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Research Paper

SSR Marker Based DNA Fingerprinting and Diversity Assessment in Superior Tea Germplasm Cultivated in Western Himalaya

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Twenty one microsatellites (genomic & genic) markers were used to evaluate genetic diversity and DNA fingerprinting of 15 popular tea accessions. Each accession had a unique marker profile, indicating that microsatellite markers were useful in differentiation studies among the tea collections. A total of 127 polymorphic alleles were scored with an allele frequency of 6.05 per primer. The polymorphism information content (PIC) ranged from 0.2 (CamsinM1) to 0.60 (TUGMS12), with an average of 0.359. SSR markers analysis detected a high level of heterozygosity (av *Ho* 0.775; *He* 0.847) in tea. The Jaccard's similarity coefficients ranged from 0.15 to 0.56 with an average similarity index (ASI) of 0.234. The first two coordinate explained 54.33% of the total variance. The unweighted pair group method with arithmetic mean (UPGMA) dendrogram and the PCoA (Principle coordinate analysis) indicated that the populations formed two major groups with exclusive China and China hybrids (I) and Assam types (II). The collections from western Himalayan possessed a moderate to high level of genetic diversity which could provide valid guidelines for genetic improvement of tea

Key words: AFLP: Amplified Length Polymorphism; He: Expected Hetrozygosity; Ho: Observed Heterozygosity; TUGMS: Tea Unigene Micro Satellite; PIC: Polymorphism Information Content

1. Introduction

Conserving biodiversity is critical for sustaining population growth and enhancing global nutrition (Frankel 1977). The assessment of the genetic variability existing in the field gene banks is of interest not only in the organization and conservation of genetic resources, but also for broadening of the genetic base of targeted species.

Tea is one of the oldest and most popular natural beverages worldwide because of its taste, attractive aroma and health benefits. Owing to specific soil and climatic requirement, majority of the tea cultivation is confined to Southeast Asian countries. In India, tea cultivation is largely confined to three geographical regions namely northeast India comprising states of Assam, West Bengal, Bihar, Tripura, Sikkim, Manipur, Nagaland, Meghalaya, Arunachal Pradesh, and Mizoram, southern regions include Kerala, Karnataka, and Tamil Nadu and the northwest regions represented by the hills of Himachal Pradesh and Uttarakhand. Among the prevailing tea cultivation regions in India, Northwest Indian tea cultivation has seen many ups and downs. During its introduction at Holta, near Palampur during 1852 by Dr Jameson, it flourished tremendously in Kanga region of Himachal Pradesh and covered 4000 ha. However, natural disaster, in the form of an earthquake struck the industry in the year 1905 that destroyed the organized tea cultivation industry in the valley and thereafter cultivation dramatically declined to 2300 ha. More than 1100 ha tea cultivation falls under the abandoned category. Tea plantations in Dehardun valley of Uttrakhand region is also in a

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very bad shape. Further, benchmark survey during 1993 revealed that about 60 % planters were not getting any income from these abandoned tea gardens (Gupta, 1995). Tremendous technical and scientific efforts of Himachal Pradesh Government and CSIR-Institute of Himalayan Bioresource Technology have helped local planters to rejuvenate the abandoned tea gardens in this region. During the process, promising tea accessions were collected, multiplied and evaluated for quality, yield and other stresses. Superior tea selections were then distributed to the local growers for cultivation. Based on agroclimatic conditions, some potential tea areas have also been identified in the valleys of Sihunta, Chowari, banikhet and Chamba. Considering the limitations of conventional tea breeding, simultaneous efforts were also made to characterize potential tea accession at molecular and biochemical level (Sharma *et al*., 2010; Karthigeyan *et al.*2008). Among the various markers, microsatellite markers due to co-dominance inheritance, locus specificity, hyper-variability coupled with genome wide distribution gained considerable importance for evolutionary, plant genetics and breeding studies (Gupta *et al*., 2000; Woodhead M *et al*. 2005). In the present study, promising tea accessions collected from the abandoned tea gardens of western Himalayan region were evaluated with polymorphic genomic and genic microsatellite markers, not only for conservation point of view but also for identification of diverse parental genotypes to broaden the genetic base in this region.

