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Full Length Research Paper

DNA landmarks for genetic diversity assessment in tea genotypes using RAPD markers

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Tea (*Camellia sinensis)* is one of the most important non-alcoholic beverages of the world. Natural genetic diversity in tea has been reduced due to continue selection in favor of desirable traits. The present study was conducted to estimate genetic diversity in tea genotypes cultivated in Pakistan using 20 randomly amplified polymorphic DNA (RAPD) primers. 75 tea accessions from 13 tea genotypes were analyzed. Genetic distance estimates ranged from 0-100% showing high level of diversity among screened genotypes. Unweighted pair group method using arithmetic averages (UPGMA) based cluster analyses of RAPD data clustered all the genotypes into five main groups which showed mixed grouping profile of the samples. However, samples from different plants of particular genotypes were segregated into independent sub-clusters. Broad and narrow leaved genotypes were accommodated in separate sub-clusters. Similarly, samples from narrow leaved genotypes collected from different sites were grouped in different main clusters reflecting the geographical origins of tea samples. Results obtained confirm that RAPD methodology is practically applicable for evaluation of genetic diversity and relationship in tea genotypes.

Key words: *Camellia sinensis*, genotypes, genetic diversity, randomly amplified polymorphic DNA (RAPD), dendrogram, cluster analysis.

INTRODUCTION

Tea belongs to genus *Camellia,* family Theaceae and has been consumed by human for thousands of years. It is economically one of the most important non alcoholic caffeine containing beverage crop of the world. Annual production of tea in the world is more than 3,400,000 tons from an area of approximately 2,561,000 ha (Chen et al., 2007). India, China, Sri Lanka, Indonesia and Kenya are major producers of tea. *Camellia sinensis* var. *sinensis* and *Camellia sinensis* var. *assamica* are two main taxa for commercial cultivation. Recently, tea is gaining further popularity as an important “health drink”. It helps in controlling high blood pressure, reducing risk of breast cancer and believed to have anti-oxidant and anti-obesity activities (Bonner et al., 2005; Hirose et al., 1994).

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Tea is a predominantly out-crossing species; selected genotypes are propagated vegetatively and released as clone varieties. Therefore, thousands of cultivars have been developed worldwide. It is estimated that about 15,000 *Camellia* cultivars have been identified (Gao et al., 2005). Harvestable yield of tea is confined to the terminal two-three leaves and a bud, which constitute less than 20% of the total biomass of the plant (Hackett et al., 2000). As the available land for tea cultivation is limited, improved breeding strategies with reduced production costs are getting increasing importance. Natural genetic diversity in tea has been reduced at an alarming rate mainly because of selection and breeding for desirable traits (Kaundun and Park, 2002). Estimation of existing genetic diversity in the available tea germplasm may be helpful to identify genotypes with high production potential which could be used to improve the commercially grown tea cultivars. In the past, various markers including morphological, cytological and biochemical

markers were used to estimate genetic diversity in various crop species including tea. These markers though successful, were not considered suitable for large scale screening mainly because such markers are influenced by environment and are limited in number. Recent introduction of molecular biology revolutionized the process of screening germplasm and assessment of genetic diversity by offering practically unlimited number of molecular markers which cover the entire genome of a species (Paterson et al., 1991). Among molecular markers, RAPD marker comparatively features sensitiveness, convenience and rapidness in detection, therefore, it was widely used in many fields such as analysis of genetic relationship (diversity), cultivars identification and gene localization in genome (Lima et al., 2007; Gupta et al., 2008; Ebrahimi et al., 2009; Thiago et al., 2009; Vural and Dageri, 2009; Wang et al., 2010). So far, very little genetic information on tea (*Camellia sinensis*) is available because of limited reports by RAPD technology or by other molecular markers (Wachira et al., 1997; Kaundun et al., 2000; Kaundun and Park, 2002; Xiao et al., 2008; Kunjupiliai et al., 2009). Previously, RAPD based genetic diversity in tea genotypes available in Pakistan has been reported using few markers and very few tea samples (Gul et al., 2007). Thus, the present study aimed to investigate all the 13 tea genotypes available in the country, using RAPD markers. The study would be helpful for further investigation of *Camellia* genome diversity, cultivars identification, classification and protection, molecular breeding, etc.

