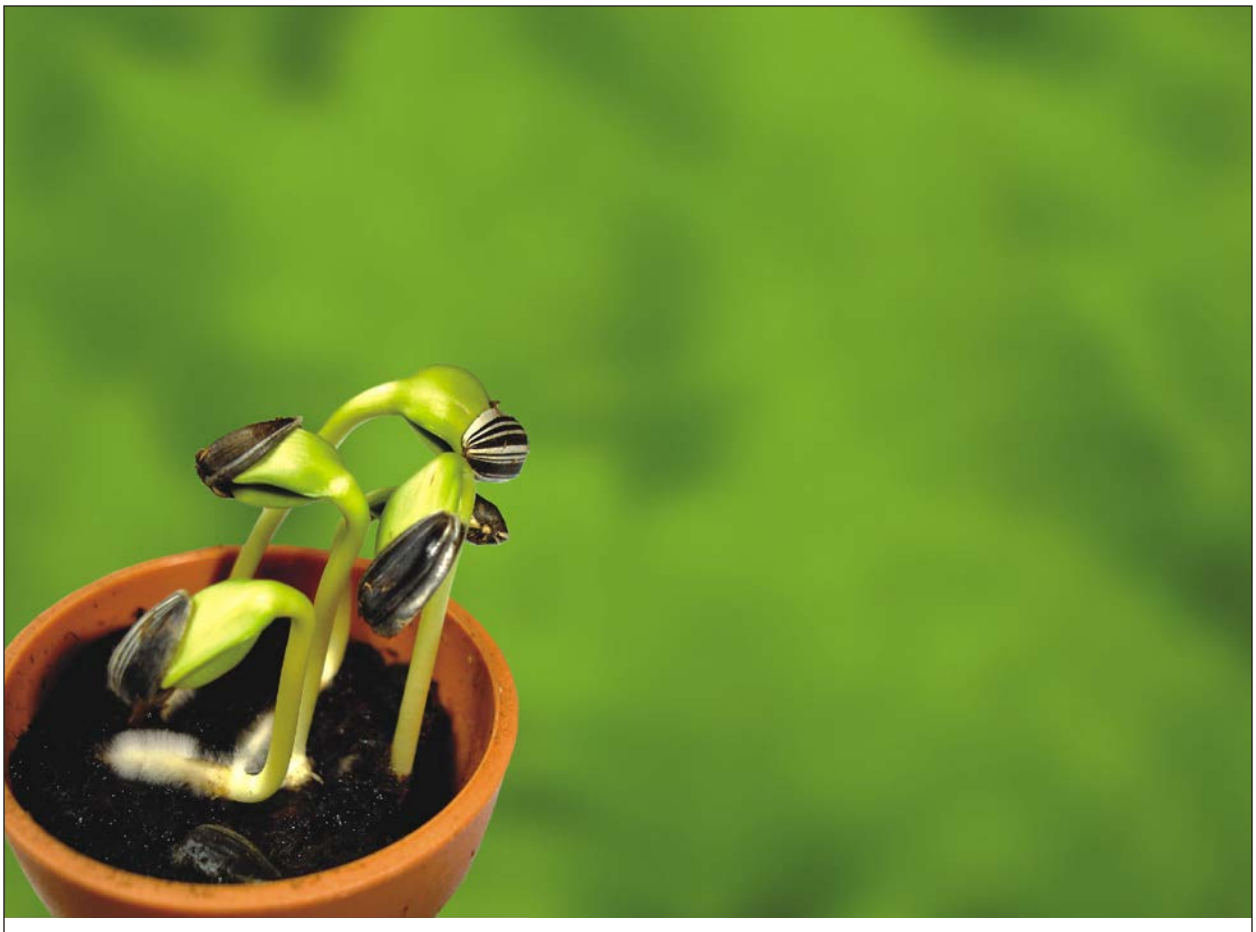

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Molecular Marker Technology in Genetic Improvement of Tea

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ABSTRACT

Tea, one of the most popular beverages in the world, is loosing its genetic diversity due to mass vegetative propagation. Genetic improvement of tea is urgently necessary to combat biotic and a biotic stress. Molecular markers [Randomly Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Inter Simple Sequence Repeat (ISSR) and Simple Sequence Repeat (SSR)] studies have been done mainly for genetic diversity analysis. There are attempts to study marker trait association and construct genetic linkage maps. Efficiency and cost effectiveness of Marker Assisted Selection (MAS), future perspectives for development of core collections from germplasm and association mapping for genetic improvement has been discussed.

