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Molecular marker - a new approach for forest tree improvement

Manoj Kumar Jhariya*, Abhishek Raj, Parmeshwar Sahu[1], Nongmaithem Raju Singh and K.P. Sahu

Department of Forestry, College of Agriculture, I.G.K.V., Raipur 492 012 (C.G.), India
[1]Department of Genetics and Plant Breeding, College of Agriculture, I.G.K.V., Raipur 492 012 (C.G.), India

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ABSTRACT

Molecular markers are important tools for forest tree improvement. Forest tree improvement has therefore been a slow and arduous process by virtue of the large size and long generation times of trees. The most important markers are hybridization based DNA markers RFLP and, PCR based DNA markers such as Randomly Amplified Polymorphic DNAs (RAPDs), Simple Sequence Repeats (SSRs) or Microsatellites/ Sequence Tagged Sites (STS), Amplified Fragment Length Polymorphisms (AFLPs), Inter-Simple Sequence Repeats (ISSR) and Cleaved Amplified Polymorphic Sequences (CAPS). Recent advances in molecular biology offer considerable scope to understand and evaluate population structure, levels of genetic variation, and relatedness and mating systems of tropical tree species. Various molecular techniques are being investigated for a quick and reliable assessment of genetic diversity and related parameters for a number of timber and non-timber tree species in the country. Their application is very important in case of estimating polymorphism and relatedness, genotype characterization and marker-assisted selection. Isozymes generally provides ample information and are inexpensive, rapid, and technically easy to apply. The large sized, long-regeneration cycle and sporadic seed production of trees cause numerous problems in tree improvement programs. However, the molecular markers simply reduce the rotation period to attaining a merchantable size, so it can be sold in a shorter period of time with a disease resistance, pest resistance, and phenotypicaly and genotypicaly good tree species which satisfied the national needs of the country.

***Key words** : Molecular markers, Tree improvement, Rotation period.*

Introduction

It was a time, when forest covered the maximum geographical areas of the whole landscape (mother land). But due to increasing population, which creates a huge pressure on the natural forest for satisfying national needs by getting forest property. So, for reducing pressure on natural forest, plantations of tropical forest tree species are generally being established using unimproved materials to supplement timber supply from the depleting natural forests quickly but this has resulted in poor yields and high incidence of diseases in some instances. The problems encountered in tree breeding are the large size of the genome, scarcity of multigenerational pedigrees and long generation times (Tulsieram *et al.*, 1992), this is combined with the non availability of adequate number of morphological markers in conventional tree breeding. These problem can be overcome by some extent through the use of biochemical markers where the product controlled by genes.

Recent developments in molecular biology and biotechnology offer rapid characterization of genotypes and detection of genetic variation using a range of molecular markers from isozymes, restriction fragment length polymorphisms (RFLPs), ran-

***Corresponding author's email:** manu9589@gmail.com

domly amplified polymorphic DNAs (RAPDs), directed amplification of mini-satellite regions (DAMD), amplified fragment length polymorphisms (AFLPs) to simple sequence repeats (SSRs). Their application is studied in Walnut, Poplar, Yellow Poplar (*Liriodendron tulipifera*), Sweet gum (*Liquidambar styraciflua*), Red maple (*Acer rubrum*), Eucalyptus species etc. for tree improvement program. The other most studied tree species are Loblolly pines, Monterey pine, Scots pines, Slash pines, Western white pines, Douglas-fir, Norway spruce etc. Some of these molecular markers can also be effectively used in early selection for hybridity, disease resistance and wood quality, long the hope of tree breeders. Prior knowledge of the population genetics of indigenous tree species is, thus, imperative for successful implementation of genetic conservation programmes. So, with this context we are highlighting the important molecular markers system which can be utilized in tree breeding programs with their applications, strategies and available evidences.

Why forest tree improvement is essential?

The genetically improved trees are generally disease resistant, most durable, and produce good seeds which results in a phonotypicaly and genotypicaly superior progeny, this can be possible through the plantation work with the help of proper molecular genetics treatment and reduces the pressure on the natural forests and the satisfying the national needs in terms of public benefits. Like improved varieties of corn, beans, wheat, vegetables and fruits, tree researchers can select for desirable traits for trees being planted for conservation purposes. Many landowners have a goal of establishing tree plantings with vigorous conservation seedlings with the genetic programming to grow high quality timber. For example, we may want fast growing trees to overcome susceptibility to depredation from some biotic factors (animals, parasites and insect pest) or simply reduce the amount of time (rotation period) it takes to attaining a merchantable size, so it can be sold in a shorter period of time. The large sized, long-regeneration cycle and sporadic seed production of trees cause numerous problems in tree improvement programs. However, DNA recombination and genetic transformation, and RFLP/RAPD markers, provides the opportunities to solve the above problems and to accelerate tree improvement.

