J. Phytol. Res. **17**(2) : 123-128, 2004

PCR-BASED DNA FINGERPRINTING OF DARJEELING TEA CLONES (*CAMELLIA SINENSIS*) -FOR EVALUATION OF GENETIC DIVERSITY

SUBHAS CHANDRA ROY*
Molecular Cytogenetics & Plant Biotechnology Laboratory, P.G. Department of Botany, Darjeeling Govt. College, Darjeeling-734 101,WB. India.
* E-mail : subhascr@rediffmail.com

Darjeeling tea regarded as the champagne of tea comes from the foothills of the Eastern Himalayas. The cultivated varieties that are found in and around Darjeeling Himalayas are rich in quality tea production but they are highly heterozygous due to intercrossability among the three tea species. So, the traditional methods using morphological traits for classification are largely unsuccessful in establishing the diversity and relationships among different cultivated tea species because of environmental influence on traits of interest. As an alternative, PCR based marker assay, the Random amplified polymorphic DNA (RAPD) technique was applied to assess the genetic diversity among the four tea clones (*Camellia sinensis*) of Darjeeling Hills namely HV39, T383, TtV3 and T78. A set of 4-oligonucleotide decamer primers was used to detect the polymorphisms among the four cultivars. Total number of amplified bands varied from 2 to 7 according to the primer and template used. The four primers generated a total of 36 reproducible bands, ranging from 250bp to 2500bp. Twenty-two (22) were polymorphic for those four clones. The results demonstrated that RAPD markers could be used successfully to determine the genetic diversity among the tea clones which otherwise morphologically indistinguishable. All the four primers detected polymorphism among tea genotypes with an average of 9 markers per primer. Clonal differences were assessed in relation to the RAPD markers.

Keywords: *Camellia sinensis*; Cluster analysis; Dendogram; Genetic diversity; PCR-primer; RAPD markers.

Introduction

Tea, the oldest known beverage, is made from the tender leaves of three cultivars based on morphological parameters viz. *Camellia sinensis* (L) O. Kuntze (China type) ; *Camellia assamica* Shneider (Assam type) and *Cammellia assamica* sub-species lasiocalyx (Cambod type) . All three cultivars are highly cross-pollinated and intercrossable, so the existing population is a mixture of three categories of tea[1,2]. Assam type was developed in India and China type in China; they were subsequently introduced in to Japan. Indo - China region being a primary center of origin, India harbors a large tea biodiversity. However, introgression of pest and disease resistance genes from related wild species into modern tea cultivars over the past several decades has broadened the germplasm base of the cultigens, in different parts of the country viz-TV-series, and UPASI-series etc. Conventionally the tea breeders often select the parent on the basis of a few parameters i.e., morphology, yield and resistance to biotic stress without considering any genetic basis, however these parameters are highly dependent on environmental conditions. Moreover germplasm conservation must aim to conserve the minimum number of populations but at the same time maximize genetic diversity. The plant breeders urgently need some of the unchangeable genetic markers of the varieties, which can be used for the development of new improved varieties in all respects. So, it can be employed DNA markers because they are effective at evaluating genetic diversity and the data are easier to obtain than classical morpho-anatomical descriptors. The advantage of genetic markers is that they are least affected by environmental factors and are almost unlimited in number. They also offer a possibility to observe the genome directly, and thus eliminate the shortcomings inherent in a phenotype observation. A number of DNA based markers are now available for the effective quantification of genetic variation in plant species. Restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) have been applied successfully and have provided considerable genetic information in a number of plant species[3-5]. These techniques are, however, slow and expensive and are not amenable for assessment of genetic variation in large-scale population genetic studies. More recently PCR-based RAPD and simple sequence repeat (SSR) markers requiring small amounts of DNA have also

