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Applications of Molecular Characterization in Fruit Crops

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ABSTRACT
In recent years, there is growing public awareness and increasing demands for greater dietary diversity, as well as future production challenges leading to exploded population. Also the increased environmental variability resulting from climate change, food crisis situation seen in last few years implies that in the future, farmers and plant breeders will need to be able to access an even wider range of plant genetic resources for food and agriculture than today. Exact and quick identification of the cultivars and to recognize groups with similar genotypes for determining the distinctness and uniqueness of the phenotypic and genetic formation of genotypes is very important to improve the performance of cultivars in practical breeding programme as well as for the protection of proprietary rights. Laborious and very lengthy procedures in conventional plant breeding ,heterozygous nature and high degree of outcrossing in tropical fruit crops,perennial nature and specific climatic needs of fruit crops, difficulty in detecting and transferring the complex characters like disease resistance ,biotic stress , mineral deficiencies, etc. by traditional breeding method, extinction of semi-wild and wild species of underutilized fruit crops, unavailability of systematic morphological characterization and evaluation of these species,low morphological variation and lack of differentiating characters among some closely related species andvarieties,all these reasons have led to the emergence of new technique i. e. molecular characterization. Also the Controversy about genetically modifiedorganisms (GMO) demands for the determination of the origin of the product and the rawmaterials used. It has led to requirement of a well-documented traceability system (to identify and trace a product at all stages of production and marketing). This has also demanded for the molecular characterization. Characterization with the help of DNA markers is called as molecular characterization. It is the most advanced and most reliable techniqueamong various characterization methods like molecular, morphological, biochemical characterization. The molecular method of characterization is the most reliable technique. In this method various markers viz. RAPD, RFLP, AFLP, VNTR,ISSR, SSR, SNP, STR,SEP,SCAR are used for characterization. Molecular markers have diverse applications in fruit crop improvement, particularly in the areas of genetic diversity and varietal identification studies, gene tagging, identification of QTL, gene cloning, disease diagnostics, pedigree analysis, hybrid detection, sex differentiation and marker assisted selection.
KEYWORDS: *Applications, Molecular characterization, Fruit crops*

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INTRODUCTION

Characterization is the description of plant germplasm. It determines the expression of high heritable characters ranging from morphological features to seed proteins or molecular markers. There are three major types of genetic markers/characters, viz., i) morphological markers which themselves are phenotypic traits orcharacters, ii) biochemical markers, which include allelic variants of enzymes called isozymes and iii) DNA (or molecular) markers, which reveal sites of variation in DNA.

Markers that are located in close proximity to genes (i.e. tightly linked) may be referred to as gene 'tags'. Such markers themselves do not affect the phenotype of the trait of interest because they are located only near or 'linked' to genes controlling the trait. Genes are the

units of heredity. DNA i.e. deoxyribonucleic acid is a nucleic acid that contains the genetic instructions used in the development and functioning of all known living organisms and some viruses. DNA is a set of blueprints needed to construct other components of cells, such as proteins and RNA molecules.

Characterization with the help of DNA markers is called as Molecular characterization.Most advanced and most reliable technique is utilized for characterization of numerous cultivars known as molecular characterization. Molecular markers are the heritable differences in the nucleotide sequence of DNA from two different individuals

Significance of molecular characterization in fruit crops

- The conventional breeding methods are laborious and very lengthy and in fruit crops it takes more than 15 years to develop a variety due to perennial nature and specific climatic and edaphic needs of fruit crops. Tropical fruit species are mostly heterozygous due to high degree of outcrossing. Some of the semi-wild and wild species of underutilized fruit crops are on a way of extinction. At the same time, growing public awareness has increased demands for greater dietary diversity, as well as future production challenges.
- In conventional breeding whole genome is transferred. Moreover, some of the characters like complex disease resistance reaction, biotic stresses, mineral deficiencies/toxicity that show continuous variation and do not fit into Mendelian ratios are most difficult to detect and transfer through conventional plant breeding. In addition, there is low morphological variation and lack of differentiating characters among some closely related species and varieties
- Unavailability of systematic morphological characterization and evaluation of the fruit species.
- Controversy about genetically modified organisms(GMO) demands for the determination of the origin of the product and the raw materials