Other traits like form, mast production, pest resistance or adaptability to grow on a drier site versus wetter site can be selected, depending on the goals. Once a trait is identified through testing, the best selections for that trait can then be propagated and planted into seed orchards that will then be able to cross pollinate with one another, producing seed to meet reforestation goals. Social benefits arise largely from the substantial contributions that forest trees make to both the environment, and to economic growth. Economic benefits are becoming increasingly obvious in particular when tree biotechnology leads to the deployment of highly improved trees with genetic improvement in plantation forestry (Walter *et al.*, 2007).

Marker System

Now a day's genetic markers have become indispensable tools for understanding, managing, and improving natural and planted populations of trees species. The discriminatory power provided by molecular markers can be used to resolve and understand hybridization and species differentiation. Although each marker system is associated with some advantages and disadvantages, the choice of marker system is dictated to a large extent by the intended application, convenience and the cost involved. An ideal DNA marker should have following properties-

- Easily available.
- Assay is easy and rapid.
- Highly polymorphic and reproducible.
- Co-dominant inheritance and recurrent occurrence in genome.
- Selectively neutral to environmental conditions or management practices.
- Data exchange between different laboratories should be easy.

Depending on the type of study undertaken, a marker system can be recognized that would fulfill the above characteristics. Different types of molecular markers are utilized to evaluate DNA polymorphism and are classified as:

1. Hybridization based DNA markers such as Restriction Fragment Length Polymorphisms (RFLPs).
2. PCR based DNA markers such as Randomly Amplified Polymorphic DNAs (RAPDs) which can also be converted into Sequence Characterized Amplified Regions (SCARs), Simple Sequence Repeats (SSRs) or Microsatellites/Sequence Tagged Sites (STS), Amplified Frag-

ment Length Polymorphisms (AFLPs), Inter-Simple Sequence Repeats (ISSR) and Cleaved Amplified Polymorphic Sequences (CAPS).

1. Hybridization based DNA markers

a) RFLPS (Restriction Fragment Length Polymorphism)

RFLPs, being co dominant markers, can detect the coupling phase of DNA molecules, as DNA fragments from all homologous chromosomes are detected. They are very reliable markers in linkage analysis and breeding and can easily determine if a linked trait is present in a homozygous or heterozygous state in an individual, which is information highly desirable for recessive traits. RFLP markers that are used for high density genomic mapping (Botstein *et al.*, 1980) provided a new technique which overcame some of the problems associated with isozymes and proteins.

RFLPs were recovered from many loci and extended the linkage maps in poplar (Bradshaw *et al.*, 1994) and Loblolly Pine (Devey *et al.*, 1994). However, RFLP markers are found to be powerful tools forcomparative and synteny mapping (Xu, 2010).

2. PCR based molecular markers-

a) AFLPS (Amplified Fragment Length Polymorphism)

AFLP is a highly sensitive method for fingerprinting genomic DNA within any organism. Restriction endonucleases such as *Mse1* and *EcoR1* are used to digest the DNA before amplification. Applications of this technique reach far beyond agricultural applications, ranging from agronomic trait analysis, diagnostics, pedigree analysis, forensics, parental heritage and may be used as a universal fingerprinting system (Pereira *et al.*, 2010). AFLP analysis is not popular as much as other PCR based markers like RAPD; it was employed in genetic studies of tree species like Larch (Arcade *et al.*, 2000) and Neem (Singh *et al.*, 2002).

(b) RAPDS (Random Amplified Polymorphic DNA)

A Random Amplified Polymorphic DNA (RAPD) technique is based on the polymerase chain reaction and has been one of the most commonly used molecular techniques to develop DNA markers.

RAPDs are much simpler and less expensive to work with than RFLPs because no prior knowledge of sequences is required and there is no need for radioactive probes. RAPDs produce DNA profiles of varying complexity, depending on the primer and template used. The RAPD markers are well exploited in tree genetics and breeding by various workers, in Olive (Gemas *et al.*, 2004), *Morus* sp (Awasthi *et al.*, 2004), neem (Deshwal *et al.*, 2005; Bhatt *et al.*, 2011), *Eucalyptus* sp (Grattapaglia *et al.*, 1994), Larch (Arcade *et al.*, 2000) etc. A major drawback of the technique is that because of the random nature of their generation, and short primer length, they cannot be easily transferred between species. An additional drawback is that of poor reliability and reproducibility, and their sensitivity to experimental conditions.