been developed[6-8]. RAPD methodology overcomes all the above limitations; a considerable number of polymorphic markers can be obtained with relative ease from minute amounts of genomic DNA without prior knowledge of sequence information. One frequently reported drawback associated with RAPD is the lack of reproducibility that may arise if experimental conditions are not standardized carefully[9]. Despite this fact, RAPD methodology has provided informative data consistent with other markers, especially at the intraspecific level[10,11] and it is cost effective for large-scale population genetic analysis. The usefulness of genetic technologies, including RAPD, in the characterization and identification of plant germplasm has great advantage over the phenotypic characterization because of their independent from environmental fluctuation[12]. These RAPD markers based on the amplification by the PCR (polymerase chain reaction) of random DNA segments, using single primers of arbitrary nucleotide sequences. The amplified DNA fragments, referred to as RAPD markers (Random amplified polymorphic DNA), were shown to be highly useful in the construction of genetic maps (RAPD mapping). In the present study, RAPD assay has been adapted to tea, using a set of 4 oligonucleotide primers, for the amplification of a genome-specific fingerprinting of DNA fragments for their genetic diversity analysis.

Material and Methods

Plant material : Four elite tea genotypes, namely HV39, T383, TtV3 and T78 were collected from the Happy Valley Tea garden, Darjeeling, WB, India and used in the present study.

DNA Extraction: The total genomic DNA was extracted by a modified CTAB method[13]. Five hundred milligrams of fresh unfolded tender leaf tissue were frozen in liquid nitrogen and ground into a fine powder with a mortar and pestle. The pulverized material was transferred to 15ml Tarson centrifuge tube and 4ml of preheated CTAB extraction buffer (65°C) was added (2% CTAB, 1.4M NaCl, 20mM EDTA, 100mM Tris-HCl; pH-8.0 and 20μl β-mercaptoethanol, 500mg PVP-40 was added just before making slurry). Then incubated at 65°C temperature for 45minutes with occasional gentle shaking. Following incubation, equal volumes of chloroform: isoamyl-alcohol (24:1) was added, and the mixture was shaken vigorously. The mixture was centrifuged at 10,000rpm for 10 minutes and supernatant was transferred to a new Tarson tube, and added double volume of chilled (-20°C) absolute alcohol for DNA precipitation, followed by addition of 1/10th volume of 3M Sodium acetate and kept at -20°C for over night for total DNA precipitation. The precipitated DNA was spooled out and washed 2-3 times in 70% ethanol and dissolved in 500μl 1X TE (10mM Tris and 1mM EDTA, pH-8.0) buffer containing 0.001μg/l RNase A for purification. The DNA was finally dissolved in 250μl sterile distilled water and quantified by gel analysis using uncut λDNA as standard, diluted to 2ng/μl to use in PCR amplification and kept at -20°C until further use.

RAPD Analysis: A total of 4 decamer (10-mer) primers were screened: all from Bangalore Genei Pvt. Ltd, Bangalore, India. PCR reactions were carried out in a 25-μl reaction mix containing approximately 25 ng template DNA, 100μmol of each dATP, dCTP, dGTP, and dTTP, 30 pmol of a single 10-base primer, 1.5units of Taq DNA-polymerase . The buffer [10 mM Tris-HCl, pH 8.8 at 25°C, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% (v/v) Triton X-100] used was the one provided by the manufacturer of the enzyme. Each reaction mixture was overlaid with 50μl mineral oil to prevent the evaporation. The PCR reactions were run in a Bio-Rad thermal cycler (Model-version 1.5) programmed for an initial denaturation step of 92°C for 2 min followed by 35 cycles of 1 min at 92°C, 1 min at 36°C, and 1 min at 72°C. A final elongation step of 5 min at 72°C was included. The PCR products were separated on 1.5% (w/v) agarose gel run in 1x TAE buffer (0.04 M Tris -acetate, 1 mM EDTA, pH=8.0) at 100 volt for 50 min and subsequently stained with ethidium bromide solution (0.5μg/ml) for 10 min. The gels were visualized with a UV transilluminator and photographed. A DNA ladder (Lambda/HindIII & Eco R1 cut) was used as a molecular size marker. All PCR reactions were run in triplicate, and only reproducible and clear bands were scored.