Principles of DNA isolation & purification

DNA isolation is a procedure to collect DNA for subsequent molecular analysis. There are three basic steps in a DNA extraction:

i. **Cell disruption** - This is commonly achieved by grinding or sonicating the sample.
ii. **Extracting DNA** - It is done by adding pre-heated extraction buffer to sample and heating it at 65 º C for 60 minutes, centrifuging mixture and collecting supernatant and by adding phenol : chloroform : isoamyl alcohol to it.
iii. **Precipitating the DNA**: Usually ice-cold ethanol or isopropanol is used. Since DNA is insoluble in these alcohols, it will aggregate together, giving a pellet upon centrifugation. This step also removes alcohol soluble salt.Wash DNA pellet to remove excess salt in 70% Ethanol and air-dry. Resuspend the pellet in sterile distilled water (PH 7.4 or in TE) and store it at 4 ˚C or frozen it at -20 ˚C for long term.

iv)Quantifying the DNA

The purity of the DNA is reflected in the OD260: OD 280 ratio and must be between 1.6 and 2.00. If it is <1.6, then it is protein contaminated and if it is >2.0, then it is chloroform/phenol contaminated. Such samples need to be repurify.

Polymerase chain reaction (PCR)

The polymerase chain reaction is an extremely versatile technique for copying DNA**.** It wasinvented by Kary Mullis in 1983 (Nobel prize in 1990).PCR allows a single DNA sequence to be copied (millions of times) or altered in predetermined ways.PCR has many variations like reverse transcription PCR (RT-PCR) for amplification of RNA and real-time PCR (QPCR) which allow for quantitative measurement of DNA or RNA molecules. It is relatively inexpensive requiring small amounts of DNA. It gives quicker results.

Components of PCR

The master mix for PCR contains various components as DNA Template, primer, dNTP mixture, Taq DNA polymerase, $MgCl_2$, 10x PCRbuffer and sterile deionized water.

PCR Analysis

PCR is an in-vitro method for enzymatic amplification of a specific DNA segment from the genomic DNA.A common application of PCR is the study of patterns of gene expression and gene mapping. PCR also assist in the task of DNA sequencing and the phylogenic analysis of DNA from ancient sources.PCR can also be used in parental testing, where an individual is matched with their close relatives.

Gel electrophoresis

Here, the basic principle is that DNA, RNA and proteins can all be separated by means of an electric field.In agarose gel electrophoresis, DNA and RNA can be separated on the basis of size by running the DNA through an agarose gel. Proteins can be separated on the basis of size by using an SDS-PAGE gel or on the basis of size and their electric charge by using what is known as a 2D gel electrophoresis.

Primer

A primer is a strand of nucleic acid that serves as a starting point for DNA synthesis.These primers are usually short, chemically synthesized oligonucleotides, with a length of about twenty bases. They are hybridized to a target DNA, which is then copied by the polymerase.Minimum primer length used in most applications is 18 nucleotides.Replication starts at the 3'-end of the primer, and copies the opposite strand

Molecular markers

Molecular or genetic markers are sequences of DNA which have been traced to specific locations on the chromosomes and associated with particular traits.Molecular marker are based on naturally occurring polymorphism in DNA sequence(i.e. base pair deletion, substitution,addition or patterns). Anideal DNA marker must be highly polymorphic with co-dominant inheritance. It should be reproducible and randomly and frequently distributed throughout the genome. It should not be affected by pleiotropism and epistatic interactions

Types of genetic markers

- RFLP (or Restriction fragment length polymorphism)
- AFLP (or Amplified fragment length polymorphism)
- RAPD (or Random amplification of polymorphic DNA)
- VNTR (or Variable number tandem repeat)
- ISSR (Inter-Simple sequence repeat)
- Micro satellite polymorphism, SSR (or Simple sequence repeat)
- SNP (or Single nucleotide polymorphism)
- STR (or Short tandem repeat)
- SFP (or Single feature polymorphism)
- DArT (or Diversity Arrays Technology)
- RAD markers (or Restriction site associated DNAmarkers)

Present Status in Fruit Crops

Lot of molecular characterization work has been done for genetic diversity assessment in fruit crops [1-7]. Grape rootstock identification [8],Papaya sex determination [9],isolation of gene MaCOL1 responsible for fruit ripening and stress management in banana, identify major QTLs for resistance to grapevine powdery mildew and their use in marker assisted breeding [10], technique of simultaneous detection of 2-3 viruses at NRC, Banana are the important works carried out through molecular characterization. In recent years, molecular characterization technique has been started to be exploited for disease diagnosis.

