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REVIEW ARTICLE

MOLECULAR MARKERS AND THEIR ROLE IN MULBERRY IMPROVEMENT

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ABSTRACT

Mulberry (genes Morus) a perennial tree or shrub in an economically important plant used for sericulture and is the sole food plant for the domesticated silkworm, *Bombyx mori*. Mulberry is widely distributed in Asia, Europe, North and South America and it is cultivated extensively in East, central and South Asia for silk production. Genetic characterization of germplasm resources is necessary for their effective management and efficient utilization, especially for species like mulberry in which the available germplsm exhibits rich phenotypic diversity with almost no information about its genetic base. Molecular markers are useful complements to morphological and phenological characters because they are plentiful, independent of tissue or environmental effects and allow accession identification in the early stages of development. Such techniques reveal polymorphisms at the DNA level and are very powerful tool for characterization and genetic diversity estimation. Many molecular markers such as RAPD, SSR, AFLP and SRAP have been successfully used in identification and genetic diversitv analvsis in mulberrv.

INTRODUCTION

Mulberry (Morus) is an important crop plant in sericulture. Its foliage is the exclusive food of domestic silkworm (*Bombyx mori* L.) which produces the natural silk. The queen of textiles. Mulberry is believed to have originated at the feet of Himalayan ranges. According to watt (1873) certain forms of morus were truly wild in India, but according to Vavilov (1926) the primary centre of origin of mulberry was the China, Japan gene center, which includes East China, Korea and Japan. Presently, morus species are grown in warm and moist climates between lat. 50^0 N and 10^0 S, which includes the Southeastern tip of Asia and Japan. Taxonomically, the genus was placed in urticales under the family Moraceae. A number of species have been reported within this genus. Initially, linneaus (1753) divided the genus morus into seven species: *Morus alba* L; *Morus nigra* L; *Morus rubra* L; *Morus tarterica* L; *Morus indica* L; *Morus papyrifera* and *Morus tinetoria*. But the last two where later shifted to the genera Broussonetia and chlorophora respectively. However, Brandis (1874) described four species and classified the genus into two section based on style length and subdivided both the sections again according to the length and shape of the syncarp and a few leaf characters. Koidzumi (1923) identified 25 species and classified them into 2 sections, dolichostyle and macromorus, based on the length of the style each section was then subdivided into two subsections pubescentae and papillosae based on stigma hairiness on the basic of leaf anatomical and wood characters. Shah and Kachroo (1979) classified the genus into tow sections (i) *Morus nigra* L; (ii) *Morus alba* L; *Morus bombycis koidz*., and *Morus latifolia* P. However, after assessing the protein and Allozyme profiles of *M. alba*, *M. bombycis* and *M. latifolia*, Hirano (1982) suggested grouping all three spices together. The confusion over the classification of Morus into species remains a major bottleneck for formulating appropriate strategies to conserve the previous materials for future breeding programs.

Genetic characterization of germplasm resources is necessary for their effective management and efficient utilization, especially for species like mulberry in which the available germplasm exhibits such phenotypic diversity with almost no information about its genetic base. Genetic diversity in the genus Morus have been developed using morphological characters and molecular techniques. Molecular markers are useful complements to morphological and phenological characters because they are plentiful, independent of tissue or environmental effects and allow accession identification in the early stages of development. Such techniques reveal polymorphisms at the DNA level and are a very powerful tool for characterization and genetic diversity estimation. Many molecular marker

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techniques have been successfully used in identification and genetic diversity analysis in mulberry, such as RAPD (Xiang et al., 1995; (Zhao and Pam, 2004), SSR (Aggarwal et al., 2004), ISSR (Zhao et al., 2006) and AFLP (Sharma and Sharma 2000). RAPD in simple, convenient and inexpensive but poor consistency and low reproducibility limit in its utilization (Roodt et al., 2002). AFLP technology is now widely used for genomic fingerprinting (Karaca et al., 2002) due to its high polymorphism (Vos et al., 1995). However AFLP is complex, requires multiple steps and shows pseudo-polymorphism when methylation-sensitive restriction enzymes are used. SSRs are stable, abundant, highly polymorphic and reproducible, but they require the development of working primers for each species which makes the method laborious and costly (Xiao et al., 2008). Sequence- related amplified polymorphism (SRAP) is a novel molecular marker technique based on two primer amplification that preferentially amplifies open reading frames [(ORFS) Li and Quiros, 2001]. The observed polymorphism originates in the variation in the length of extrons, introns, promoters and spacers both among individuals and species. SRAP markers are more powerful for revealing genetic diversity among closely related cultivars than SSR, ISSR or RAPD markers.