Data analysis : Bands were scored as 1 for their presence or 0 for their absence across the cultivars for the RAPD to generate a binary matrix. A genetic similarity (GS) was computed based on Jaccard's coefficient of similarity [(Jaccard[14]; GS (ij) = 2a/(2a+b+c), where GS (ij) is the measure of genetic similarity between individuals i and j, a is the number of polymorphic bands that are shared by i and j, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i]. The data was subsequently used to construct a dendogram using the unweight pair group method of arithmetical average (UPGMA) algoritham. All the computations were carried out using the NTSYS-pc software.

Results and Discussion

Genetic variation : The protocol of the sensitive RAPD technique was carefully optimized for various experimental parameters and a subset of three replicas from each of the clones were used to select the reproducible bands with each primer. Only clear, repeatable and reproducible bands were scored and used in further genetic diversity analysis. Four cultivars of *Camellia sinensis* were subjected to RAPD analysis. RAPD assay of the total

genomic DNA from *Camellia sinensis* was performed using 4 random primers (Table 1). The assay revealed a large amount of polymorphism, and the size of amplification product ranged between 250 - 2500bp. Figure 1 shows the amplification profile obtained with four primers L5, OPA-16, OPA-18 and OPA-19. The bands were scored as either monomorphic or polymorphic. Primer L5 generated a total of 9 products of which 5 (55.55%) were scored as polymorphic, primer OPA-16 generated a total of 7 products of which 5 (71.42%) were scored as polymorphic. With primer OPA-18, 6 out of 11 bands (54.54%) were polymorphic and with primer OPA-19, 6 out of 9 bands (66.66%) were polymorphic. Using 4 decamer primers, a total of 36 bands were scored (an average of 9 bands per primer) and about 62.04% were found to be polymorphic between the tea cultivars (Table 2 and 3). The number of scorable bands per primer ranged between 2 to 7 with an average 4.5. The percentage polymorphism varied from 54.54 % (OPA-18) to 71.42% (OPA-16).

Genetic similarity matrix and cluster analysis : The bands obtained with RAPD analyses were used to compute the Jaccard's similarity coefficient using the NTSYS-pc program[15]. The data set with 36 bands was employed for cluster analysis. With RAPD data matrix, the highest genetic similarity value of 0.55 was obtained between HV39 and T78. The lowest value obtained was 0.42 between T383 and TtV3. The genetic similarity matrices thus obtained with the RAPD data set were used to cluster the cultivars using the un-weighted pair group method of arithmetic averages (UPGMA) algorithm (NTSYS-pc, Rohlf[15]). The resulted dendogram is shown in Figure 2. All the four cultivars were grouped into one cluster with RAPD data set (Fig. 2). In this cluster, cultivars HV39 and T78 are grouped together at a similarity value of 0.55, cultivar HV39 and TtV3 are joined together at the similarity value of 0.52 and least similarity between cultivars T383 and TtV3 at the value of 0.42 are grouped together. Inspite of the genetic dissimilarity presents among the tea cultivars, they are grouped into one cluster due to the existence of genetic similarity according to the Jaccard's similarity index.

Table 1. Nucleotide sequences of primers used to generate amplification products and detect polymorphism.

S.No.	Primer code	Sequences (5' to 3')
1	L5	acgcaggcac
2	OPA-16	agccagcgaa
3	OPA-18	aggtgaccgt
4	OPA-19	caaacgtcgg

Table 2. Analysis of the polymorphism obtained with random primers among four cultivars of *Camellia sinensis*.

Primer	Total no. RAPD bands(a)	Number of polymorphic bands(b)	Polymorphism =b/a x 100(%)	Aprox. band size(bp) Mini	Maxi
L5	9	5	55.55	250	2500
OPA-16	7	5	71.42	250	2500
OPA-18	11	6	54.54	250	2500
OPA-19	9	6	66.66	250	2500
Total =4	36	22	Average 62.04%		

Table 3. Distribution of amplified fragments among the tea cultivars.

Total no. of primers screened - 4
Number of primers producing polymorphism -4
Total number of loci screened -36
Total number of polymorphic loci- 22
Total number of monomorphic loci-14
Average markers produced by individual primers - 9
Size of amplified bands - 250 -2500bp.
Percent of total bands, which are polymorphic 62.04%

The present work evaluates the genetic diversity and relationships among the four tea clones of Darjeeling Hills using RAPD markers. RAPD markers have been earlier used to study taxonomic relationships[16] and shown to detect higher polymorphism than RFLP markers[17]. The difference in the generation of bands per primer was probably due to the differences in the primer sequence and primer- template DNA interaction[18]. According to Rafalski *et al.*[19] variation in the number of bands in RAPD profiles is independent of the complexity of the genome.