MATERIALS AND METHODS

Materials

Plant material used during the present study comprised 75 accessions (Table 1) grown at National Tea Research Institute (NTRI), Shinkiari, and Uniliver Tea Pvt. Ltd., (UTSI) Icherian, Mansehra, Pakistan (Latitude 34°20'N, Longitude 7°15'E, Altitude 1066 m). Approximately, 0.5 g fresh leaves were collected and placed in a 1.5 ml Eppendorf tube which was then immediately dropped in liquid nitrogen.

Genomic DNA extraction

Total genomic DNA was isolated from tender leaf tissues using modified CTAB procedure, described by Doyle and Doyle (1987) and Matasyoh et al. (2008) with minor modifications. The DNA quality and quantity was checked through 1% agarose gel electrophoresis.

Polymerase chain reaction (PCR)

The PCR amplifications were carried out following the procedure of Williams et al. (1990). Annealing was carried out at 34°C and 40 cycles were used for amplification of genomic DNA. Initially, 42 RAPD primers (obtained from Gene Link, Inc, NY 1052, USA) were tested and finally 20 primers were selected for further analysis. The PCR amplifications using 20 decamer primers were carried out using Creacon Thermal Cycler (model 00005.400). The amplification products were separated on 2% Agarose/TBE gels. A 100 bp DNA ladder was used to estimate the size of DNA fragments amplified. Results were documented using "Uvitech" gel documentation system.

Statistical analysis

For statistical analysis, binary (1-0) data matrices were generated by scoring presence (1) or absence (0) of DNA fragments amplified. Unweighted Pair Group method using Arithmetic Averages (UPGMA) was used for the estimation of genetic distances (GD) among all the possible combinations (Nei and Li, 1979). A dendrogram was constructed using computer program, POPGENE 3.2 (Yeh et al., 1999).

RESULTS

Giving due consideration to the concern regarding poor reproducibility of RAPDs, all the amplifications were repeated twice. Only reliably scoreable and reproducible bands were included in the analysis. An example of PCR amplification profile of 12 tea accessions using RAPD primer GLA-07 is presented in Figure 1.

A total of 6300 DNA fragments were amplified in 75 accessions using 20 RAPD primers giving an average of 4.0 bands per accession per primer. Amplified DNA fragments ranged in size from 100 bp to 1400 bp. Average genetic diversity (GD) estimates (based on all the RAPD primers used during present study) ranged from 0 to 100% (data not shown). In total, more than 80% comparisons showed high level of genetic diversity (GD = 51-100%). The 75 accessions were clustered in five main groups viz; A, B, C, D and E comprising 12, 43, 4, 6 and 7 accessions, respectively (Figure 2). Group A was further subdivided into subgroups A1 and A2 each comprising 6 accessions. Subgroup A1 predominantly comprised accessions belonging to genotype CL-01-05 with leaves of medium size, collected from NTRI. Subgroup A2 comprised accessions obtained from IN-01-07 genotype which is predominantly broad leaved (*C. sinensis var. assamica*) and is under experimental trial. Cluster B contained the largest number of samples (43) and was further divided into subgroups B1, B2, B3 and few small groups of 2-3 samples from a particular genotype. Sub cluster B1 predominantly comprised 16 samples of different small leaved genotypes collected from NTRI Shinkiari. Sub-cluster B2 comprised 7 accessions among which 4 were from broad leaved SL-01-05 genotype (Indian type) and 3 from small leaved Qi-Men genotype which is predominantly China type. Four tea samples of mixed genotypes from same location (UTSI, Icherian Mansehra), characterized by small leaves were grouped together in sub-cluster B3. The rest of the 16 genotypes in main cluster B were scattered in a mixed pattern in groups of 2 or 3. Cluster C comprised 4 accessions, 2 from Ich-05 and 1 each from Ich-04 and

Table 1. Samples of tea genotypes used in present study using RAPD analysis.