Key words: Tea, genetic diversity, molecular markers, marker assisted selection, association mapping

INTRODUCTION

Tea is one of the most popular beverages and an important revenue source for the tea producing countries in the world. It is widely cultivated in countries of Asia (India, China, Srilanka, Japan, Vietnam and Indonesia) and Africa (Kenya, Uganda and Malawi). There are three races of tea plant which have contributed significantly to the cultivated genetic pool throughout the world: China [*Camellia sinensis* (L.) O. Kuntze], Assam [*Camellia assamica* (Masters)] and Cambod [*Camellia assamica* sp. *lasiocalyx* (Planch. MS)] (Wight, 1962).

Tea is a highly heterozygous, out-crossing crop with most of its morphological, physiological and biochemical descriptors show continuous variation and high plasticity. Mass vegetative multiplication of elite clones was done for higher productivity and better quality (Richards, 1966; Bandyopadhyay and Das, 2008). The total production of tea in India has been increased from 354.4 million Kgs in 1961 to 987.02 million kgs in 2007-08 (http://www.teaboard.gov.in/pdf/stat/Production07.pdf). The increase in yield can be largely attributed to the cultivation of well defined clonal tea plants which has become popular with advent of vegetative propagation. Clones are genetically uniform and give uniform yield and quality. Clonal selection is an important and widely adopted method of tea plant improvement because of wide heterogeneity in the existing seedling population (Barua, 1963, 2008; Shanmugarajah, 1994). The drawbacks of mass vegetative multiplication are yield variability under stress conditions and relative proneness to pathogens, whereas seed populations which have high genetic variability are less prone to stress condition and of average quality (Bandyopadhyay and Das, 2008). At a local scale, tea plants are usually highly homogenous in terms of spatial distribution (Sarmah and Bandyopadhyay, 2009). Over-reliance on a limited number of clones may lead to a loss of valuable genetic resources (diversity) and an increase in potential risks of natural hazards like pests and diseases. So, genetic variability is

desirable because it provides a buffer against co-evolving factors of natural hazards like diseases, pests and changing environment (Barua, 1963; Wachira *et al*., 1995). Investigation of genetic diversity of natural, old seedling tea populations is required to preserve these diverse populations as *in situ* conservation sites, prior to replace them with genetically more similar modern tea cultivars. Genetic variability with trait related molecular markers, genetic linkage maps with association mapping is urgently necessary to improve tea.

CROP IMPROVEMENT AND GENETIC EROSION

Crop improvement has been started since cultivation began by human. Genetic bottlenecks imposed during early domestication and modern breeding activities result in cultivated varieties, which carry only a fraction of the variation present in the gene pool (Tanksley and McCough, 1997).

At any point in time, the level and distribution of genetic diversity in a crop species depends on three types of variables: (1) the biological characteristics of the species, including its reproductive system, ploidy level and further genetic characteristics, (2) the biotic and abiotic environments and (3) the human environment (Gepts, 2004). It is the existence of the third set of factors, namely the human environment, which sets apart crop evolution from natural evolution.

The amount of variability in a crop varies depending on the type of genetic resource dealing with, e.g., whether it is present from wild genetic stalk or whether it is a highly selected material from a breeding programme. There is, generally speaking, a relationship between the gain in yield in the broad sense (production, desirable characteristics) and the amount of variability. In the sequence of selections only a portion of the most advantageous, inheritable traits are favored. The genetic variation will change and narrowed down, if the selection intensity is high. Improvement in yield may thus be obtained at the cost of genetic variability depending on the improvement strategy. This is important to bear in mind in the context of gene resource conservation (Keiding and Graudal, 1989).

In tea, clonal identification has traditionally been based on morphological descriptors such as plant shape, leaf shape and young leaf type. Vegetative propagation (clones) began to replace seed propagation in the 1960s and reduced genetic diversity within tea cultivation (Visser, 1976). Vegetatively propagated plants lead to great uniformity in crop which is a very useful agricultural feature. Clonal degeneration, a gradual loss of vigor and yield with age of a variety, is also a well known phenomenon in vegetatively propagated crops (Forbes and Watson, 1992).