2. Materials and Methods

(a) Plant Materials

Fifteen accessions of tea germplasm maintained at the CSIR-IHBT gene bank were used for SSR marker analysis. Of these nine were collected from abandoned tea gardens of western Himalayan states of Himachal Pradesh and Uttrakhand. Four popular released Assam clones from Tocklai, UPASI, Coimbatore, Darjeeling and TRI, Sri Lanka and single accessions from NIVOT, Japan were included for comparison purpose. Single ornamental *Camellia japonica* was also included as related *Camellia* species.

(b) DNA Isolation

DNA from young leaves was extracted by CTAB method (Doyle and Doyle 1990) with minor modifications. The DNA concentration was estimated spectrophotometrically (ND1000) and quality was ensured on 0.8% agarose gel electrophoresis. The DNA was diluted according to the requirement of the technique.

(c) SSR Analysis

Genetic diversity was assessed using 12 UGMS primers (Sharma *et al*. 2009) and 9 genomic primers (Freeman *et al*., 2004) (Table 2). PCR amplification of all the primers were performed in 10 µl reaction volume consisting 1 x PCR buffer (10 mM Tris pH 9.0, 50 mM KCl, 0.01% Geletin, 1.5 mM MgCl2), 200 µM of each dNTPs, 15 ng each of forward and reverse primers, 0.2 U Taq DNA polymerase (Bangalore Genei) and 20 ng of template DNA. The PCR protocol was consisted of one denaturation cycle at 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, annealing at optimum temperature (*Ta*) for 1 min, and extension at 72°C for 2 min. The final extension cycle was carried out at 72°C for 7 min. All the PCR reactions were carried in I-Cycler (Bio-Rad).

PCR fragments were separated on denaturing polyacrylamide gels consisting of 7% polyacrylamide, Bis acryalimed (AA: BIS = 19:1) and 7 M urea in 1 x TBE buffer. The PCR reactions were mixed with equal volume of loading buffer (98% formamide containing 0.8 mM EDTA and 0.025% of each bromophenol blue and xylene cyanol), denatured at 94°C for 3 min and snap cooled on ice. Samples were loaded in preheated Sequi-Gen GT sequencing cells (Bio Rad, Australia), which run at 60 W for 1.5 up to 2.0 hrs depending on the size of amplified product. The fragments were visualized by silver staining with Silver Sequence kit (Promega, USA) as per the manufacturer directions. The size of the fragments was estimated using 20bp DNA size standard (Cambrex Bioproduct, USA).

(d) Data Analysis

Bands were scored as present (1) or absent (0) across

Table 1: Details of tea accessions utilized for SSR analysis

S.No.	Accession details	Varietal types	Source
1.	KangraAsha	China hybrid	Himachal Krishi Viswavidhalaya, Palampur, India
2.	KangraJat	China hybrid	Institute of Himalyan Bioresource Technology, Palampur, India
3.	CSIN-303536	China	National Research Institutes of Vegetables, Ornamental Plants and Tea (NIVOT), Japan
4.	UPASI 9	Assam	Brookland Estate, The Nilgiris, India
5.	CEF 01	China	Institute of Himalyan Bioresource Technology, Palampur, India
6.	BS-53	China hybrid	Institute of Himalyan Bioresource Technology, Palampur, India
7.	Mahalpat 2	China	Baijnath, Himachal Pradesh, India
8.	Raipur 3	China	
9.	TV1	Assam China hybrid	Tocklai Experimental Station, Jorhat, Assam , India
10.	TRI 2024	Assam	Tea Reseach Institute,Sri Lanka
11.	Teenali-17/154	Assam	Teenali, Assam, India
12.	BS-54	China hybrid	Institute of Himalyan Bioresource Technology, Palampur, India
13.	KM8	China hybrid	Gwoladam, Kumaon Hills, Uttrakhand, India
14.	KM9	China hybrid	Vijaypur Tea Estate, Kumaon Hills, Uttrakhand, India
15.	CJAP (D/S)	*Camellia japonica*	Institute of Himalyan Bioresource Technology, Palampur, India

all the genotypes. The entry was done into a binary data matrix as discrete variables. Estimates of genetic similarity between the genotypes were calculated using Jaccard coefficient, which is based on number of shared bands between a pair of OTUs. Genetic similarity data was used for cluster analysis using unweighted pair group method of arithmetic mean (UPGMA) and a dendrogram was obtained using NTSYS-PC ver. 2.10 (Rohlf, 1993). Bootstrap analysis was performed on the binary set with 1000 replicates using TREECON. The allelic SSR data was used to calculate the heterozygosity ($H_e=1-\Sigma P_i^2$, where, Pi is the frequency of i^{th} allele) by using software POPGENE (Yeh *et al*., 1999). Principal coordinate analysis was performed for multivariate analysis and first five vectors were used to construct a three dimensional coordinate plot using software DARwin. Polymorphic information content (PIC) was calculated according to Anderson *et al*.1993.

$$PIC_i = 1-\Sigma P_{ij}^2$$

where, P*ij* is the frequency of j^{th} pattern for marker i and summation extends over n patterns.

