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Differentiation and identificaiton of tea clones using RAPD and ISSR markers

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ABSTRACT: The genetic differentiation and clonal identification of eight tea [*Camellia sinensis* (L.) O. Kuntze] clones selected from different agroclimatic zones of India were investigated in the present study using morphological and molecular markers (RAPD; Random Amplified Polymorphic DNA and ISSR; Inter Simple Sequence Repeats). Major morphological parameters (leaf area, leaf length, average leaf width and plant height) were considered and based on cluster analysis of leaf area two different groups were formed; clones BSS-449 and BSS-379 formed one group and remaining clones formed another. RAPD analysis produced 82.14% polymorphism among the clones. Two main groups were recognized from cluster analysis, clones UPASI-9 and T-383 formed one cluster and the remaining clones another; similarity within the clones ranged from 0.33 to 0.91. ISSR data also revealed a pattern similar to that of RAPD markers. Maximum similarity was observed between the clones P-312 and AV-2. Although ISSR similarity matrix analysis showed two different clusters these markers were able to separate the Assam hybrid BSS-449 and it formed another clusters with clone BSS-379. ISSRs were found to be more discriminative in cultivar identification than RAPDs since clones that could not be distinguished by RAPD markers were easily differentiated by the ISSR markers. The results of this study may serve as basic information for rapid and easy characterization and subsequent identification of tea clones.

Keywords: *Camellia sinensis*; Characterization; ISSR marker; RAPD marker

Introduction

The genus *Camellia* is well known for its beautiful flowers and grown world over as ornamentals. Among the different species of Camellia, tea [*Camellia sinensis* (L.) O. Kuntze] is one of the most important beverages in the world and a major cash crop in a number of countries including India.[1,2] It contributes significantly to the economy of the country. India is the largest producer of black tea as well as the largest consumer of tea in the world. Currently, India produces 23% of total world production and consumes about 21% of total world consumption of tea – nearly 80% of the tea produced is consumed within India.[1,3] The production of high quality tea has nevertheless remained a highly profitable business. Breeding of the new high quality varieties and conservation of tea germplasm resources continue to be important for sustainable cultivation of tea in India.

Tea being a long-lived crop, it is important that high yielding and quality planting material should be used for raising plantations. Although not much land is available for tea plantation in other regions of India, central Himalaya has sufficient land and the suitable agro-climatic conditions. It is also suitable for producing high-quality tea, except during the cold and dry winter months when bushes undergo dormancy. Tea plantations particularly the hilly areas of Uttarakhand state grow under rainfed conditions; often suffer from soil moisture stress, which in turn affects crop productivity. Due to poor economy, difficult terrain, unavailability of water and inaccessibility, it prohibits the use of expensive high-tech controlled irrigation plan; therefore, the ever-increasing demand for tea can be met to a certain extent by increasing the area under tea. Another option would be to increase the production per unit of cultivated land by growing high-yielding good quality clones most suitable for this climatic region. In this context, characterization of different tea clones collected from different agro-climatic zones of India have been carried out earlier on the basis of some physiological parameters, namely, chlorophyll 'a' fluorescence technique[4,5,6] and resistance to biotic and abiotic stresses.[4] These attributes have been found to be useful for quick selection from a wide range of clones. However, these traits are dependent on environment as well as developmental stage of the plant, and hence may not reflect their true genetic relationship. Therefore, in continuation to earlier studies on physi-

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Table 1: Details of tea clones used in the study

Tea clones	
BSS-449	Biclonal Seed Stock-449, Assam hybrid, Tocklai Experimental Station, Assam.
BSS-379	Biclonal Seed Stock-379, China hybrid, Tocklai Experimental Station, Assam.
P-312	Phoobshering-312, China hybrid, Darjeeling Tea Research and Development Center, West Bengal.
UPASI-9	United Planter's Association of Southern India, China hybrid, Brooklyn Tea Estate, Nilgiri hills, Valparai, Tamil Nadu.
T-383	Tukdah-383, China hybrid, Tukdah Tea Estate, Darjeeling, West Bengal.
RR-17	Rungli Rungliot-17, China hybrid, Darjeeling Tea Research and Development Centre, West Bengal.
AV-2	Balai, China hybrid, Darjeeling Tea Research and Development Center, West Bengal.
T-78	Tukdah-78, China hybrid, Tukdah Tea Estate, Darjeeling, West Bengal.

ological assessment, genetic characterization and relationship of cultivated clones deserve investigation. The present study was, therefore, carried out to characterize the eight tea clones using morphological and molecular markers such as random amplified polymorphic[7] DNA (RAPD) and inter simple sequence repeats (ISSR).[8]

Materials and Methods

Plant Material

Leaf samples were collected from eight tea clones selected from different agroclimatic zones of India like Assam, Tamil Nadu and West Bengal. Details of the sample material are presented in Table 1.

Morphological Analyses

Plant Height

The total plant height, measured from the base to the tip of the plant[9] from ten randomly selected plants of each clone was recorded and the average value was calculated.

Leaf Characteristics

Leaf characteristics were determined with the help of an automatic leaf area meter (model LICOR-3000A, LICOR, USA). Ten leaves from each clone were randomly collected and length (cm), maximum width (cm), average width (cm) and average leaf area (cm^2) were calculated and converted in per plant basis.

Data Analysis

Distance matrix was estimated by single linkage method. Cluster analyses were carried out using the SYSTAT Version 9 Software.