Molecular markers

Several types of molecular markers which have been developed and used in plants are restriction fragment length polymorphism (RFLP), sequence tagged sites (STS), Expressed sequence tags (ESTS), Simple sequence repeats (SSRs) or microsatellites, randomly amplified polymorphic DNA (RAPDs), sequence characterized amplified regions (SCARs), amplified fragment length polymorphic (AFLP) and Sequence-related amplified polymorphism (SRAP) markers.

1) Hybridization based markers

i) Restriction Fragment length polymorphism (RELPs)

The variation at DNA level is assessed by shearing the entire DNA with restriction enzymes. Such enzymes are available in bacteria for use as defense mechanism against viruses. Each restriction enzyme identifies a specific site of DNA usually 4-8 base pairs at which the enzymes act upon to cut the both stands of DNA. The restriction sites for a particular enzymes are present at several places throughout the entire genome with the result that a large number of segments of DNA are produced. The length of each segment depends upon the distance between two adjacent restriction sites. The electrophoresis of stained DNA can detect fragments with different lengths. But the number of all fragments being very large gives a continuous smear which makes it very difficult to observe individual fragments. The number of fragments is thus reduced by screening through probes which hybridized with only some of the fragments. Only the fragments complementary to the sequences of probe hybridize with it which are observed through autoradiography. Each individual genotype has a fixed pattern of distribution of fragments for a given enzyme and probe. Another individual with difference at DNA level will differ for restriction sites as a result of which a different pattern of fragment distribution is observed. The term restriction fragment length (RFLP) has been coined to describe this variation for length of fragments from digestion of DNA form two or more organism with same endonuclease (Helentjaris et al., 1985). The presence of polymorphism depends on the restriction enzyme used and nucleotide sequence of the probe. So a number of probes and restriction enzymes are used. All the individuals in the mapping population like F_2 are evaluated for each enzyme/probe combination. Banding pattern among individuals is compared and variation in one DNA fragment obtained with a specific enzyme is treated as one RFLP. Probes may be obtained from either cDNA or genomic DNA libraries but probes with unique sequence or interspersed low copy number portion of the genome are most useful as these produce only resolvable number of bands. The sequences with high copy number will detect a large number of fragments which are difficult to interpret and may even give a smear on autoradiograph. A unique sequence probe will hybridize to a single restriction fragment and low copy number may hybridize to as many as five loci. Technical complexity of performing RFLP analysis and difficulties with the use of short-lived radio-isotopes have led to the development of some other, mostly PCR based techniques. However (RFLPs) has not been used in mulberry due to some limitations.

- ☞ The assay is time consuming and labour intensive.
- ☞ Requirement of radioactive isotopes makes the analysis expensive and hazardous.
- ☞ Satellite and highly respective sequences are inaccessible with naturally occurring probes.

ii) Polymerase chain reaction (PCR) based markers

Sequence tagged sites (STS)

The RFLP probes linked to desirable traits can be converted to polymerase chain reaction (PCR) based markers. In this the RFLP probes are end-sequenced and complementary primers are synthesized. These primes (generally 20 mers) are then used for amplifying specific genomic sequences using PCR. One major limitation of these markers is the reduced polymorphism hence they are not used in mulberry.

Expressed sequence tags (EST)

These markers are developed by end sequencing of random cDNA clones. The cDNA markers are first mapped as RFLP markers and then partially sequenced to convert them into PCR based markers. These can be used for synteny mapping and cloning of specific genes.

Randomly Amplified Polymorphic DNAs (RAPDs)

Williams *et al.* (1990) originally developed the technique. This is a PCR based technique where a single short oligonucleotide primer which binds to many different loci, is used to amplify random sequences from a complex DNA template such as a plant genome. For most plants the primes that are 9-10 nucleotide long are expected to generate 2-10 amplification products. The primers are generally of random sequence, based to contain at least 50% GC content and to lack internal repeats. The products are easily separated by standard electrophoretic techniques and visualized by UV illumination of ethidium bromide stained gels. Polymorphism results from changes in either the sequence of the primer binding site (e.g. point mutation) or from changes which alter the size or prevent the amplification of target DNA (e.g. insertions, deletions, inversions) in inheritance studies, the amplifications products are transmitted as dominant markers(Waugh and Powell, 1992).