Applications of molecular markers in Fruit Crops

1. Assessment of genetic diversity

By using molecular markers characterization and identification of cultivars, varieties and natural populations is done to understand the genetic variability of the population. A number of reports are available on the use for DNA markers to assess genetic diversity among species of several horticultural crops, as well as validation of genetic relatedness among them. This has significant application, especially for difficult to breed woody perennials

In mango, attempts are made to exploit molecular analysis for genetic distinctiveness and relationships of indigenous landraceswith popular cultivars of mango in Andhra Pradesh, India [11]. They selected 20 indeginous land races of mango and further characterized for their genetic distinctiveness and relationship with the choisest juicy cultivars of mango in Andhra Pradesh (Peddarasam, Chinnarasam, Cherukurasam, Panchadarakalasa and Suvarnarekha). 109 mango specific SSR markers were used out of which 57 were polymorphic and 10 were highly polymorphic and showed 31- 60% variability.

Large morphological and genetic diversity detected due to free pollination among plants.
Large morphological and genetic diversity detected due to free pollination among plants.

Various types of DNA markers, including RFLPs, RAPDs, ISSRs, and SSRs have been used to characterize citrus germplasm and relationships. Researchers have identified putative hybrid accessions based on high heterozygosity and lack of unique alleles, and studied their probable parentage. ISSR (inter-simple sequence repeat) markers are useful for distinguishing hybrid seedlings from apomictic seedlings because they provide a complex "DNA fingerprint" that targets rapidly evolving sequences. A graduate student, Noelle Barkley, has completed a study of 24 SSR markers in 370 mostly sexually derived accessions from the Citrus Variety Collection and determined population structure and identified putative hybrid accessions.A collaborative project with Dr. T. E. Mirkov (Texas A&M University) is directed toward cloning a Poncirustrifoliata gene that causes resistance to citrus tristeza virus. They have developed high-density linkage maps of the region surrounding this gene and sequenced a 282 kb BAC coating that should contain the gene. Candidate genes are now being tested by transformation. (http://plantbiology.ucr.edu/faculty/roose.html)

Baig M.N.R et. al. [12] carried out molecular characterization and genetic diversity analysis of citrus cultivars by RAPD markers. RAPD markers were used to evaluate genetic similarity and interrelationship among 18 citrus cultivars, including 13 species and 5 hybrids. Out of 40 primers screened, 25 were selected which produced 250 markers; of which 231 were polymorphic and some species or cultivar specific RAPD markers. The average genetic similarity value observed across all the genotypes was 0.63, with the 2 sweet orange cultivars, Jaffa and Blood red, showing maximum similarity (82%). The Jatti-Khatti and King Mandarin were found to be genetically most diverse. Dendrogram separated Jatti-Khatti from all major clusters at a similarity coefficient of 0.61. The genetic variation between cultivars was quite high and revealed their different origins.

Genetic variability of Musa spp. in Sri Lanka is rich and needed characterization for its conservation and use. Accordingly, morphological and molecular characterization of *Musa Germplasm in* Sri Lanka and selection of superior genotypes was conducted by Samarasinghe W.L.G.[13].In this study, 27 cultivars (*M. acuminate (AA* genome),*M. balbisiana (BB* genome),polyploids and interspecific hybrids) *were* characterized using morphological traits and SSRs. From the SSR analysis, 2-11 alleles were observed, reflecting the genetic diversity among cultivars and hence an important reason for their conservation

Molecular characterization and genetic relationships among some grape(*Vitisvinifera*L.) cultivars as revealed by Nagaty M. A. and El-Assal S.E. [14] by using RAPD and SSR markers. Objective was to use these fingerprints to identify molecular markers that co-segregate and could be used in isolating gene(s) which controlling some important traits, thereafter can be used in breeding programs(marker assisted selection). In this study, 8 SSR pairs of primers specific for grape and 10 RAPD primers were used. This work focused on estimating of intra- and inter-cultivar polymorphisms among eight seedlings (one year old) of some grape cultivars : Superior; Early Superior; Thompson seedless-1; Thompson seedless-2; Thompson seedless-3; Fayomi; RoumiAhmer and Bez El-Anza, (collected from Agricultural Research Center (ARC) farm, Giza, Egypt). Genetic similarity ranged between 87% and 89% which were recorded among Thompson seedless-1, Thompson seedless-2 and Thompson seedless-3 cultivars.