Genetic erosion was first applied to highly breed agricultural crops like wheat, maize, rice etc. in which very high yields and other advantages were achieved through systematic breeding and special combinations of a limited part of the genetic potential. The high-yielding strains quickly spread replacing less productive but more variable cultivars. As a result higher yields were obtained with much more genetically uniform crops but at the same time also more vulnerable to large-scale attacks by pests or diseases. Adaptability or 'resistance' was not taken sufficiently into account in the endeavor to improve production which is also applicable in tea. A broad genetic base has been replaced by narrow one and the old genetic diversity is disappearing. This trend is inevitable with the need for highly efficient and uniform cultivars in advanced and sophisticated agriculture system (Hawkes, 1981). Genetic diversity is important to a species fitness, long-term viability and ability to adapt to changing environmental conditions. Also, plant populations that are less genetically diverse may be more susceptible, in some cases, to pathogens or other environmental

stresses. Genetic erosion, or the reduction in genetic diversity in crop plants, takes on various shapes depending on one's standpoint, including the reduction in the number of different crop species being grown and the decrease in genetic diversity within crop species.

Plant breeding, with its emphasis on elite X elite crosses in the incessant pursuit of higher performance (Kelly *et al*., 1998) and close adherence to norms imposed by the market, is a strong force in the reduction in genetic diversity. Plant breeders have become aware of this situation and have attempted to rectify this situation by broadening the genetic basis of their cultivar gene pool (Duvick, 1984; Smale *et al*., 2001; Kelly, 2004). However, it remains that the genetic diversity represented in the elite gene pools is only a small fraction of that present in the entire gene pool of crop plants. Hence, there is an enduring concern about the disappearance of genetic diversity over the long term.

TEA GERMPLASM CONSERVATION AND DEVELOPMENT OF CORE COLLECTION

Tocklai Experimental Station, Jorhat is having more than 2,500 accessions maintained at three main centres (Singh, 2006). Germplasm acquisition of tea in India is based mainly on phenotypic characters which results in duplicates. To avoid that marker assisted acquisition is very important after selection of plants. Tea planters are now uprooting the old seed populations for better productivity and replace them with high yielding quality cultivars. It is important to conserve some unique plant materials from those old seed populations which may have certain characteristics to enrich the germplasm pool before losing them forever. Trait related phenotypic characters is lacking in tea. A core collection of tea which is defined as a representative sample of the whole collection with minimum repetitiveness and maximum genetic diversity (Frankel and Brown, 1984a, b; Brown, 1989) will have to be established exclusively based on molecular data. The core collection will serve as a working collection that could be evaluated and utilized preferentially, which could solve the problem of large size of collection hindering the preservation and utilization of germplasm resource (Frankel, 1984). A core collection constructed based on genotypic values will be more representative than that constructed based on phenotypic values (Hu *et al*., 2000). Performing genetic experiment in multiple environments will draw more accurate results in constructing core collections.

Genetic introgression to enhance germplasm innovation: Germplasm innovation by distant hybridization has important role of broadening the genetic base. Ackerman (1973) attempted a large number of interspecific crosses involving 20 *Camellia* species. Tea could be easily crossed with 10 different species. In Japan, the distant hybridizations of tea plant and 26 species in the genus Camellia were conducted. An interspecific hybrid between tea plant (*C. sinensis*) and flower camellia (*C. japonica*) named Chatsubaki was obtained. It proved highly resistant to tea anthracnose, gray blight and cold damage in winter and furthermore had low caffeine content (Takeda *et al*., 1987). It has become one of the three promising parental materials for tea breeding in Japan. A clone TV 24 in Assam, India was produced by crossing between F1 hybrids (*C. irrawadiensis*×*C. assamica*) and TV1, an Assam-China hybrid (Bezbaruah, 1987).

Molecular markers in genetic diversity assessment: Molecular markers may be broadly divided into three classes based on the method of their detection: (1) hybridization-based; (2) Polymerase Chain Reaction (PCR)-based and (3) DNA sequence-based (Gupta *et al*., 1999; Jones *et al*., 1997; Joshi *et al*., 1999; Winter and Kahl, 1995). These markers are selectively neutral

because they are usually located in non-coding regions of DNA. Unlike morphological and biochemical markers, DNA markers are practically unlimited in number and are not affected by environmental factors and/or the developmental stage of the plant (Winter and Kahl, 1995). The study of genetic diversity is a prerequisite to design the most suitable strategy for conservation programme, either *in situ* or *ex situ* (Bandyopadhyay *et al*., 2005).

