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DNA based elaboration of tea genotypes cultivated in Pakistan

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Abstract

Tea, obtained from *Camellia sinensis* L. is one of the most important non-alcoholic beverages of the World. Continuous selection in favor of desirable traits has reduced natural genetic diversity in tea. Present study was conducted to estimate genetic diversity in a world collection of tea germplasm cultivated in Pakistan using Random Amplified Polymorphic DNA (RAPD) primers. A total of 42 tea accessions were characterized using 21 RAPD primers. The data obtained from PCR analysis was used for making average genetic distances matrix based on Unweighted pair group method using arithmetic averages (UPGMA) and for construction of a dendrogram as well. A high amount of genetic diversity (G.D=0-100%) was estimated among the germplasm accessions. Accessions were clustered into 2 main groups and 2 subgroups according to conventional classification of tea taxa and geographical origin of the genotypes. Tea genotypes were also separated clearly according to their main taxa (i.e. *C. sinensis* and *C. assamica*) in the world. The results obtained, will help in establishing conservation strategies for tea in Pakistan. RAPD methodology proved practical for evaluation of genetic diversity and relationship among tea genotypes.

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Introduction

Tea belongs to genus *Camellia*, family *Theaceae*. It is economically one of the most important non alcoholic beverage crops 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 hectares (Chen *et al*., 2007). Major producers of the tea are India, China, Sri Lanka, Indonesia and Kenya. *Camellia sinensis* var. *sinensis* and *Camellia sinensis* var. *assamica* are two main taxa for commercial cultivation (Sealy, 1958). 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 (Hirose *et al*., 1994; Sayama *et al*., 2000; Bonner *et al*., 2005).

Tea is a predominantly out-crossing species; selected genotypes are propagated vegetatively and released as clonal varieties. 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 (Magambo and Cannell, 1981). 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 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/or 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 entire genome of a species (Paterson *et al*., 1991).

A large number of molecular markers including RAPD has been used worldwide for discriminating tea genotypes/accessions (Chen *et al.,* 2007; Yao *et al.,* 2007; Liu *et al*., 2008; Borchetia *et al*., 2009; Liu *et al*., 2012; Murty *et al., 2013*).

Pakistan is the 3rd largest importer of tea in the world having more than 1kg per capita annual consumption. It spends a very large amount of its national exchequer to import most of its tea. Its import bill for the year 2010 alone was approximately 22billion PKR (Anonymous, 2011). In Pakistan tea cultivation was started about 2 decades ago, and very little work has been reported regarding the agronomy and soil research of this crop in the country. A limited data about the biotechnological improvement and screening of existing genotypes using molecular markers is reported (Gul *et al.,* 2007; Afridi *et al*., 2011). Hence genetic diversity study of tea germplasm available in Pakistan is of prime importance for future breeding programs. During the present study, genetic diversity in 42 accessions of tea in Pakistan representing the world collection of tea was estimated using 21RAPD primers.

Materials and methods

Plant materials

Plant materials used in this study comprised 42 tea accessions (Table 1) grown at National Tea Research Institute, Mansehra, Pakistan (Latitude 34°20'N, Longitude 7°15'E, Altitude 1066 meter). All accessions were obtained from 8 different genotypes including 2 broad leaved (Sri Lankan and Indonesian genotypes), 5 narrow leaved (Chinese genotypes) and 1 Clonal genotype. All the accessions selected for the study were of same age and height.

DNA isolation

Approximately 0.5 g fresh young shoots with two leaves and a bud were randomly excised from each plant in 1.5 mL eppendorf tubes and immediately frozen in liquid nitrogen till DNA extraction. Total genomic DNA was isolated using procedure described by Kobayashi *et al*. (1998) with minor modifications according to our lab conditions.

The extracted DNA samples were subjected to gel electrophoresis in order to check the purity and quality of DNA. The DNA extracted in this way was of high purity and gave good quality bands after running on 1% agarose gel.