Several types of molecular markers have been employed previously to quantify the genetic diversity within tea germplasm collections in different countries[20-24]. These include RFLP, RAPD, and AFLP markers, which all revealed a very narrow genetic base of the different collections. Wachira *et al.*[21] have used this molecular marker system to determine genetic diversity and differentiation within and between cultivated tea and related *Camellia* species. Paul *et al.*[23] studied the diversity and genetic differentiation of Indian and Kenyan tea using AFLP markers. Lai *et al.*[25] studied the genetic relationships in cultivated tea clones and native wild tea in Taiwan using RAPD and ISSR markers. A few Tocklai variety (TV clones) has also been characterized on the basis of RAPD analyses by Bera and Saikia[26]. Matsumoto *et al.*[20] characterized the genetic diversity in tea cultivars on the basis of PAL (phenylalanine ammonia lyase) cDNA probe

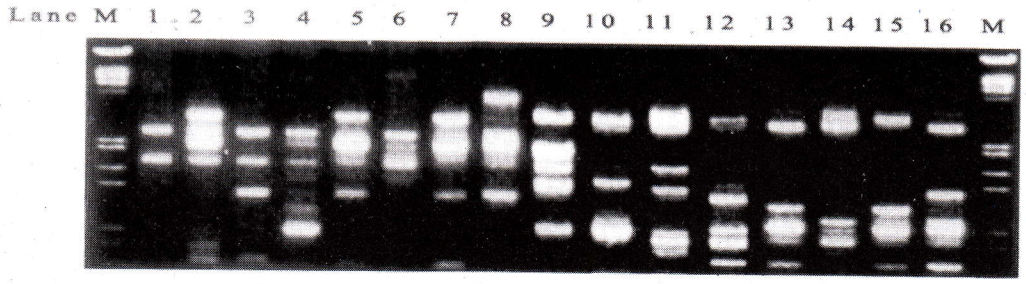

Fig. 1. RAPD profile of the four selected genotypes generated by four different primers (Lanes 1-4 for L5; 5-8 for OPA - 16; 9-12 for OPA - 18 and 13 - 16 for OPA - 19). Lanes 1, 5, 9 & 13 for clone HV 39; 2, 6, 10 & 14 for clone T383; 3, 7, 11 & 15 for clone TtV3; 4, 8, 12 & 16 for clone T78. Lane M, means for molecular size marker Lambda DNA *Eco*R1&*Hind* III cut.

analysis. Raina *et al.*[27] evaluated the genetic integrity of micropropagated diploid and triploid elite tea clones on the bases of RAPD, ISSR and RFLP fingerprinting. Kaundun *et al.*[24] also studied the genetic diversity among elite tea accessions using RAPD markers. Six Korean tea populations were analysed on the basis of RAPD-PCR to reveal their genetic diversity among the tea populations by Kaundun *et al.*[28].

In this study it has also been found that there are considerable amount of genetic variation present among the four cultivars of Darjeeling Hills viz. HV39, T383, TtV3 and T78 on the basis of DNA polymorphism in RAPD fingerprinting, which is very much consistent with the earlier report[21,25,26]. The degree of DNA polymorphism obtained from these studies will help in the detection of genetic variability among tea cultivars and their phylogenetic relationship. The cluster analysis has shown only 42% to 55% genetic similarity indicating a wide range of genetic base among the four tea cultivars (HV39, T383, TtV3 and T78). Therefore, the wide genetic base observed in the present study is an account of the wide range of outbreeding nature which has evolved as preventive mechanism for selfing increases genetic exchange and diversity. Similar observation has been made in *Camellia sinensis*[23], which is outcrossing/ outbreeding in nature. It is presumed from the phenetic dendogram (Fig. 2) that the cultivars HV39 and T383 are closely related, cultivars HV39 and TtV3 are moderately related and T383 and TtV3 are distantly related cultivars. Such information can be very useful in plant system where *a priori* knowledge on breeding habits are not available.