In tea, the molecular markers Random Amplified Polymorphic DNAs (RAPDs) by Wachira *et al*. (1995, 1997), Kaundan *et al*. (2000), Chen and Yamaguchi (2002), Young-Goo *et al*. (2002), Chen *et al*. (2005a) and Mewan *et al*. (2005), Inter Simple Sequence Repeat (ISSR) by Mondal (2002) and Yao *et al*. (2005), Amplified Fragment Length Polymorphism (AFLP) by Paul *et al*. (1997), Wachira *et al*. (2001), Balasaravanan *et al*. (2003), Mishra and Sen-Mandi (2004a, b) and Sharma *et al*. (2009), Cleaved Amplified Polymorphic Sequence (CAPS) by Kaundan and Matsumoto (2003), Simple Sequence Repeats (SSRs) or microsatellites developed by Freeman *et al*. (2004), Zhao *et al*. (2007) and Hung *et al*. (2008) have been mainly used for genetic fingerprinting and phylogenetic study. Tanaka and Taniguchi (2002) have developed emphasized RAPD by adding nucleotides to the 3' end to make the RAPD bands clearer in tea.

Devarumath *et al*. (2000) used RFLP, RAPD and ISSR for identification of very closely related tea cultivars and evaluation of genetic fidelity in micro propagated plants. RAPD and microsatellite markers were also used to determine the genetic fidelity (Gangopadhyay *et al*., 2004a, b; Roy *et al*., 2006) of three micro propagated tea plants for commercialization (Borchetia *et al*., 2009). Genetic relationship study by RAPD and ISSR of cultivated tea clones and wild tea in Taiwan has been done by Lai *et al*. (2001). Singh and Ahuja (2006) showed that 5S rRNA gene can be used as a useful molecular marker that can be utilized in phylogenetic studies of tea. Intra clonal genetic variability in vegetatively propagated tea by RAPD has shown by Singh *et al*. (2004). Genetic diversity study of cultivated teas has also been done by comparing the nucleotide sequence of ribosomal RNA maturase (matK) regions in chloroplast (cp) DNA (Katoh *et al*., 2003). Polymorphisms among varieties of tea with heterologous nuclear and chloroplast primers have been studied by Kaundan and Matsumoto (2002). Phenylalanine Ammonia Lyase (PAL) cDNA has been used in classification of varieties and cultivars of tea by Matsumoto *et al*. (1994).

TRAIT SPECIFIC MOLECULAR MARKERS AND GENETIC LINKAGE MAPS

The narrow genetic base of tea cultivars is the serious obstacle to sustain and improve productivity due to rapid vulnerability of genetically uniform cultivars by potentially new biotic and abiotic stresses. Molecular markers are rapidly being adopted for crop improvement as an effective and appropriate tool for basic and applied studies addressing biological components in agriculture production systems (Jones *et al*., 1997; Mohan *et al*., 1997; Prioul *et al*., 1997). Molecular markers offer specific advantages in assessment of genetic diversity and in trait specific crop improvement.

As a part of the gene sequencing projects, the construction of cDNA libraries for tea (Li *et al*., 2007; Chen *et al*., 2005b, c) and Expressed Sequence Tags (ESTs) from these cDNA libraries have been generated (Park *et al*., 2004; Chen *et al*., 2005b, c). Primers can be developed that provide a unique sequence tagging the gene. The EST marker is detected as size difference in amplified product and inherited in a co-dominant manner. These markers are good for mapping, discovery of genes associated with specific QTLs to understand a specific trait. Data-mining for SSRs in ESTs and the development of EST-SSR markers also appeared recently (Jin *et al*., 2006).

The main goal of genetic mapping is to detect neutrally inherited markers in close proximity to the genetic causatives or genes controlling the complex quantitative traits. Genetic mapping

can be done mostly in two ways: (1) using the biparental mapping population that is called QTL-mapping as well as genetic mapping or gene tagging and (2) using the diverse lines from the natural populations or germplasm collections that is called LD-mapping or association mapping.

Tea germplasm resources worldwide are the important reservoirs of natural genetic variations. The efficient exploiting these conserved genetic diversities are vital to overcome future problems associated with narrowness of genetic base of modern tea cultivars. However, productivity and quality, tolerance to environmental stresses and pest/disease resistance are controlled by polygenes and multifactorial that greatly depends on genetic×environmental (G×E) interactions. These complex traits are referred to as quantitative trait loci (QTLs) and it is challenging to identify QTLs based on only traditional phenotypic evaluation. Identification of QTLs of agronomic importance and its utilization in a crop improvement further requires mapping of these QTLs in a genome of crop species using molecular markers (Collard *et al*., 2005; Ross-Ibarra *et al*., 2007).