Most of the fruit characteristics of the genotypes within the same group were variable. Therefore, the results showed that molecular characterization is necessary to get reliable relationships among pomegranate genotypes and AFLP markers can be used effectively in pomegranate. It seems that there are no relationships betweenmorphological and molecular data among pomegranate genotypes from the Coruh Valley. These discrepancies werepreviously reported by several authors working on pomegranate. The high level of phenotypic differences observed among pomegranate genotypes might be the result of environmental conditions, which greatly affect expression of quantitative traits.

The molecular characterization is a very efficient, effective and reliable technique to study diversity amongst sugar apple genotypes. Genetic diversity in *Annonasquamosa by* morphological, biochemical and RAPD markers was studied by Bharad et al. (2009).RAPD markers have proved to be suitable for characterizing sugar apple (*Annonasquamosa) genotypes*. The results as obtained using biochemical and RAPD markers showed that there was considerable diversity amongst the genotypes collected from different locations. The

genotypes AKCa 05, AKCa 07 and AKCa 10 can be conserved based on true genetic diversity.

Rodrigues M.G.F. [15] studied genetic characterization of fig tree mutants with molecular markers. The improvement programs of fig trees using conventional procedures in order to obtain new cultivars are rare in many countries, such as Brazil, especially due to the little genetic variability and to the difficulties in obtaining plants from gamete fusion once the wasp *Blastophagapsenes*, responsible for the natural pollinating, is not found in Brazil. In this way, the mutagenic genetic improvement becomes a solution of it. For this reason, in an experiment conducted earlier, fig plants formed by cuttings treated with gamma ray were selected based on their agronomic characteristics of interest.

Researchers determined the genetic variability in these fig tree selections, using RAPD and AFLP molecular markers, comparing them to each other and to the Roxo-de-Valinhos, used as the standard. For the reactions of DNA amplification, 140 RAPD primers and 12 primer combinations for AFLP analysis were used. The selections did not differ genetically between themselves and between them and the Roxo-de-Valinhos cultivar. Techniques that can detect polymorphism between treatments, such as DNA sequencing, must be tested. The phenotypic variation of plants may be due to epigenetic variation, necessitating the use of techniques with methylation-sensitive restriction enzymes.

Sane et al. [16] studied morphological and molecular (ISSR) characterization of jackfruit (*Artocarpusheterophyllus Lam).* They distinguished 18 accessions of jackfruit (Artocarpus heterophyllus Lam.) maintained in the field gene bank of Indian Institute of Horticultural Research Bangalore at the DNA level by ISSR analysis.Primers that generate accession specific profiles were identified and found useful for generating DNA profiles unique to the jackfruit accessions studied.

This study revealed the ability of ISSR markers to detect polymorphism and also to evaluate genetic variability and differentiate accessions of jackfruit. According to Hamrick et al. 1992, most of the morphological traitsare influenced by environmental factors and many of qualitativetraits which are of polygenic inheritance are expressed only afterseveral years of growth. As a result, the leveland pattern of genetic diversity determined only by morphologicaltraits are less accurate and questionable.

Biodiversity in Pears (*Pyrus Spp.)* was studied by Ahmed *M. (2008).* A survey of five pear growing districts of Azad Jammu and Kashmir i.e. Rawalakot, Bagh, Muzaffarabad, Sudhnoti and Kotli was conducted during the year 2003 and 60 accessions of distinct characters of horticultural importance were selected from 48 sites of these districts for characterization.