A total of 12 oligonucleotide random primers were used for RAPD analysis against the 12 collections of mulberry of each of Mysore local and V-I. All the primers produced distinct polymorphic banding pattern between the two cultivators. A total of 73 markers were generated of which 40 were monomorphic and rest 33 were polymorphic (45%). The size of the amplified markers ranged from 500-2500 bp with 3-10 markers per primer as expected of asexually propagated material, no difference in banding pattern was observed within the collections of the same cultivator (Naik et al., 2002). Similar result was obtained by Mulcahy et al. (1993) in vegetatively propagated apple and concluded that the different accessions of same cultivar yielded identical fingerprints.

RAPD analysis showed the genetic similarity among the different collection ranged from 76% (between v-1 and RFs-175/Anantha) to 100% (among duplicate collections). However the third suspected group of duplicate collection namely, Kousen and Xuan-10 did not show complete similarity in DNA amplifications profiles (Naik et al., 2006).

Simple Sequence Repeats (SSRs)

In multicellular organisms about 90% of DNA is redundant which comprises 'satellite' or repeat regions which may be present at one or more sites through out the geneome. The tandem repeats of sequences ranging from 9 to 100 bp are called minisatellites or variable Number of Tandem Repeats (VNTR). The tandem repeated sequences of DNA with a repeat size ranging from 1-6 bp are called microsatellites or simple sequence Repeat (SSR). DNA sequences containing SSRs can be amplified by PCR and SSR variants can be detected by gel electrophoresis of the amplified fragments (Bligh et al., 1195).

The analysis of six microsatellite across 43 genotypes revealed significant ($P<0.05$) deviation from HWE and significant LD for two pairs of markers (MULSTR1/MULSTR2 and MULSTR2/MULSTR3) using ARLEQUIN and four pairs (MULSTR1/ MULSTR2, MULSTR2/ MULSTR3, MULSTR1/MULSTR5 and MULSTR2/MULSTR5) with GENEPOP (Aggrawal et al., 2004).

Inter-simple sequence repeats (ISSR)

ISSR marker have been used for the genetic study of mulberry germplasm characterization, crop improvement and molecular systematics. Genetic variability of clonal mulberry within field and tube seedling. Fifteen ISSR primers were used with five mulberry individuals, eight primers generated bright amplification products, six primers failed to generate any amplification products and one generated weak or ambiguous amplification products. The 8 ISSR primers produced amplification products that were monomorphic within the population of mulberry field and tissue culture. Only using the primer $(GAG)_4$ GC, one polymorphic band was present within two populations. Within the natural cultivated mulberry population, a total of 33 reliable fragments were obtained, one bands were polymorphic with a polymorphic ratio of 3.03 per cent, while within the population of *in vitro* culture plant, 35 bands were amplified among 27 individuals with the polymorphic ratio of 2.89 per cent, indicating the polymorphism detected by ISSR markers (Zhao *et al*., 2007).

Sequence characterized Amplified Region Markers (SCAR)

A scar marker represents a specified genomic region that is amplified by PCR using a pairs of specific oligonucleotide primers. 'Scar' markers are considered better than RAPD because these are identified as distinct single bands in agarose gels and some of these show codominance which differentiates heterozygotes from both types of homozygotes (Paran et al., 1993).

Amplified Fragment Length Polymorphism (AFLP)

This technique was developed by Vos et al. (1995). In this technique restriction fragments generated by a frequent (4 base) and a rare (6 base) cutter are anchored with oligonucleotide adapters of a few oligonucleotide base. This method generates a large number of restriction fragments facilitating the detection of polymorphism. The number DNA fragments amplified can be controlled by choosing different base numbers and composition of nucleotides in the adapters. This technique is more reliable since stringent reaction conditions are used for primer annealing. This technique thus shows an ingenious combination of RFLP and PCR techniques and is extremely useful in detection of polymorphism even between closely related genotypes. Characterization of 43 *Morus* accessions originating from distinct regions of Turkey using fluorescent dye amplified fragment length polymorphism (AFLP) markers and capillary electrophoresis. The accessions belonged to *M. alba*, *M. nigra* and *M. rubra*, *M. alba* consisted of white and purple fruited samples. Eight primer combinations generated a total of 416 bonds, 337 of which were polymorphic (80.5%). Resolving powers of the AFLP primers ranged from 0.410 to 0.924 making a total of 5.015, where as the polymorphic information content ranged from 0.662 to 0.898 with an average of 0.812. Unweighted pair group method of arithmetic mean (UPGMA) clustering of the accessions showed three major groups representing to *M. nigra*, *M. rubra*, and *M. alba* accessions. The *M. alba* group had two subgroups that were not correlated with fruit colour. The UPGMA dendrogram of average taxonomic differences confirmed these results (Salih et al., 2008).