(c) SSRs (Microsatellites or Simple Sequence Repeats)

These are short segments of DNA that are derived from short (usually < 6 base pairs) tandemly repeated sequences such as (GA) n, (AAT) n, (GT) n and are known as microsatellites. These are an ideal genetic markers, would provide the specificity and the rapidity of PCR with more information per locus examined. SSRs have been characterized in many tree genomes such as Eucalyptus sps (Brondania *et al.*, 1998), Olive (Rallo *et al.*, 2000), rubber tree (Feng *et al.*, 2009) etc. SSRs are the markers of choice due to their codominant expression, multiallelism and high PIC value.

(d) Minisatellites or Variable number tandem repeats (VNTRs)

A minisatellite (also referred as VNTR) is a section of DNA that consists of a short series of bases about 10–60 bp in length. Minisatellite consists of repetitive, generally GC-rich, variant repeats. These variant repeats are tandemly intermingled,which makes minisatellite ideal for studying DNA turnover mechanisms and have been used extensively in many areas of genetics.

Minisatellites have been associated with chromosome fragile sites andare proximal to a number of recurrent translocation break points. Minisatellite markers have been used for a variety of purposes in several tree species including fingerprinting in rubber tree (Besse *et al.*, 1993) and diversity analysis and linkage map construction in pedunculate oak (Barreneche *et al.*, 1998).

(e) CAPS (Cleaved Amplified Polymorphic Sequences)

CAPS are DNA fragments amplified using specific primers, which are afterwards digested by restriction enzymes. Sequence polymorphisms result inthe

cutting of products in different places, and these variants are revealed as length differences on agarosegels. The CAPS approach is sometimes known as restriction fragment length polymorphism (RFLP) PCR, and the technique bears similarities to the non-PCR-based older RFLP method.

CAPS can be applied to organism-specific nuclear sequences,or to organelle DNA using universal primers. As with SSRs, sequencing is generally required in the former case in order to develop primer pairs. Similarly to SSRs, CAPS assess variation at one locus only in a particular PCR (Kadu *et al.*, 2006).

(f) SNPs (Single Nucleotide Polymorphisms)

SNPs markers are known as the third generation markers, which are now a days extensively used in various genomic studies for individual genotyping. Single nucleotide polymorphisms or SNPs are DNA sequence variations that occur due to point mutations when a single nucleotide (A, T, C, or G) in the genome sequence is altered (Gupta *et al.*, 2001). The association analysis and SNP genotyping analysis was successfully utilized in species like Pinus (Neale, 2007) and Vitis (Vezzulli *et al.*, 2008).

(g) ESTs (Expressed Sequence Tags)

An Expressed Sequence Tag or EST is a short (300–500 bp) sub-sequence of a cDNAs sequence. The cDNAs used for EST generation are individual clones from a cDNAs library which are complementaryto mRNA, so the ESTs represent portions of expressed genes. ESTs are used to identify gene transcripts, and play an important role in gene discovery and gene sequence. Since the ESTs are often partial sequences that correspond to the same mRNA of an organism, they are assembled into contigs so as to reduce the number of expressed sequence tags for downstream gene discovery analysis.

Applications of molecular markers for forest tree improvement

In tree improvement, molecular markers are most popularly used for estimation of polymorphisms, relatedness and mating system parameters, genotype characterization and marker-assisted selection. Whereas in conservation related activities molecular markers would be useful for estimation of polymorphisms, relatedness, mating system parameters and gene flow. The choice of molecular markers is dependent on the biological information already available for the species, the preciseness of the information required and the technical capabilities/facilities already available. There are several important applications of molecular markers for forest tree improvement-

1. Estimation of polymorphisms and relatedness

An assessment of the level of genetic variation between and within populations is a prerequisite for tree breeding and selection programmes for both in situ and ex situ conservation. The use of DNA markers in addition to the cheaper usage of isozymes together with phenotypic traits (provenance performance) as indicators of genetic diversity has immediate applications in sampling and selection guidelines to the selected species. RAPDs are currently the forerunners among the PCR-based techniques for estimation of genetic diversity of forest species mainly because of their rapidity and ability to handle large sample sizes, RAPDs have been successfully used for the estimation of genetic diversity and relatedness.

2. Estimation of mating System parameters and gene flow

Mating system parameter estimates e.g. out-crossing rates (TR, t), inbreeding coefficients (Fi, F") and extent of pollen dispersal are useful factors for designing seed orchards for optimal seed yield. Isozymes are currently the most suitable and cheapest marker for mating system and gene flow studies because of their co-dominant mode of inheritance.

3. Genotype characterization

It is possible using molecular markers to determine the genetic identity of individuals including inter and intra-specific hybrids, inbred lines and clones. . Clone identification in forestry using both RFLP and PCR based markers have been demonstrated in poplars (Vijay Rani *et al.*, 1995), willows (Change *et al.*, 1995) and RAPD markers in Neem tree (Bhatt *et al.*, 2011).