3. Results and Discussion

(a) SSR Analysis

Twenty one SSR primers (12 TUGMS + 9 CamsinM) produced 127 reproducible alleles with 100 % polymorphism in tested tea accessions (Table 2). The number of reproducible alleles generated per primer varied from 4 (CamsinM1 & CamsinM5) to 10 (TUGMS15, TUGMS73 & TUGMS82) with an average of 6.05. The ideal molecular approach for population genomics should uncover hundreds of polymorphic markers that cover the entire genome in a simple and reliable experiment (Luikart *et al*., 2003). The mean polymorphism information content (PIC) calculated from the frequency of polymorphic alleles across all genotypes was 0.359 (Table 2). However, PIC ranged from 0.2 (CamsinM1) to 0.60

Table 2: Characteristics of SSR markers including number of alleles, percentage polymorphism, PIC and heterozygosity among the 15 cultivated tea accessions

Locus name	SSR Motif	Alleles numbers	Heterozygosity		PIC	Unique alleles
			He	Ho		
CamsinM1	(GT)16	4	0.71	1	0.2	1
CamsinM3	(CA)18	8	0.85	1	0.28	
CamsinM4	(GA)19	6	0.73	1	0.47	
CamsinM5	(GT)15(GA)8	4	0.86	0.86	0.42	1
CamsinM6	(TG)12(T)15	5	0.83	1	0.25	
CamsinM9	(CT)15(CA)12	3	0.82	1	0.21	2
CamsinM11	(CA)12	4	0.65	0.68	0.33	
CamsinM12	(GT)12(GA)18	6	0.84	0.87	0.57	1
CamsinM14	(GA)16	7	0.77	0.88	0.55	2
TUGMS 102A	$(GGAAA)_{12}$	5	0.63	0.66	0.12	2
TUGMS12	$(TA)_{12}$	4	0.52	0.60	0.60	
TUGMS 108	$(CAAAAA)_{6}$	7	0.87	0.86	0.47	
TUGMS 27	$(GA)_{20}$	6	0.77	1	0.40	1
TUGMS 82	$(CAT)_{8}$	9	0.82	0.80	0.31	3
TUGMS 73	$(TAA)_{12}$	9	0.87	1	0.41	2
TUGMS 23	$(TC)_{13}$	7	0.86	0.86	0.47	1
TUGMS 35	$(TC)_{11}$	6	0.66	0.46	0.31	1
TUGMS 15	$(GA)_{14}$	9	0.85	0.60	0.53	2
TUGMS 34	$(TTC)_{18}(GA)_{10}$	8	0.86	0.80	0.50	1
TUGMS 22	$(GA)_{13}$	6	0.84	1	0.27	
TUGMS 13	$(TG)_{32}(TC)_{24}$	4	0.68	0.86	0.3	1
Total		127				21
Mean		6.05	0.775	0.847	0.359	

(TUGMS12). As the PIC provides a measure that is influenced by the number and frequency of alleles, current PIC values suggested that most of the tested SSR loci captured rare alleles in tested germaplsm. Further, high level of hetozygosity (av *Ho* 0.775; *He* 0.847) revealed by SSR markers analysis suggested high level of out crossing in tea. These results are congruent with previous AFLP analysis of selected tea collections from western Himalaya (Karthigeyan *et al*., 2008; Sharma *et al*., 2010). Present SSR analysis revealed that China/China hybrids and Assam accessions shared 22 % average GS. Recently, AFLP based fingerprinting of 1644 tea accessions with large number of intergrades also revealed the high level of heterozygosity in Indian tea (Raina *et al*., 2011).