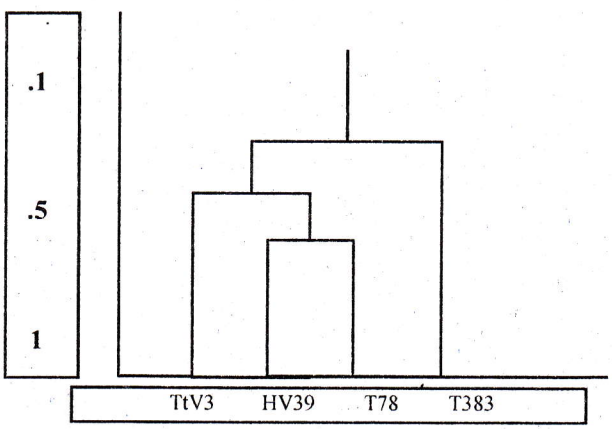

Fig. 2. Phenetic dendogram generated by UPGMA analysis based on RAPD data showing the genetic relationships among different cultivars of *Camellia sinensis*.

The present study reveals that PCR based fingerprinting technique, RAPD is informative for estimating the extent of genetic diversity as well as to determine the pattern of genetic relationships between different cultivars of *Camellia sinensis*, with polymorphism levels sufficient to establish informative fingerprints with relatively fewer primer sets. However, other primers should also be tried to provide better understanding of the genetic relationship of tea cultivars. But an extensive screening of large number of primers with more number of cultivars is necessary before any concrete conclusion is drawn of the degree of diversity existing among the Darjeeling tea cultivars. This study shows that if assay conditions are carefully controlled, the RAPD methodology may provide a cheap, rapid, and effective means to evaluate the genetic diversity among a large number of tea populations and help devise sampling strategies to complement classical morpho-agronomic descriptors. Detailed DNA characterization is also necessary for the protection of own tea cultivars.

Acknowledgements

The work was supported by financial assistance from the University Grants Commission, Govt. of India, New Delhi, for Minor Research Project for the promotion of research

on Tea at Darjeeling Hills. I would like to thank Professor T. Midya, Head of the Deptt. Of Zoology, Darjeeling Govt. College, Darjeeling, for providing Thermal Cycler facilities in the Department.