PCR analysis

Twenty one decamer RAPD primers (obtained from Gene Link, Inc, NY 1052, USA) were selected for present molecular characterization. PCR reactions were carried out in a 25 microlitre reaction mix containing approximately 20 ng template DNA, 2.5 picomoles each of dATP, dCTP, dGTP and dTTP (Promega, Madison, Wis.), 5 picomoles of a RAPD primer, 0.5 units of *Taq polymerase* enzyme (Farmentas) and buffer (10mM Tris-HCl pH 8.8 at 25 ^{o}C, 1.5mM MgCl2, 50 mM KCl). The PCR amplification followed Williams *et al.* (1990) were carried out using Creacon Thermal Cycler (model 00005.400) programmed for an initial denaturation step at 94 ^{o}C of 4 min followed by 40 cycles of 60 s denaturation at 94 ^{o}C, 60 s primer annealing at 34 ^{o}C and for 120 s extension at 72 ^{o}C. Final extension step was added at 72 ^{o}C for 7 minutes followed by holding at 4 ^{o}C. 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. Unweighed Pair Group method using Arithmetic Averages (UPGMA) was used for the estimation of genetic distances (GD) among all the possible combinations (Neiand 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 scorable and reproducible bands were included in the analysis. An example of PCR amplification profile of 5tea accessions using RAPD primer GLB-18 is presented in Fig. 1. A total of 3969 DNA fragments were amplified in 42 accessions using 21 RAPD primers giving an average of 4.5 bands per accession per primer. Amplified DNA fragments ranged in size from 100bp to 1400bp. Genetic diversity (GD) estimates ranged from 0 to 100 % (Table2). In total, 80.1 % comparisons showed high level of genetic diversity (GD=50-100%).

Table 1. Details of 42 accessions of 8 tea genotypes used during present study.

S. No.	Clone/code	genotype/origin	Plant type	leaf size
1.	C1 - C7	Clonal, China	Bushy	medium
2.	C8 - C14	Chauy, China	Bushy	small
3.	C15 - C 21	Raupi, China	Bushy	small
4.	C22 - C26	Qimen, China	Bushy	small
5.	C27 – C29	Turkey	Bushy	small &narrow
6.	C30 – C31	Japan	Bushy	small &narrow
7.	C32 – C35	Sri Lanka	Erect	large & narrow
8.	C36 – C42	Indonesia	Erect	large & broad

The dendrogram clustered all the accessions into2 main groups A and B comprising 12 and 30 accessions, respectively. Group A contained all the small leaved accessions belonging to pure China or China like genotypes.

Group B clustered genotypes in a mixed pattern containing both narrow and broad leaved genotypes but placing them in separate subgroups B1 and B2.Subgroup B1 clustered 16 genotypes together all belonging to small leaved genotypes obtained from China (Raupi and Qimen) (Fig. 2).

Accessions in group B1 revealed very low genetic diversity within the B1 subgroup. Subgroup B2 predominantly comprised accessions belonging to Indonesian and Sri Lankan genotypes. The accessions clustered in the subgroup B2 solely belonged to broad leaved tea species known as *C. assamica* (Indian type)but geographically these genotypes belonged to two different areas of origin i.e. Sri Lanka and Indonesia.

Though both type had some variation in their morphology but clustering of both in the same group based on their DNA sequence confirmed that they belong to the same species of genus *Camellia*. Accession no. 11was out grouped in the dendrogram. From dendrogram analysis it was also revealed that 3 accessions (C29, C30and C31), 1 belonging to Turkish and latter 2 belonging to Japanese genotypes, showed no diversity at all.

Table 2. Range of genetic distance estimates among 42 accessions of tea using 21 RAPD primers.

S. No	Range of GD	%age of comparisons
1	0-10%	1.7
2	11-20	2.3
3	21-30	1.5
4	31-40	1.7
5	41-50	12.7
6	51-60	11.4
7	61-70	18.3
8	71-80	29.7
9	81-90	17.0
10	91-100	3.7

Discussion

Among molecular marker techniques, 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).