Genotype	Number of sample	Sample Id	Present Status
CL-01-05	7 (1-7)	CL-01-05-1,2, 3, 4, 6, 7 & 9	Cloned from pure Chinese genotypes. promising
Chuye	7 (8-14)	Ch-01, 02, 03, 05, 06, 07 & 10	Chinese type C. *sinensis.* promising
Roupi	4 (15-18)	Rp-01, 04, 05 & 08	Chinese type C. *sinensis* promising
Qi-Men	8 (19-26)	Q-01, 02, 03, 04, 05, 06, 08 & 10	Chinese type C. *sinensis* promising
T-05-10	3 (27,28 & 31)	T-01, 02 & 05	Unknown origin with small leaved
JP-01-05	2 (29 & 30)	J-01 & 02	Unknown origin with small leaved
SL-01-05	4 (32-35)	SL-01, 02, 03 & 05	Broad leaved C. *assamica* needs improvement
IN-01-07	7 (36-42)	IN-01, 02, 03, 04, 05, 06 & 07	Broad leaved C. *assamica* poor adaptation to environment
Ich-04*	7 (43-49)	Ich-04-1, 2, 3, 4, 7, 8 & 9	Unknown origin with small leaved
Ich-05*	6 (50-55)	Ich-05-1, 2, 3, 5, 6 & 7	Unknown origin with small leaved promising
Ich-07*	7 (56-62)	Ich-07-1, 2, 3, 5, 6, 7 & 8	Unknown origin with small leaved
Ich-08*	6 (63-68)	Ich-08-2, 3, 4, 5, 7 & 8	Unknown origin with small leaved promising
Ich-09*	7 (69-75)	Ich-09-1, 2, 3, 5, 6, 7 & 8	Unknown origin with small leaved

*= Genotypes from Uniliver Tea Station Icherian (UTSI).

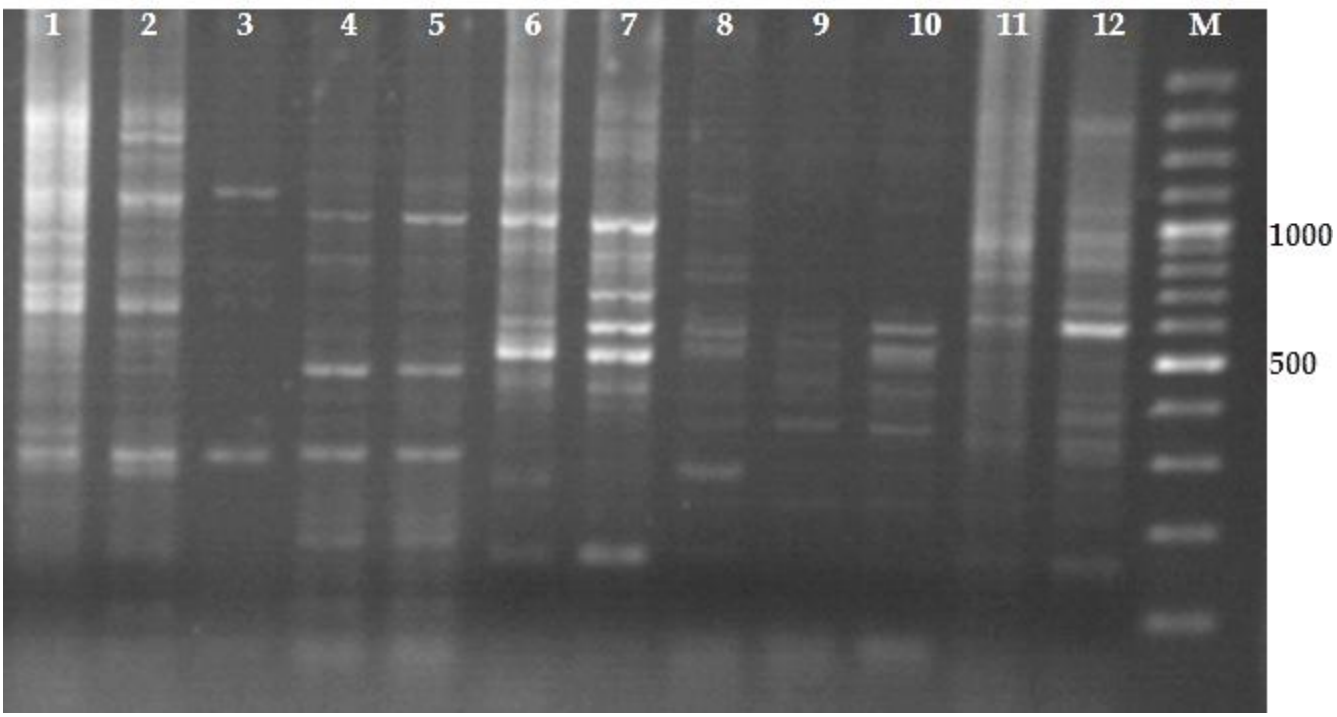

Figure 1. An example of PCR amplification of 12 accessions of tea using RAPD primer GLA-07. M = Molecular weight marker (100 bp DNA ladder). Size of DNA fragments (in bp) is presented on right.

