Review Article

Chapter 4

MOLECULAR MARKERS AS TOOLS FOR CHARACTERIZATION AND IMPROVEMENT OF TEA GERMPLASM

Shashi Bhushan Tripathi[1] and Madan Singh Negi

INTRODUCTION

Tea (*Camellia sinensis* [L.] O. Kuntze) is one of the most common non-alcoholic beverages of the world used since ancient times. India is the largest producer and exporter of processed tea with over 30% share in global tea production. Thus, tea holds a key position in Indian economy generating over 660 million US dollars of foreign exchange annually.

The Indo-Burma region adjacent to the courses of Irrawaddy River is a rich source of diversity for different types of tea and justifies the suggestion that this region is the primary centre of origin for tea (Singh and Bera, 1994). Three species of cultivated teas namely, *C. sinensis*, *C. assamica* and *C. assamica* spp. *lasiocalyx* have been taxonomically recognized in India based on leaf morphology and growth habit (Wight, 1962).

Till the advent of clonal technology and clonal selections, planting material was produced using seeds obtained from seed orchards or "Seed Baris". The individual shrubs in these seed orchards were sourced from plants growing in 'wild' and therefore the planting material obtained from these sources used to be highly heterogeneous in their morphology and field performance due to genetically diverse individuals in seed baris (Bezbaruah and Dutta, 1977). Till recently, not many efforts had been made to evaluate the genetic diversity of tea germplasm in India or abroad. With recent focus on germplasm conservation and improvement, numbers of studies on germplasm characterization on tea have been reported. This area of research experienced a logarithmic surge during early 90's and afterwards, due to technical advances made in the field of molecular markers during this period. This article discusses various molecular techniques that have been used in tea so far and the future potential of these technologies in its genetic improvement.

Importance of Genetic diversity studies

Conservation of genetic diversity present in bioresources is important to meet the future requirements of planting material. Estimates of intra- and inter-population genetic diversity help in developing suitable strategies for conservation. For example, genetically rich natural resource areas for *in situ* conservation can be identified based on diversity estimates. A high level of genetic heterogeneity exists in tea presumably due to frequent outcrossing as a result of self-incompatibility (Neog *et al*., 2004). Pollination in tea is chiefly by insects, which usually migrate to only short distances. Yet wide spread natural hybridization at intra- and inter-specific level is common in natural populations. This has made the taxonomy of *Camellia* species confusing. The present germplasm of tea at best may be considered as a complex mixture of several compatible species of *Camellia* with overlapping morphology and therefore pure archetypes of tea

1. Biotechnology and Management of Bioresources Division, The Energy and Resources Institute, Darbari Seth Block, Lodhi Road, New Delhi-110003. e-mail: sbhushan@teri.res.in

may be non existent at present (Visser, 1969). However, this heterogeneity of tea germplasm is economically very important because it has served as the only source of improved planting material of tea. Most of the presently cultivated tea clones have been developed through selection from natural populations. In spite of these inter-specific hybridisations, the tea varieties are still conventionally attributed to one the three types (China, Assam and Cambod) solely based on their morphological features (Konwar, 1999).

As is the case with agricultural crops, the biodiversity of tea germplasm is decreasing rapidly due to cultivation of only a few vegetatively propagated cultivars (clones) many of which have shared parentage. Thus the genetic base that is in use currently has become narrow. This necessitates the inclusion of genetically diverse genotypes with important traits in *ex situ*-conserved germplasm. Although the accommodating capacity of *ex situ* conservation is very limited yet these are important for improvement programs because they act as easily accessible germplasm material to plant breeders. Moreover, background information on these germplasm for important traits and genetic diversity provides helps the breeder in selecting the right material. To reduce the cost of maintenance of *ex situ* germplasms, the strategy is to maintain a "core collection", which is a subset of total accessions (usually less than 30%) harboring maximum (over 90%) of overall genetic diversity. Significant portions of germplasm collections are commonly genetically redundant which can be detected by genetic diversity studies.