Though these primers are not considered reproducible as banding patterns have been found to be very sensitive to varying experimental conditions but this drawback of RAPD primers can be overcome by standardizing all experimental conditions.

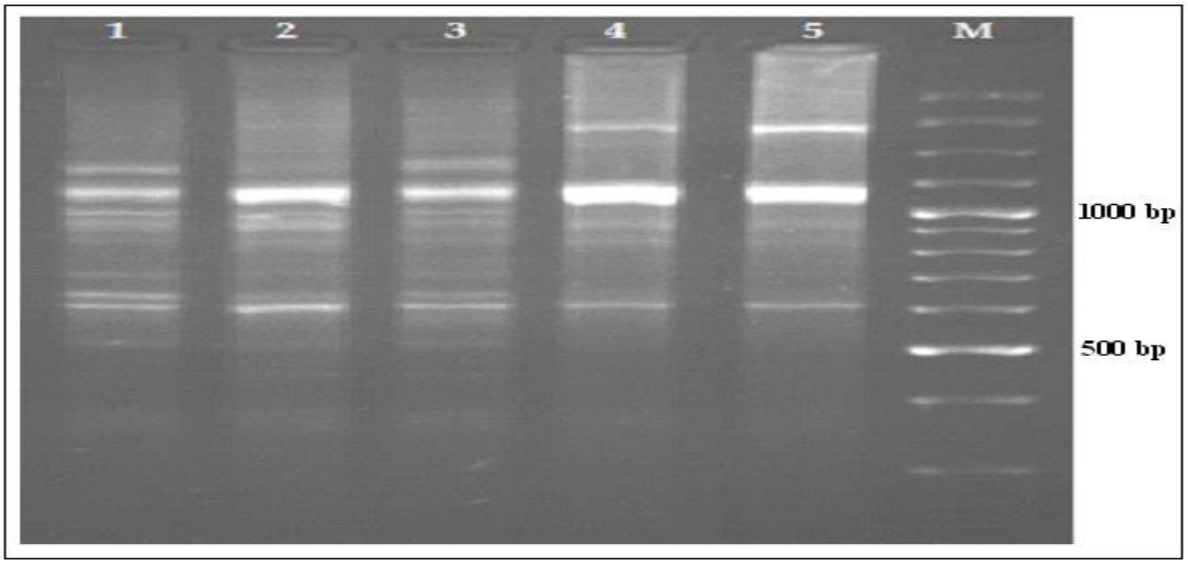

Fig. 1. An example of PCR amplification of 5 accessions of tea using RAPD primer GLB-18. M = Molecular marker (100 base pairs). 1= C27, 2= C28, 3= C29, 4= C30 and 5= C31.

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*., 2007; Yao *et al*., 2007; Liu *et al*., 2008; Borchetia *et al*., 2009; Wang *et al*., 2010; Liu *et al*., 2012). The average number of amplified fragments during present study were 4.5 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.