Ich-07 characterized as small leaved genotypes collected from UTSI Icherian. Cluster D had 6 samples out of which 5 were from Ich-08 genotype collected from UTSI Icherian and is characterized as a high yielding promising among the genotypes under trial in UTSI Icherian Mansehra. Cluster E comprised all the tea samples belonged to narrow leaved China genotype "Chuye" collected from NTRI Shinkiari.

DISCUSSION

Previously morphological, cytological and biochemical markers were used for estimation of genetic diversity in

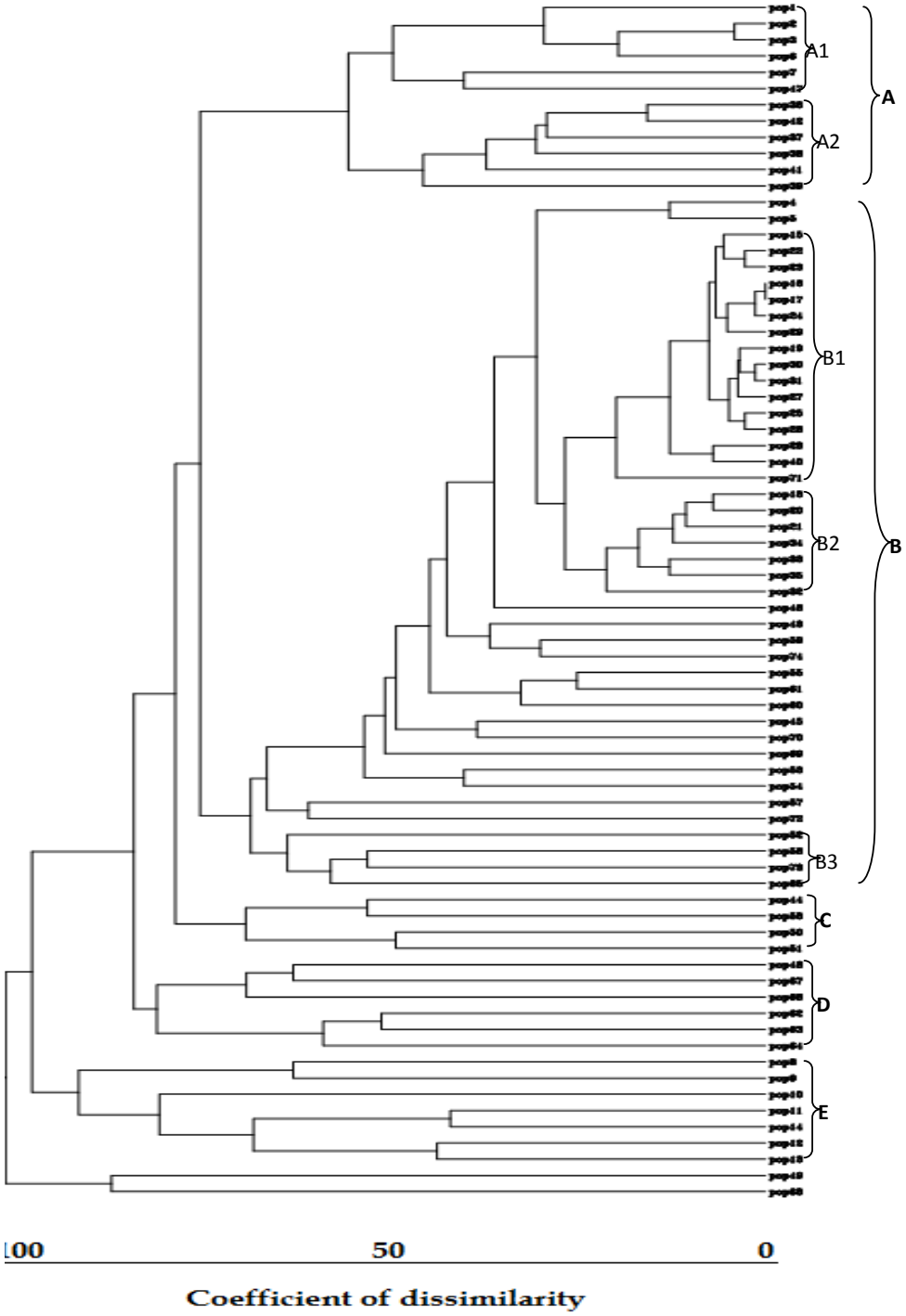

Figure 2. Dendrogram constructed for 75 tea genotypes using data obtained from PCR with 20 RAPD primers.