The reliability and reproducibility of marker systems used for genetic diversity studies is indispensable for the accuracy of the results. Conventionally, morphological (such as leaf, flower and fruit morphology) cytological (such as chromosome number and morphology) and biochemical markers (such as content and composition of sugars, amino acids, fats and secondary metabolites) have been used for estimation of genetic diversity of genetic resources of several plant species including tea (Wickremaratne, 1981). Protein based markers such as isozymes, have also been used to estimate genetic diversity in *Camellia* species (Sen *et al*., 2000). However, the major problem of these markers is their poor reproducibility, which is primarily due to the fact that they are severely influenced by environmental factors and developmental stages of the plant. Further, most of these markers display continuous variations, if at all, that make it difficult to assign them to any discrete group. Besides, these markers are available in limited numbers. All of these problems have been alleviated with development of DNA based markers making them the most commonly used tools for genetic diversity analysis.

DNA based markers

During past two decades, a series of DNA based marker techniques have been developed and used for germplasm characterization. These markers can be primarily classified in two major categories:

a) *Specifically primed markers* from known and characterized regions of the genome. The examples are restriction fragment length polymorphism (RFLP), cleaved amplified polymorphic sites (CAPS), sequence-tagged microsatellite sites (STMS) and sequence characterized amplified regions (SCAR) markers. The development of these markers requires extensive cloning and sequencing and designing of specific PCR primers to amplify target sequences. All of these are co-dominant single locus markers and therefore highly

expensive during application also. These markers could be developed from coding as well as from non-coding regions of the genome.

b) *Arbitrarily primed anonymous markers*, which are dominant. The examples of these markers are randomly amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR) and amplified fragment length polymorphisms (AFLP) markers. These markers are very commonly used because no prior sequence information is required prior to their use. In addition, these markers are multilocus markers and they provide information from several loci per assay, which reduces the cost per locus.

Sequence characterized markers

RFLPs were the first DNA based markers used for genetic diversity studies. Although RFLP markers are codominant and therefore highly informative, their use in genetic diversity analysis has been less common. Matsumoto *et al*., (1994) cloned the phenylalanine ammonia-lyase (PAL) cDNA from tea, and used it as RFLP probe. They were able to distinguish Assam hybrids and Japanese tea varieties with low and high levels of catechins. The PAL gene was shown to be single copy and multi-allelic. The major disadvantage of RFLPs is that they are highly expensive and time consuming both during development and application. The advent of polymerase chain reaction (PCR) led to development of another codominant marker, known as cleaved amplified polymorphic sites (CAPS) or PCR-RFLP.

Kaundun and Matsumoto (2003) analyzed the genetic diversity of sinensis and assamica cultivars of tea using CAPS markers for three genes, namely, PAL, chalcone synthase (CHS2) and dihydroflavonol 4-reductase (DFR), which are involved in phenylpropanoid pathway leading to biosynthesis of catechin and tannin. The study showed that polymorphisms in PAL and DFR genes was due to indels in the introns of both genes as well as due to point mutations as revealed by restriction digestion of amplicons. Although they did not find any particular CAPS profile specific to sinensis or assamica, the combined dataset from all the CAPS markers could group the cultivars according to their respective varietal status. A greater level of variation was observed in CHS2 gene, which is expected due to fact that CHS is a multigene family comprising of three non-allelic genes with high sequence similarity (Takeuchi *et al*., 1994).

As more and more genes from tea are being cloned and sequenced, there is great future potential for CAPS markers. The expressed sequence tags (EST) projects in tea have produced a large number of sequence data (Park *et al*., 2004). Presently, there are over 2000 ESTs for tea available in NCBI database (www.ncbi.nlm.nih.gov). CAPS markers based on these ESTs can be also used for linkage analysis and marker- assisted selection in tea because these are from the expressed regions of the genome. Although majority of these are uncharacterized sequences, these can still be used for development of CAPS markers in future. Several important genes controlling quality traits in tea such as beta-primeverosidase (Mizutani *et al*., 2002), caffeine synthase (Kato *et al*., 2000) and polyphenol oxidase (Genebank accession number AF269192) have been already cloned and sequenced. Identification of polymorphisms in these genes can help in developing varieties with improved quality traits through marker-aided selection.

