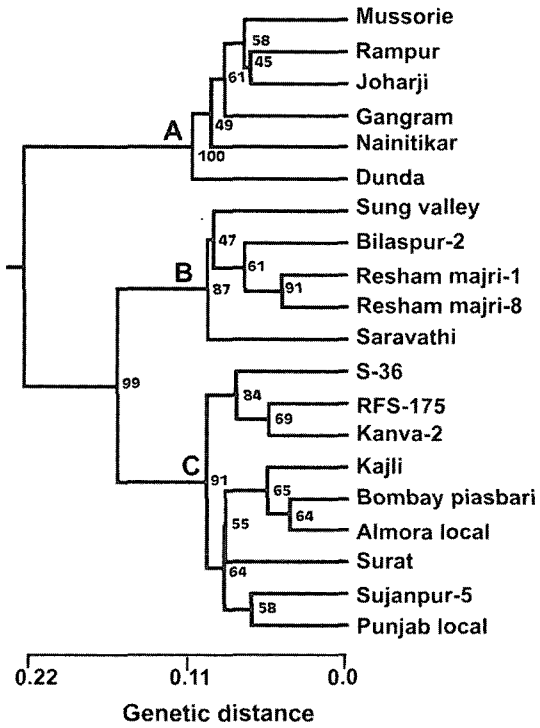

Fig. 3. Phylogenetic relationship of *M. serrata* with other Indian mulberry species; A, *M. serrta*; B, *M. laevigata*; C, *M. indica* and *M. alba* (Vijayan *et al.*, 2005b).

together and treated as sub-species as suggested earlier by Gururajan (1960) and Hirano (1977, 1982). Awasthi *et al.* (2004) also worked out the inter-species relationships among 15 mulberry species using ISSR markers on polyacrylamide gel electrophoresis (PAGE) as well as on agarose gel electrophoresis. This study could also separate wild mulberry species such as *M. laevigata, M. sraata and M. tiliaefolia* from the domesticated mulberry species *viz.*, *M. latifolia*, *M. nig*ra, *M. australis*, *M. sinensis*, *M. indica*, *M. bombycis*, *M. rubra*, *M. multicaulis*, *M.lh*ou, *M. cathayana*, *M. rotundiloba* and *M. alba*. However, the results of this study differ from that of Vijayan *et al.* (2004c) in the placement of *M. alba* in a group away from *M. indica*. Similarly, the study contradicted the contention of Koidzumi (1917) that *M. lhou*, *M. multicaulis* and *M. latifolia* are similar and belong to a single species. Thus, the results of the above studies show that ISSR markers are not very effective in determining the systematic position of mulberry genotypes; hence, more reliable marker systems like mitochondrial DNA sequences and chloroplast DNA sequences are to be used.

To identify markers associated with agronomic traits

Genetic markers associated with agronomically important traits are quite useful for marker-assisted selection to identify hybrids with desired traits at an early stage. This is very important in tree plants like mulberry where the juvenile and adult plant correlation on character expression is very poor. Hence, attempts have also been made in this direction by Vijayan and Chatterjee (2003). Using stepwise multiple regression analysis two specific markers *viz.*, $UBC825_{1400}$ and $UBC835_{750}$ associated with leaf yield in mulberry were identified, though their inheritance has not been ascertained in F_1 or F_2 progenies. Research in other crops like chick pea (Rantnaparkhe *et al.*, 1998), rice (Hussain *et al.*, 2000), maize (Domeniuk *et al.*, 2002) has revealed that ISSR markers are associated with disease resistance, temperature sensitive male sterility and agronomic traits. Using multiple regression analysis ISSR markers Chatterjee and Mohandas (2003) identified a number of markers associated with cocoon productivity in silkworm *Bombyx mori*. ISSRs have been ISSR markers have been used for linkage map construction in wheat (Nagaoka and Ogihara, 1997; Kojima *et al.*, 1998), potato (*Solanum tuberosum* L.) (Prevost and Wilkinson, 1999), citrus (*Poncirus trifoliata* L.) (Sankar and Moore, 2001), watermelon (*Citrullus lanatus* L.) (Hashizume *et al.*, 2003) and chickpea (*Ascochyta rabiei* L.) (Flandez-Galvez *et al.*, 2003). All these studies clearly indicate that ISSR markers associated with economically important traits can be identified for their utilization in marker-assisted selection (MAS) breeding in mulberry.

Future perspectives of ISSR in mulberry genome analysis

Since ISSR is one of the simplest, quickest marker system with high reproducibility, they will have an important role in securing plant variety rights by virtue of its unique efficiency in distinguishing even closely related germplasm (Reddy *et al.*, 2002). It is clear from the literature that ISSR primers could detect more polymorphism than with mtDNA, cpDNA, RAPD and isozymes in closely related plants (Gupta *et al.*, 1994; Salimath *et al.*, 1995; Virk *et al.*, 2000). Thus, ISSR profiles can be of much use in developing molecular IDs for each mulberry accessions for its proper identification, registration and conservation. Since mulberry genome analysis has been taken in a big way in India, more data on the occurrence and distribution of SSR motifs will become available shortly. Aggarwal *et al.* (2004) has reported six SSR primers from mulberry along with its microsatellite details. These primers can be used for amplifying the microsatellites motifs from different mulberry species, which in turn would help to design more ISSR primers for genome analysis in mulberry. Species and genotype specific ISSR marker can also be sequenced and used for synthesizing sequence characterized amplified region (SCAR) primers for their

utilization in taxonomic identification of the genotypes. Similarly, markers linked to the traits of agronomic importance can be sequenced and used as sequence tagged sites (STS) markers for their utilization in marker assisted selection. Another advantage in the use of ISSR markers lies in their linkage to SSR loci. Although microsatellites themselves are probably nonfunctional and selectively neutral, they are known to be linked to coding regions and, hence, ISSRs are likely to mark gene rich regions (Kojima *et al.*, 1998). Thus, ISSR techniques would be quite promising for mulberry genome analysis in the future.

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