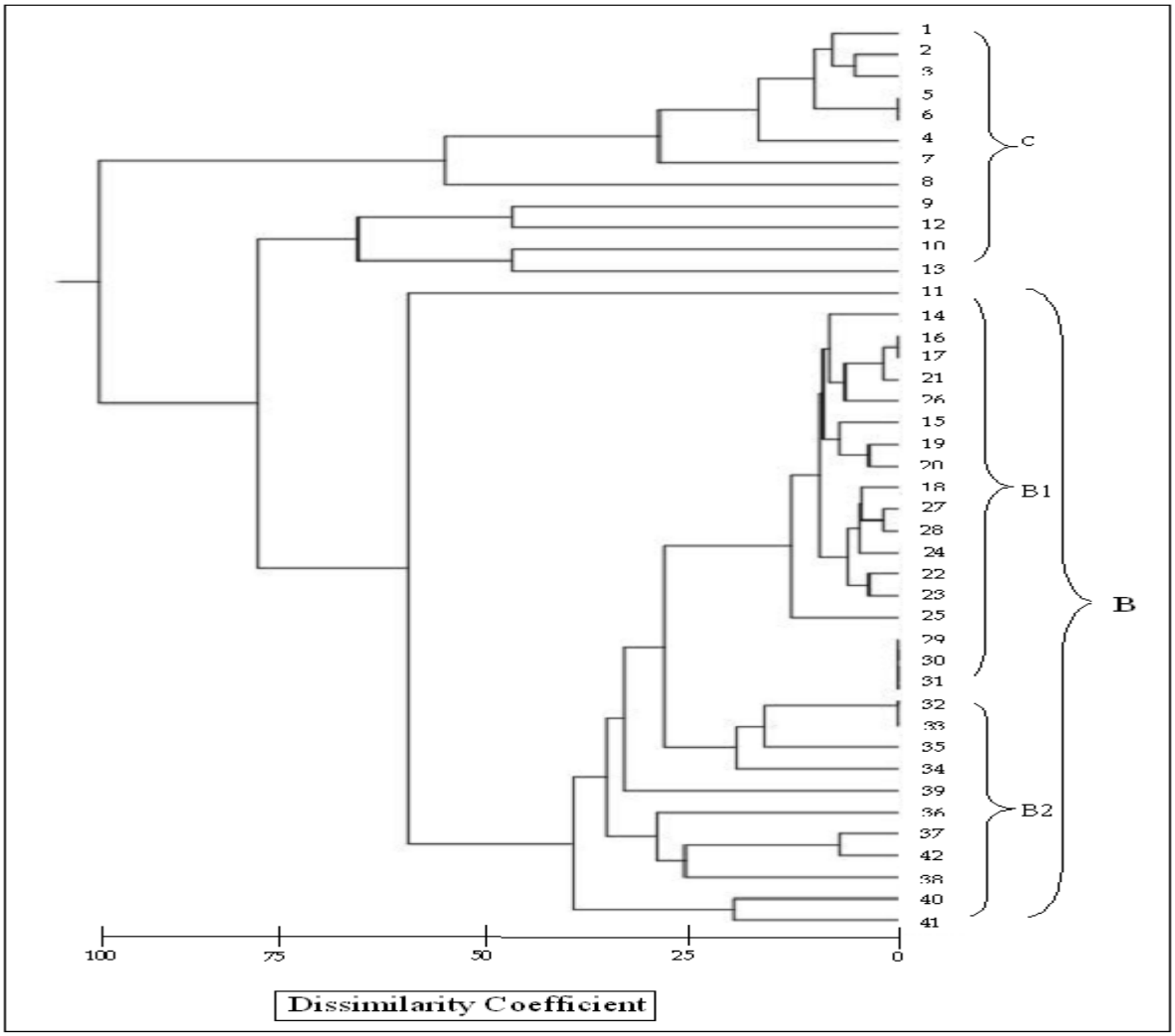

Fig. 2. Dendrogram showing relationships among 42 tea genotypes using 21 RAPD primers based on UPGMA clustering method.

It may be because in an earlier study selected elite genotypes were used where most favored alleles are retained as compared to the rare ones. Relatively higher genetic distances estimated during present study could result from the fact that tea genotypes used belonged to various geographical regions in contrast to previous study e.g. Chen *et al*. (2005a) who used Chinese genotypes which were established from a limited gene stock. Results of cluster analyses, showing different genotypes in separate groups, were in contradiction with previous reports (Chen *et al*., 2005b; Ariyarathna and Gunasekare, 2006) where various taxa tend to cluster in one group. During present study genetic diversity revealed was higher among main groups than within group diversity.

These results were contradicted with the findings of Lai *et al.* (2001), who reported higher genetic diversity within populations than among populations. The reason might be a broader range of geographical origins of plant material in present analysis. It might be due the utilization of a small number RAPD primers as well as small sample size. Overall, the data shown by dendrogram were in comparison with the conventional classification of tea taxa. The data presented here will help in establishing conservation strategies for tea crop improvement in Pakistan. In this respect priority should be given to the marginal genotypes (belonging to sub group B1 and B2 Fig. 2) which showed high genetic diversity.

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