STMS markers are another type of codominant markers, which are the most desirable markers for genetic studies due to inherently high levels of allele

diversity. These markers are based on flanking sequences of microsatellites, which are tendem repeats of 2-5 base pairs. Development of STMS markers is highly expensive because it requires isolation, cloning and sequencing of portions of genome containing these repeats. Till date, only 15 microsatellite sequences from *C. sinensis* have been reported (Freeman *et al*., 2004). Few microsatellites sequences from *C. japonica* have also been reported earlier (Ueno *et al*., 1999). These markers are non-transferable between species except very closely related ones and therefore need to be developed for each species separately. Kaundun and Matsumoto (2002) attempted to transfer four nuclear microsatellites from C*. japonica* and seven chloroplast microsatellites from *Nicotiana tabacum* into *C. sinensis.* They tested these markers on twenty-four "sinensis" and "assamica" cultivars of tea. While all four microsatellites from *C. japonica* produced polymorphism in *C. sinensis*, only four out of seven microsatellites from tobacco produced amplification products and only one showed polymorphism between different cultivars.

Arbitrary primed anonymous markers

In recent years, several studies have been done in tea using PCR based anonymous markers such as RAPD, ISSR and AFLP for analysis of genetic diversity (Wachira *et al*., 1995; Paul *et al*., 1997; Lai *et al*., 2001; Balasaravanan *et al*., 2004) and for development of genetic linkage map (Hackett *et al*. 2000). The comparatively high number of publications using these arbitrarily primed markers reflects their ease of application and cost effectiveness in such studies. Using RAPD, Kaundun *et al*. (2000) observed higher level of genetic diversity in Korean cultivated accessions of tea as compared to accessions from Japan and Taiwan. However, the intra-population genetic diversity of six Korean tea populations was found to be relatively lower than the expected diversity of natural populations (Kaundun and Park, 2002). This was attributed to narrow genetic base of the tea samples introduced from China and considerable reduction in population size following mass destruction of tea populations during fourteenth century.

Wachira *et al*. (1995) used RAPD to estimate genetic diversity and taxonomic relationships in 38 clones belonging to the three tea varieties, assamica, sinensis, and assamica ssp. lasiocalyx. Extensive genetic variability was detected in these clones 70% of which was partitioned within varieties. Cluster analysis using RAPD data could separate the three populations in a manner consistent with both the present taxonomy of tea and with the known pedigrees of some clones. The study also showed the usefulness of RAPD in discriminating the commercial clones, even those that cannot be distinguished on the basis of morphological and phenotypic traits.

A major limitation of RAPD markers is their low reproducibility, which could be due to competition for primer binding sites during first few cycles of PCR (Powell *et al*., 1996; Archak *et al*., 2003). This is further compounded by the low resolving power of agarose gels that are used to separate the RAPD fragments. The number of RAPD bands produced per assay (multiplex ratio) is usually between 3-10. Therefore, the genetic diversity estimates in many RAPD studies have been made with few numbers of loci, which may produce sub optimum results (Kaundun *et al*., 2000).

The AFLP technology developed by Vos *et al*., (1995) is a more reliable and robust technique. The multiplex ratio of AFLP is between 40-100 and more importantly this number can be modulated by using

appropriately designed primers. This marker technology has received great attention in genetic diversity studies during recent years.

The first paper on genetic diversity of tea using AFLP was by Paul *et al.,* (1997) who studied diversity of 32 tea clones comprising of Indian and Kenyan origin. A total of 73 bands generated with 5 AFLP primer combinations revealed that the intra-morphotype genetic diversity was higher in Chinary types than in Assam or Cambod types. The clustering obtained in this study was consistent with the morphotypes of the accessions used in this study. The principal component analysis revealed that the Assam clones from India and Kenya were highly related which supports the history of migration of tea from India to Kenya (Singh and Bera, 1994).