References

1. Wight W 1962, Tea classification revised. *Curr. Sci.* **31** 298-299.
2. Banergee B 1992, Botanical classification of tea. p. 25-51. *In* : K.C. Wilson and M.N. Clifford (ed.) *Tea: Cultivation to consumption.* Chapman and Hall, London.
3. Zhang Q, Saghai Maroof M A, Lu T Y and Shen BZ 1992, Genetic diversity and differentiation of indica and japonica rice detected by RFLP analysis. *Theor. Appl. Genet.* **83** 495-499.
4. Vos P, Hogers R, Bleeker M, Reijans M, Van Der Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M and Zabeau M 1995, AFLP : A new technique for DNA fingerprinting. *Nucleic Acids Res.* **23** 4407-4414.
5. Xu R Q, Tomooka N and Vaughan D A 2000, AFLP markers for characterizing the Azuki bean complex. *Crop Sci.* **40** 808-815.
6. Williams, J G K, Kubelik A R, Livak K J, Rafalski J A and Tingey S V 1990, DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18** 6531-6535.
7. De Bustos A, Cassanova C, Soler C and Nouve N 1998, RAPD variation in wild populations of four species of the genus *Hordeum. Theor. Appl. Genet.* **96** 101-111.
8. Welsh J and McClelland M 1990, Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* **18** 72138.
9. Prenner GA, Bush A, Wise R, Kim W, Dommier L, Kasha K, Laroche A, Scoles G, Molnar S J and Fedak G 1993, Reproducibility of random amplified polymorphic DNA (RAPD) analysis among laboratories. *PCR methods Applic.* **2** 341-345.
10. Dos Santos J B, Nienhuis J, Skroch P, Tivang J, and Slocum M K 1994, Comparison of RAPD and RFLP genetic markers in determining genetic similarity among *Brassica oleracea* L. genotypes. *Theor. Appl. Genet.* **87** 909-915.
11. Lerceteau E, Robert T, Petiard V and Crouzillat D 1997, Evaluation of the extent of genetic variability among *Theobroma cacao* accessions using RAPD and RFLP markers. *Theor. Appl. Genet.* **95** 10-19.
12. Varghese Y A, Knaak C, Ecke W, Saraswathymma, C K and Sethuraj, M R 1998, Application of molecular markers with special reference to RAPDs for genetic analysis in *Hevea.* In : *Development in Plantation crop research,* PLACROSYM-XI, eds, Mathew, N.M. and Jacob, C.K., pp. 40-45.
13. Murray and Thompson 1980, Rapid isolation of high-molecular-wt plant DNA. *Nucleic Acid Res,* **8** 4321-4325.
14. Jaccard P 1908, Nouvelles recherches sur la distribution florale. *Bull. Soc. Vaud. Sci. Nat.* **44** 223-270
15. Rohlf F J 1989, NTSYS-pc Numerical taxonomy and multivariate analysis system, version 1□ 50 (New York: Exeter)
16. Demeke T, Adams R P and Chibbar R 1992, Potential taxonomic use of random amplified polymorphic DNA (RAPD): a case study in *Brassica. Theor. Appl. Genet.* **84** 990-994
17. Thormann C E, Ferreira M E, Carmago L E A, Tivang J G and Osborn T C 1994, Comparison of RFLP and RAPD markers to estimate genetic relationships within and among cruciferous species. *Theor. Appl. Genet.* **88** 973-980
18. Weir B J, St. Pierre R G and Chibbar R N 1997, RAPD marker polymorphism among Saskatoon cultivars, clones and seedlings. *Hort. Sci.* **32**(6) 1109-13.
19. Rafalski J A, Tingey S V and Williams J S K 1999, RAPD markers- A new technology for genetic mapping and plant breeding. *Agr.Biotech.News Info.* **3** 645-8.
20. Matsumoto S, Takeuchi A, Hayatsu M and Kondo S 1994, Molecular cloning of phenylalanine ammonia-lyase cDNA and classification of varieties of tea plants (*Camellia sinensis*) using tea Pal cDNA probe. *Theor. Appl. Genet.* **89** 671-675.
21. Wachira F N, Waugh R, Hackett C A and Powell W 1995, Detection of genetic diversity in tea (*Camellia sinensis*) using RAPD markers. *Genome* **38** 201-210.
22. Tanaka J I and Yamaguchi S 1996, Use of RAPD markers for the identification of parentage of tea cultivars. *Bull.Natl.Res.Instt.Veg.Orn.Pl. and Tea. Series-B(Tea)* No.**9** 31-6.
23. Paul S, Wachira F N, Powell W and Waugh R 1997, Diversity and genetic differentiation among populations of Indian and Kenyan tea (*Camellia sinensis* (L.) O. Kuntze) revealed by AFLP markers. *Theor. Appl. Genet.* **94** 255-263.
24. Kaundun S S, Zhyvoloup A and Park Y G 2000, Evaluation of the genetic diversity among elite tea (*Camellia sinensis* var. *sinensis*) genotypes using

RAPD markers. *Euphytica* **115** 7-16.

25 Lai *et al.* 2001, An assessment of genetic relationships in cultivated tea clones and native wild tea in Taiwan using RAPD and ISSR markers. *Bot.Bull.Acad.Sin.* **42** 93-100.

26. Bera B and Saikia H 1999, Molecular characterization of tea [*Camellia sinensis* (L) O. Kuntze] cultivars using DNA markers. *Two and Bud* **46**(2) 16-19.

27. Raina S N and Marimutha, S 1999, DNA fingerprinting of 3 tea clones. *Newsletter, UPASI Tea Research Institute*, Dec.issue, **Vol. 9,** No. **2** 1-2.

28. Shiv Shankhar Kaundun and Young-Goo Park 2002, Genetic Structure of Six Korean Tea Populations as Revealed by RAPD-PCR Markers. *Crop Science* **42** 594-601.