The accessions were characterized by using protein markers based on bio-chemical analysis (SDS-PAGE). The accessions differed in number of bands which ranged from 12-20. According to the banding pattern the accessions were divided into the various groupsand sub-groups showing similarities and differences among them. Out of sixty local xxi accessions, fifty six accessions along with eight varieties (used as reference control) were also characterized using DNA based SSR markers to assess genetic diversity and relationship among them. Nine out of 12 primers revealed clear and reproducible amplification banding pattern in 41 genotypes (33 accessions and 8 control varieties).

Cluster analysis based on UPGMA dendrogram, grouped the genotypes into clusters subclusters and groups on the basis of relatedness and variability. Most of the accessions were absolutely homogenous and were classified into two homogenous groups, despite the fact that these accessions differed in there morphological and physico-chemical traits.

The AFLP analysis was found useful fordetection of genetic differences among the plum cultivarsstudied. The results of the present study may also benefitbreeders in selecting the most diverse cultivars with similarfruit characteristics to begin crossing and selection programs.This may result in increased plum growing for fruit production.

Azhar Hussain [17] also experimented RAPD markers forcharacterization of Loquat (*Eriobotrya japonica Lindl.)*genotypes cultivated in Pakistan. 42 loquat genotypes identified through the morphological and physical characters were also subjected to the DNA analysis using 14 random primers todetermine the level of genetic diversity among the local loquat genotypes found in different areas of Pakistan and to assess the relationships among them. According to the dandrogram, two main groups 'A' and 'B' of the loquat genotypes have been identified having a linkage distance of 33%.

2. Varietal diagnosis, sex determination

Varietal identification is nothing but DNA fingerprinting. Singly or in groups, molecular markers are capable of producing patterns that are unique for each individual genotype. Their patterns, whether they are generated by PCR or by hybridization with single copy, multicopy, or repeated sequences are referred to as genetic fingerprintings. Few examples of DNA markers used for varietal identification are mentioned below:

As per the DARE/ICAR Annual Report-2008-9 & Bhat Z. A. et. ai. [8], at NRC for Grape, Pune twenty-one grape rootstocks were analysed with AFLP and SSR primers. Two clones of rootstock, viz. Dogridge A and Dogridge B, were distinguished. Dogridge B was grouped with *Vitischampinii,* while Dogridge A with 110R (a hybrid of *V. rupestris and V. berlendieri)* with 80% similarity. Several accessions resistant tothripsand downy mildew were identified through field screening. Eight AFLP and 5 SSR markers showing promising association with resistance/ susceptibility to disease have been identified

With the help of molecular characterization, at NRC, grapes, Pune (India), the ambiguity of Dogridge from two different sources (IIHR, Bangalore and American strain) has been solved. Dogridge from Bangalore was found to be true to type while Dogridge of America was actually a hybrid and was renamed as B-26.

Deputy *et al.*, [9] developed molecular marker for sex determination in papaya which is tightly linked to gene *sex 1*. SCAR T1 and SCAR W11 produce products in hermaphrodite and male plants and only rarely in females. SCAR T1 produces a product in all papayas regardlessof plant sex.PCR was run on DNA from papaya varieties 'Sunrise' and 'Kapoho' using primers for both SCAR T1 and SCAR W11. It was found that SCAR W11 is present in hermaphrodite plants but not in female plants.

3. Identification of QTLs, gene cloning

Many important heritable characters are a consequence of the joint action of several genes. Such characters are often referred to as polygenic or quantitative. Several characters of plant species, among which are traits of agronomic importance, are inherited quantitatively. Yield, maturitydate and drought tolerance are examples of such characters. The genetic loci for such characters have been referred to as quantitative trait loci (QTLs). The essential feature which makes feasible the finding and characterization of a QTL is its linkage with a known marker locus segregating with Mendelian ratios. DNA markers provide this opportunity by making it feasible to identify, map and measure the effects of genes underlying quantitative trait. In grape QTLs were use for features such as critical Photoperiod, growth cessation, or dormancy, bud break (BB) and winter hardiness Approximate position of 28 major genes were mapped in different populations of peach (orange background), almond (yellow background) and Myrobalan plum (green background) on the framework of the *Prunus* reference map.