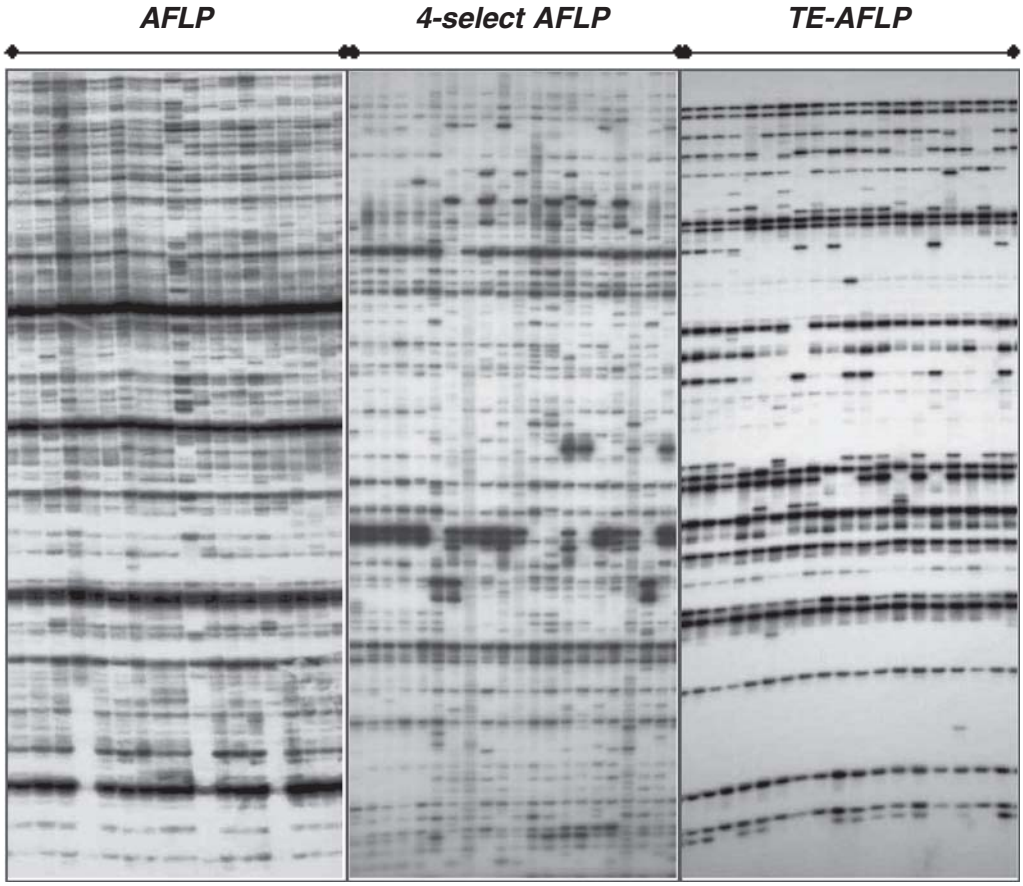
In another study, Balasaravanan *et al*. (2003) studied the genetic diversity of 49 tea accessions from UPASI comprising of Assam, China and Cambod types. A total of 1555 bands obtained with 3 primers combinations supported the findings of Paul *et al*., (1997) revealing an overall higher genetic diversity with China type accessions than Assam types. Their study also revealed a very low diversity within South Indian cultivars, which may be due to selection from a narrow gene pool.

Optimization of two AFLP based assays in tea

Although AFLP is known to be more reproducible and robust than RAPD and ISSR, the number and quality of bands in AFLP depends greatly on genome complexity of the species under investigation (Han *et al*., 1999). With large genomes, there may be severe overcrowding of bands, which are difficult to score. The tea genome is moderately large (2n= 3.8 x 10^6 Kb, Hanson *et al*., 2001) and we observed large number of bands with conventional AFLP (Figure 1, left panel). A critical review of previous AFLP studies indicates that problems were probably encountered in deed. For example, while Paul *et al*. (1997) reported only 73 polymorphic bands using 5 primer combinations, Balasaravanan *et al*. (2003) reported 1555 polymorphic bands using only 3 primer combinations. Interestingly, none of these studies have addressed the issue of genome complexity and its effects on the quality of bands.