These rapid molecular techniques for genotype characterization can be effectively employed in screening for hybrids and checking mislabeling of ramets/plantlets in clonal propagation and validity of controlled crosses.

4. Marker-assisted selection

Molecular markers offer the possibility of effective

and early selection for highly correlated commercially important genes (e.g. wood density and wood volume). Utility of RFLPs and RAPDs have been demonstrated for quantitative trait loci (QTL) mapping in trees (Grattapaglia *et al.*, 1993; Bradshaw *et al.*, 1994). Some successesin QTL mapping in forestry include resistance to white pine blister sugar pine (Devey *et al.*, 1994), wood density and wood volume in radiata pine (Devey, 1994) and rust (*Pucciniapsidii*) resistance with quasi Mendelian inheritance was found and mapped in *Eucalyptus grandis* (Junghans *et al.*, 2003a).

5. Estimation of genetic variability in domesticated populations

Isozymes have been used to assess the amounts of genetic variability in selected breeding populations versus wild stands from where selections were made, in seed orchard crops, and in commercial seed collections from wild stands (Adams, 1981b). Thus, for rapid assessments of variability in domesticated populations and outputs from such populations (*e.g.*, seed crops), isozymes are probably the best approach. However, in species in which levels of isozymes variability are low or in which estimates must be obtained from tissues where only a few loci can be resolved (e.g., seedlings in nursery crops), the application of DNA-based markers would be warranted. RFLPs would be a better marker than RAPDs for this application because multiple alleles at a locus can be detected.

6. Germplasm identification

Probably the most important application of genetic markers in tree improvement is the broad problem of germplasm identification. The array of identification problems ranges from simple to complex.

Forest trees, especially conifers, have high levels of isozymes variation, which makes these markers quite adequate for many germplasm identification problems. The most important type of marker for germplasm identification is the fingerprinting markers such as M13, AP-PCR, SSLPs, or other hyper variable sequences.

7. Controlled crosses

Controlled crossing is an important aspect of most tree breeding programs. Adams *et al.* (1988) have used isozymes to determine the accuracy of such crosses from operational programs. Their results showed that 30% or more of the Douglas-fir and loblolly pine crosses they studied were not correct and that these determinations could be made with as few as 6-10 isozymes loci and five seeds per cross.

8. Genetic mapping and breeding purposes

There is a lot of application of molecular markers in the woody plants for genetic mapping and tree breeding improvement purposes. Genetic analyses with RFLP and RAPD markers have quickly been extended to tree species to develop genetic linkage maps.

Species	Scientific names	Molecular marker
Loblolly pine	*Pinus teada*	RFLP, RAPD
Slash pine	*Pinus elliottii*	RAPD
Douglas-fir	*Pseudotsuga menziesii*	RAPD
White spruce	*Picea glauca*	RFLP, RAPD
Sitka spruce	*Picea sitchensis*	RFLP
Peaches	*Prunus spp.*	RFLP
Poplar	*Populus spp.*	RFLP
Walnut	*Juglans regia*	RFLP

Potential of molecular markers to facilitate forest gene conservation management:

The potential of the application of molecular markers for the management of forest genetic conservation could be summarized as below:

1. To clarify the identity of taxa and their relatedness as well as to infer their evolutionary histories
2. To correctly identify clones and ramets in gene banks to avoid mislabeling, duplication and contamination
3. To evaluate the amount, extent and distribution of genetic diversity within and between populations
4. To estimate mating system (selfing and out crossing rate) and gene flow
5. To evaluate the status of genetic resources as the criteria for *ex situ* and *in situ* conservation from genetic information provided
6. To maximize the efficiency of management of conservation by combining adaptive traits, ecogeographic and genetic survey for both collection programs.

Conclusion

Molecular markers and marker mapping are a part

of the intrusive new genetics that is pushing its way into all areas of modern biology, from genomics to breeding, from transgenic to developmental biology, from Systematics to ecology, and even, perhaps especially, into tree and crop physiology.These are helpful in effectively used in early selection for hybridity, disease resistance and wood quality, pest resistance adaptability to grow on a drier site versus wetter site. Some traits and markers are conserved across related species and so comparative genomics of traits and markers between trees also helps in a breeding process. Markers will probably continue to enjoy increased application in forest genetics studies (diversity and conservation, phytogeography, mating systems) and tree improvement (finger printing, paternity analysis, breeding and testing, QTL mapping, MAS, association genetics) though most effort is likely to be concentrated on a few highly valued species. These applications are becoming increasingly commercial in scope.

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