Genetic Similarity and Cluster Analysis

To determine the genetic relationship among tea collections, binary data was used to calculate pair wise genetic similarity (GS) among different tea collections. The values of GS between the different accessions ranged from 0.15 to 0.56 with average GS of 0.234. China and China hybrid tea accessions collected from the abandoned tea gardens of western Himalayan regions were found to be more diverse (average genetic distance of 75%) as compared to selected Assam clones (average GS; 0.28). The ability to differentiate morphologically similar varieties is more in SSR markers as compared to AFLP analysis (Karthigeyan *et al*., 2008), hence, these markers can be useful in establishing distinctness in tea collections. In the past, successful attempts were made for determining the suitability of SSR markers for DUS testing in different crops (Singh *et al*., 2004; Law *et al*., 2001). Two group specific markers (one each for China & Assam) revealed in cluster analysis and 21 alleles specific to different clones reported in the present study could be used for DUS testing and phasing desirable alleles in future breeding programmes after their validation in large set of tea germplasm.

Genetic similarity data obtained from SSR markers was used for cluster analysis. The collection of 15 tea accessions were grouped into two major groups (Fig. 1). The first group (I) is exclusively represented by the accessions belonging to China/ China hybrids. However, Group (II) had the representation from Assam types. These two groups (China/China hybrids; group I) had a slightly lower average similarity (22 %) in comparision to Assam accessions of included in the group II (average GS: 0.28). Related *C. japonica* was clustered as a solitary out-group. Interestingly, single accessions from (CSIN303536) was grouped with China/China hybrids from western Himalayan region as earlier reported in AFLP analysis (Sharma *et al*., 2010). Further, SSR markers used in this study could differentiate each one of China/China hybrids and Assam types in spite of the high degree of similarity in morphology and geographical locations. Principal coordinate analysis (PCoA) helped in depicting the variability among the accessions of tea as largely indicated in cluster analysis. The first two coordinate explained 54.33% of the total variance (Fig. 2).

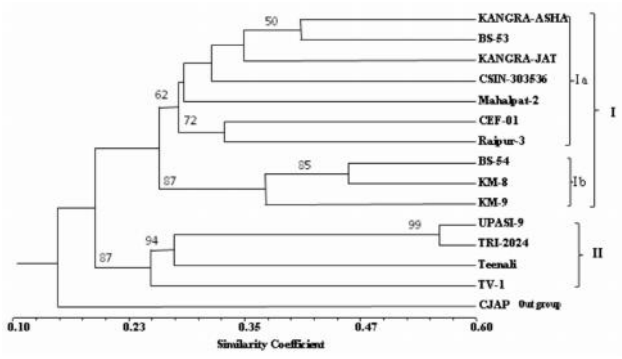

Fig. 1: Dendrogram showing genetic relationship among the 15 tea accessions based of 21 SSR markers. Tree branches with bootstrap values = 60 % are indicated

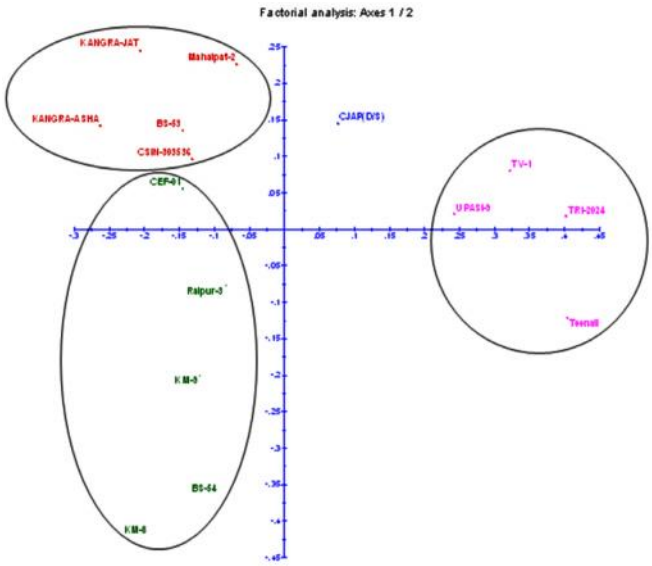

Fig. 2: Principal coordinate plot of 15 tea accessions based of 21 SSR marker data

4. Conclusion

The present study highlights the advantages of SSR marker in unique identification and diversity studies in tea. The SSR marker analyses of selected clones from abandoned tea revealed tremendous genetic diversity in tea collections. Therefore, there is an urgent need of extensive survey, collection and characterization of unexplored tea diversity prevailing in abandoned seed raised tea gardens of

Western Himalayan regions. Because of their multiple desirable attributes such as greater power of resolution, co-dominant inheritance, simplicity and hypervariable nature, the SSR markers can be a better option for conservation, characterization and identification of elite accessions for future genetic improvement so as to enhance the productivity of tea.

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