In tea, development of drought specific AFLP markers (Mishra and Sen-Mandi 2004b), EST based markers for fungal (Blister Blight) disease resistance (Agarwal and Das, 2009) and insect resistance (Ahmed and Das, 2009) has been done for future Marker Assisted Selection (MAS) in tea breeding to have resistant varieties. To achieve the ultimate goal of genetic improvement of tea, further efforts are required to construct a high density map using molecular markers and to integrate economically important traits onto the linkage map. As tea is a perennial crop and highly heterozygous F1 hybrid progenies can be used to construct genetic linkage maps by pseudo-testcross strategy (Hemmat *et al*., 1994; Grattapaglia and Sederoff, 1994; Kenis and Keulemans, 2005; Debener and Mattiesch, 1999; Yamamoto *et al*., 2002). A start has been made by constructing two frame-work maps for tea (Tanaka, 1996; Hackett *et al*., 2000; Huang *et al*., 2005) and concerted efforts are needed to locate as many markers as possible, linked to important traits.

The precision of QTL-mapping largely depends on the genetic variation covered by a mapping population, the size of a mapping population and a number of marker loci used. Once QTLs affecting a trait of interest is accurately tagged, marker tags will be the most effective tools in a crop improvement that allows the mobilization of the genes of interest from donor lines to the breeding material through MAS. Although, traditional QTL-mapping will continue being an important tool in gene tagging of crops but overall is very costly (Ross-Ibarra *et al*., 2007; Stich *et al*., 2006) and has low resolution with simultaneous evaluation of only a few alleles (Flint-Garcia *et al*., 2003) in a longer research time scale. In linkage mapping, the major limitation, hampering the fine mapping, is associated with the availability of only a few meiotic events to be used that occurred since experimental hybridization in a recent past (Jannink and Walsh, 2002).

FUTURE DIMENSIONS IN IMPROVEMENT OF TEA WITH ASSOCIATION MAPPING

Association Analysis (AA), also known as association mapping or linkage disequilibrium mapping, is a method that relies on linkage disequilibrium to study the relationship between phenotypic variation and genetic polymorphisms (Flint-Garcia *et al*., 2003).

The advantages of population-based association study, utilizing core collections from the germplasm collections, over traditional QTL-mapping in biparental crosses primarily are due to (1) availability of broader genetic variations with wider background for marker-trait correlations, (2) likelihood for a higher resolution mapping because of the utilization of majority recombination events from a large number of meiosis throughout the germplasm development history, (3) possibility of exploiting historically measured trait data for association and (4) no need

for the development of expensive and tedious biparental populations that makes approach timesaving and cost-effective (Kraakman *et al*., 2004, 2006; Hansen *et al*., 2001).

Association mapping in tea should include the following steps (1) selection of a group of individuals (core collection) from germplasm collection with wide coverage of genetic diversity, (2) recording or measuring the phenotypic characteristics (yield, quality, tolerance, or resistance) of selected population groups, preferably, in different environments and multiple replication/ trial design, (3) genotyping a mapping population individuals with available molecular markers, (4) quantification of the extent of Linkage Disequilibrium (LD) of a chosen population genome using a molecular marker data, (5) assessment of the population structure (the level of genetic differentiation among groups within a sampled population individuals) and kinship (coefficient of relatedness between pairs of each individuals within a sample) and (6) based on information gained through quantification of LD and population structure, correlation of phenotypic and genotypic/haplotypic data with the application of an appropriate statistical approach that reveals marker tags positioned within close proximity of targeted trait of interest. Consequently, a specific gene(s) controlling a QTL of interest can be cloned using the marker tags and annotated for an exact biological function (Nordborg *et al*., 2002, 2005).

Biallelic codominant types of markers Single Nucleotide Polymorphisms (SNPs) and multiallelic simple sequence repeats-SSRs) are suitable for LD to be used in plant populations (Gupta *et al*., 2005; Hedrick, 1987; Devlin and Risch, 1995). But, there are number of reports where dominantly coded (present versus absent) marker data of RAPD, RFLP, AFLP, candidate gene (CAPs) and SSRs were successfully used in genome-wide LD analysis and LD-based association mapping in plants (Kraakman *et al*., 2004, 2006; Hansen *et al*., 2001; Tommasini *et al*., 2007; Iwata *et al*., 2007; Malosetti *et al*., 2007; Gebhardt *et al*., 2004). Li *et al*. (2007) investigated the use of dominant markers in estimation of LD in diploid species and developed appropriate EM algorithm.

CONCLUSION

There is a high demand in international tea market for environment friendly, pesticide free tea as far as the public health is concerned. Mass vegetative propagation involves in loss of genetic diversity which decreases the biotic and abiotic stress tolerance in tea. Besides stress tolerance, yield and quality of tea have to be considered for its market value. Genetic introgression by interspecific hybridization has been done and will be a very crucial strategy to broaden the genetic basis of tea by marker assisted introgression. Development of core collections with association mapping will definitely give momentum for genetic improvement of tea.

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