Sequence related amplified polymorphism (SRAP)

SRAP is a novel molecular marker technique designed to amplify open reading frames (ORFs) SRAP was used to identify mulberry germplasm. Twenty three mulberry accessions were screened using SRAP techniques, with 12 primer combinations selected for their reproducibility and polymorphism. Out of 83 PCR fragments scored, 59 (71.1 %) were polymorphic, with an average of 4.9 polymorphic bands and 6.9 bands per primer combination. Mean gene diversity and PIC were 0.161 and 0.1353 respectively. The genetic similarly coefficient ranged from 0.6905 to 0.9524 with an average of 0.8330 (Weigh *et al*., 2009).

Combined RAPD and ISSR marker analysis

The genetic similarity obtained by combined analysis of RAPD and ISSR marker was more similar to the one obtained through RAPD marker analysis alone and ranged from 0.689 (between G.No.4 and Mysore local) to 0.896 (between RFS-135 and RFS-175). Clustering obtained by UPGMA analysis of the combined marker data was identical to the one obtained through RAPD analysis alone. In all the three analysis AR-11, S—1635, G.NO.2 and

G.NO.4 clustered distinctly. AR-11 was considered as an out group in all the three dendrograms. The pattern of clustering was slightly different when compared with that of the one based on ISSR marker analysis alone (Naik and Dandin, 2005).

Genetic diversity was observed among the genotypes based on the DNA markers generated by both types of primers. The band profiles generated by the RAPD primer OPA-11 and UBC-826 have clearly revealed such variability among the genotypes. The ten ISSR primers generated a total of 58 bands of which 43 were polymorphic, thus generating 74.13% polymorphism. Similarly, out of 80 bands generated by the RAPD primers, 49 were polymorphic, thus generating 60.75% polymorphism among the 11 mulberry genotypes. The results clearly showed that ISSR primers were more efficient in revealing DNA polymorphism among the genotypes than the RAPD primers (Srivastava et al., 2004).

The genetic similarity coefficient among genotypes estimated on the basic of Nei and Li (1979) varied from 0.904 to 0.544 with an average genetic similarity of 0.728 in ISSR markers. The same was in the range of 0.923 to 0.617 with a mean coefficient of 0.756 when the pooled data of both marker systems were used. The Dice coefficients (Sneath and Sokal, 1973) among the genotypes also showed considerable variation. In ISSR, it varied from 0.475 to 0.352 and in RAPD it was between 0.490 to 0.379. Incase of pooled data, the maximum genetic similarity was 0.480 and the minimum was 0.382. The similarity coefficients among the genotypes estimated on the basis of Jaccard (1901) was between 0.921 and 0.440, 0.825 and 0.374 and 0.825 and 0.272 in ISSR , RAPD and in the pooled data of ISSR + RAPD markers respectively. The Pearson's correlation coefficient between different matrices subjected to the Mantel test (Mantel 1967) were found to be highly significant (r =0.435, 0.998, p = 0.000).

The dendrograms realized from the above matrices of ISSR, RAPD and the pooled data from both marker system using UPGMA method grouped the 19 mulberry genotypes, into four clusters. The first group comprised four genotypes, two from *M. alba* and one each from *M. latifolea* and *M. bombycis* .In the 2nd cluster , seven genotypes from *M. alba*, *M. latifolea* and *M. bombycis* were again grouped together. The third cluster contained only four genotypes, all from *M. indica*. The fourth cluster included all *M. laevigata* genotypes. Another important points noticed from the dendrogram is the high genetic distances enjoyed by *M . laevigata* from the others (Vijayan et al., 2003).

CONCLUSION

Mulberry is a perennial, heterogenous out breeding tree, the leaves of which are the exclusive food of the silk secreting insect, *B. mori* L. Species identification in mulberry (*Morus*) is a great debate among scientists despite the number of criteria such as floral character, wood, leaf anatomy and biochemical characters used to identify the species within this genus. Thus it is concluded from this revealed study identification of taxa based on morphofloral characters alone often generates misleading results. Thus, an approach integrating morphological and genetic molecular parameter is required to resolve the problems pertaining to the taxonomic position of species in mulberry.

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