Chen J. [18] studied molecular characterization and expression profiles of MaCOL1, a CONSTANS-like gene in banana fruit. He identified, isolated and characterized a novel cDNA encoding CONSTANS-like gene designated as MaCOL1 from banana fruit.Real-time PCR analysis showed that MaCOL1 was differentially expressed among various banana plant organs, with higher expression in flower while in pulp it obviously increased during natural or ethylene-induced fruit ripening, suggesting that MaCOL1 might be associated with the pulp ripening of banana fruit.

Riaz, S. et.al. [10]experimented a limited genetic mapping strategy based on simple sequence repeat (SSR) marker data with five grape populations segregating for powdery mildew resistance in an effort to develop genetic markers from multiple sources and enable the pyramiding of resistance loci. They identified significant QTLs for powdery mildew resistance with overlapping genomic regions for different tissue types (leaf, stem, rachis & berry) on chromosome 18 which distinguishes the resistance in 'Magnolia' from that present in other accessions of *M. rotundifolia* and controlled by the Run1 gene on chromosome 12.

4. Marker assisted selection (MAS) and linkage map

This is one of the important applications of molecular markers. Molecular markers can potentially increase the importance and usefulness of indirect selection in plant breeding. MAS permits the breeder to make earlier decisions about the further selections while examining fewer plants. An added advantage in breeding for disease resistance behaviour is that this could be done in the absence of pathogen once marker information is available.

Earlier markers were being developed for monogenic traits but present markers are developed for traits governed by multigenes or polygenes.

5. Pedigree analysis and detection of hybrids

Isozyme analysis has been successfully employed to confirm parentage of plums, apple and mango cultivars and also to establish origin of several pineapple cultivars. Further isozyme been used for differentiating between progeny produced by self pollination and those produced via cross pollination and detection of hybrids. They are used to confirm the production of interspecific prunus hybrids, grape interspecific crosses and progeny screening for hybrid seedlings in citrus breeding programme, besides identification of zygotic and nucellar seedlings in citrus.

At NRC, grapes, Pune (India), the ambiguity of Dogridge from two different sources (IIHR, Bangalore and American strain) was solved. Dogridge from Bangalore was found to be true to type while Dogridge of America was actually a hybrid and was renamed as B-26.

Based on the phylogenetic analysis with RAPD data, 'Cambu', 'Hongnhieu', 'Liusun', 'Tieu' and 'Sanh' were clustered in mandarin cluster and *C. reticulata* was assumed to be one of their parents. 'Cam sen', 'Cam Voi', 'Trap', 'Cam Songcon', natural hybrids having unknown genetic origin appeared as genetically closer to the sweet orange *(C. sinensis*). Similarly, based on RAPD data and morphological characteristics, hybrids of *C. maxima* and *C. medica* were assumed to origin from 'Bong' and 'Bory'. Besides the above applications molecular markers are used for negative selection, estimation of genetic contribution by each parent in a segregating population and gene pyramiding.

6. Diseases diagnosis

Molecular markers have made it possible to develop diagnostic techniques to identify pathogen with an unprecedented accuracy and speed and to tap genes from as diverse sources as microbes, plants and animals to enable the researchers to develop plants resistant to diseases.

Many viral diseases have been reported in India and among them BBTV, BBrMV, CMV and BSV are considerably important. Occurrence of BBTV has wiped out large area under hill banana cultivation. BBrMV is another devastating viral disease affecting plantain and many important commercial varieties.CMV occurs in almost all banana growing states. Banana Streak Virus (BSV) is present in every banana-producing region worldwide and its infections can lead to severe decline in yield especially in Poovan varieties. The viral pathogens are a major impediment in banana tissue culture industry. Diagnosis against viruses has become an integral part in the management of viral diseases in banana. Early and correct diagnosis of viruses is very important in checking for further spread of the disease especially to a new area.

CONCLUSION

The success of DNA marker technology for bringing genetic improvement in fruit crops would depend on close interaction between Plant breeders and biotechnologists andavailability of skilled man power and substantial financial investmenton research. All the wild and semi- wild species of different fruit crops need to be fingerprinted, genes resistant to certain diseases can be identified and further utilized in breeding programme. Fruit crop specific problems like mango spongy tissue, early sex determination in papaya , kokum, etc. can be solved

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