Understanding this problem, we sought out to modify the conventional AFLP assays so that they may be applied reliably for fingerprinting tea genome. In our study, we first standardized two modified AFLP methods viz. 4-select AFLP and Three Endonuclease (TE) AFLP, and evaluated them for their utility in genetic diversity analysis of tea germplasm (Negi *et al*., 2004; Negi *et al*., 2005) These methods were compared for the quality of amplification products pertaining to their clarity and scorability on a large collection of 48 tea genotypes. The 4-selective AFLP assay involved using four selective nucleotides in *Mse* I primer instead of three, in combination with *Eco* RI primers with three selective nucleotides (Yue and Young, 2001). This optimization led to significant reduction in the total number of bands and improved the overall band readability compared to conventional AFLP (Figure 1, middle panel). However, presence of ambiguous "shadow bands" of varying intensities across multiple lanes, in few primer combinations, disallowed an error free scoring of experimental data. Such problems were effectively resolved by employing TE-AFLP assay (Figure 1, right panel). In TE-AFLP, selective adapter ligation in addition to selective amplification contributes to reducing the complexity of fingerprint pattern (Van der Wurff *et al*., 2000). We propose that the complexity of the fingerprint pattern may be modulated to improve the reliability by employing suitable AFLP assays based on the size of the genome under

Figure 1. Comparison of representative banding profiles obtained with conventional AFLP, 4-select AFLP and TE-AFLP techniques in tea.



investigation. Both methodologies yielded comparable results where China types clearly separated from Assam types. In contrast to previous reports, the dendrograms revealed an overall tendency of the accessions to cluster based on their morphotypes and their geographic origin. Our results are in line with that of Paul *et al.*, (1997) that a high fraction (~75%) of overall genetic diversity is partitioned within the population, which indicates towards predominant out-crossing in this species.

Future prospects

A fairly good amount of published work on use of DNA based markers exists in tea. Most of these studies have aimed towards estimation of genetic diversity of existing germplasm. These studies have been conducted on smaller number of accessions, which has not led to any application at the ground level such as optimization of germplasm and development of core collections. For this purpose, attempts are required at a bigger scale with a network of researchers. One example of such attempt is the ongoing network project on molecular characterization of tea, funded by Department of Biotechnology, which aims at characterizing all accessions, conserved at various germplasm banks (over 2000 accessions). With improved AFLP based markers, this project is expected to provide valuable information on genetic diversity of Indian tea germplasm.

Towards the ultimate goal of genetic improvement of tea, efforts are required to construct a high-density linkage map using molecular markers and integration of economically important traits on this linkage map. A beginning has been made by Hackett *et al*., (2000) who used RAPD and AFLP markers for construction of a linkage map of *C. sinensis*. As tea is highly cross-pollinated, conventional mapping populations such as F_2 selfed or BC_1 cannot be developed. Alternative approaches such as pseudotest cross that have been used in other tree species can also be used in tea (Grattapaglia *et al*., 1996; Hackett *et al*., 2000). Biclonal seedstock populations, which are readily available in various tea germplasm institutes, can be used for this purpose. However, they need to be first validated for their biclonal origin vis-à-vis their parents because a significant proportion of these plants may have unknown male parent or even they may be products of selfing. The study by Hackett *et al*., (2000) showed a high proportion of markers with unexpected segregation, which could only be explained if one considers that three male parents were involved in the generation of the seedstock. Nevertheless, the existing biclonal seedstocks have an additional advantage that evaluation of quality traits is possible for these plants because many of these may be in plucking stage. Besides, development of new biclonal seedstocks between selected parental clones differing in quality traits should also be given importance to generate material for future research.

Clonal seed orchards or “seed baris” can be established using genetically diverse accessions identified based on genetic diversity and morphological data. This will generate excellent raw material for future selection of clones with desirable combination of traits and for further use in research such as QTL mapping and marker-assisted breeding. Molecular markers have already proven their potential as tools for such conservation and breeding activities in agricultural crops and we can expect the same from these markers for improvement of tea as well.

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