commercially important crop species. However, with the recent developments in DNA technology, molecular markers based on the variation in DNA base sequences have been widely used in cop improvement (Paterson et al., 1991; Sumikova and Kernlova, 2010; Todorovska et al., 2009; Wang et al., 2007). Various DNA based markers commonly used to estimate genetic diversity in crops of agronomic importance include restriction fragment length polymorphism (RLFP), simple sequence repeat (SSR), amplified fragment length polymorphism (AFLP) and single nucleotide polymorphism (SNP) (Chen et al., 2006). These procedures though highly reliable,

are expensive, time consuming and some time require working with hazardous chemicals (example, use of radioactive P^{32}). In contrast, RAPD analysis is not only easier, quicker, cheaper and more user's friendly assay procedure but has an added advantage that RAPD primers do not require any prior sequence information on the target genome (Williams et al., 1990). Hence, RAPD analysis is more suitable for handling larger germplasm accessions/segregating populations of commercially important crops like tea in the developing countries where technical expertise and financial support for scientific research are limiting factors.

Genetic diversity in tea has been studied by various workers using DNA based markers (Kaundun and Park, 2002; Chen et al., 1998, 2005b; Chen and Yamaguchi, 2002; Paul et al., 1997; Shao et al., 2003; Kaundun and Matsumoto, 2003; Huang et al., 2004, 2006; Yao et al., 2007; Chen et al., 2007; Gul et al., 2007). The average number of amplified fragments during the present study were 4.0 per primer per genotype which was a little higher than reported in some earlier studies (Chen et al., 2005a) who reported approximately 3.5 alleles per primer per genotype. It may be because in earlier study (Chen et al., 2005a) selected elite genotypes were used where most favored alleles are retained as compared to the rare ones. Relatively, higher genetic distances estimated during the present study could result from the fact that material used belonged to various geographical regions in contrast to previous study example Chen et al. (2005b) who used Chinese genotypes which were established from a limited gene stock. Results of cluster analyses revealed that the cultivars belonging to the type of C. *assamica* and type of C. *sinensis* were grouped in same cluster like cluster A comprised IN-01-07 (*C. assamica*) and CL-01-05 (*C. sinensis*) genotypes together, but both were clearly separated by sub-groups A1 and A2. Similarly Cluster B also included broad leaved SL-01-05 (C. *assamica*) genotypes and small leaved tea (C. *sinensis)* genotypes but surprisingly in separated subgroups. This indicated that gene introgression occurred between these two types because of the crosspollination during long-term cultivation and improvement. High frequency of missing data (data not shown) might have a certain influence on the result of cluster analysis. This pattern of our results is in agreement with previous reports (Chen et al., 2005b; Ariyarathna and Gunasekare, 2006) where various taxa tend to cluster in one group. The data shown by dendrogram were in accordances with the conventional classification of tea taxa except that the two broad leaved genotypes (IN-01-07 and SL-01-05) belonging to type of *C. assamica* were distantly separated from each other with diversity of more than 80%.

The higher genetic diversity within C. *assamica* was in contradiction to previously observed diversity with dominant RAPD (Wachira et al., 1995) and AFLP assays (Paul et al., 1997), but also with co-dominant CAPS markers (Kaundun and Matsumoto, 2003). The conflicting results between RAPD and other markers could be due to the small sample size studied during present analyses. Another reason might be less number of RAPD primers used during present study and missing data, hence, it is suggested that more RAPD primers may be utilized for genetic diversity analysis in future. Range of diversity shows the potential for selection. The data presented here will help in establishing maximization strategies for tea in Pakistan. The range of diversity exhibited by the genotypes studied shows potential for selection. In this respect, priority should be given to the marginal genotypes (belonging to group A and group D, Figure 2) which appeared most distantly related.

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