Molecular Analyses

DNA Isolation

Total genomic DNA was extracted from the leaves using *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (CTAB) method described by Doyle and Doyle,[10] with minor modifications. In brief, fresh plant material (500 mg) was washed and then ground in liquid nitrogen. Then, 10 ml of preheated extraction buffer [2% CTAB (w/v), 0.2% β-mercaptoethanol (v/v), 100 mM Tris-HCl (pH 8.0), 20 mM ethylene diamine tetraacetic acid (EDTA) and 1.4 mM NaCl] were added to the powdered material. After incubating the homogenate for 1 hr (at 65°C) an equal volume of chloroform : isoamyl alcohol (24:1) was added and centrifuged at 10,000 rpm for 20 min. DNA was precipitated with 1/10 volume of 3 M sodium acetate and an equal volume of isopropanol followed by centrifugation at 10,000 rpm for 10 min. The DNA pellet was washed with 70% ethanol, air-dried and then resuspended in 200–300 μl Tris EDTA (TE; 1 mM). The quantity and quality of isolated total genomic DNA was determined using 0.8% agarose gel electrophoresis is 0.5× TBE buffer for mobility relative to that of known concentration of λ DNA double digested with *Eco*R1 and Hind III.

RAPD Fingerprinting

The RAPD primers were procured from Operon Tech. Inc. (Alameda, CA, USA). Initially 60 random decamer primers were screened for RAPD reactions with selected tea DNA templates. Primers resulting in discrete well-separated bands on agarose gels were selected for amplification. Polymerase chain reactions (PCRs) were carried out according to Williams *et al.*[7] in a final volume of 25 μl containing 20 ng template DNA, 200 μM each deoxynucleotide triphosphate, 10 pM of decanucleotide primers, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, 0.1%

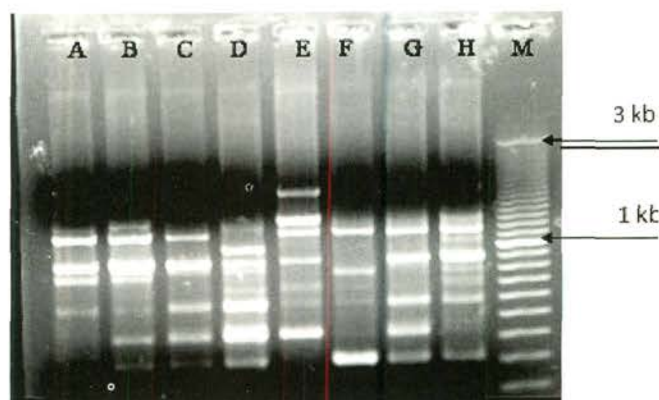

Figure 1. RAPD pattern of selected tea clones generated by primer OPA-7, where M= molecular marker, and tea clones A= BSS-449, B= BSS-379, C= P-312, D= UPASI-9, E= T-383, F= RR-17, G= AV-2 and H= T-78.

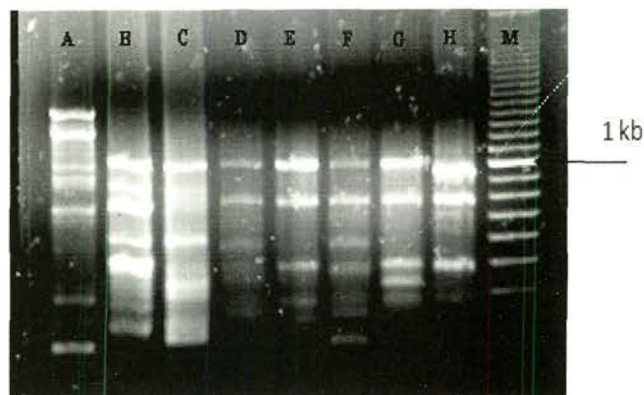

Figure 2. ISSR pattern of selected tea clones generated by primer UBC-73, where M= molecular marker, and tea clones A= BSS-449, B= BSS-379, C= P-312, D= UPASI-9, E= T-383, F= RR-17, G= AV-2, and H= T-78.

Triton X-100 and 0.5 U Taq DNA polymerase (M/s Bangalore Genei, India). Amplification reactions were carried out in a Thermocycler (Biometra, Germany) which was programmed to include pre-denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at for 30 s, annealing at 36°C for 1 min and extension at 72°C for 1 min, finally at 72°C for 10 min. A representative gel profile with OPA7 has been given in Figure 1.

ISSR Fingerprinting

A set of 100 ISSR primers procured from University of British Columbia (supplied by M/s Genetix Biotech Asia Pvt Ltd, New Delhi, India) were screened with template DNA. ISSR-PCR was carried out according to Zietkiewicz *et al.*[8] Amplification reactions were carried out same as described earlier in RAPD section except annealing temperature which was 50°C. PCRs (RAPD and ISSR) were repeated at least twice to establish reproducibility of results.

Gel Electrophoresis

Amplified products were resolved on 1.5% agarose gel using 1× TAE (Tris-Acetate EDTA) buffer at constant voltage of 60 mA for 3 hr. Gels were stained with ethidium bromide and visualized under UV light. Gel profiles were archived using Gel Doc System (Alpha Imager™ IS-2200, CA, USA). Extended DNA ladder (3 kb) was used as DNA weight marker to measure the amplified fragments. The gel images were photographed and stored as digital pictures for future processing. A representative gel profile with UBC-73 has been provided in Figure 2.

Data Analysis

Each polymorphic band was considered as a binary character and was scored 1 (presence) or 0 (absence) for each sample and assembled in a data matrix. Only intensely stained, unambiguous, and reproducible bands were scored for analysis. Similarity index was estimated using the Dice coefficient of similarity.[11] Cluster analyses were carried out on similarity estimates using the unweighted pair-group method with arithmetic average (UPGMA) method using Gene Profiler 1-D Phylogenetic analysis and data basing software. Polymorphic information content (PIC) values were calculated for each RAPD primers according to the formula:

$$PIC = 1 - \Sigma(P_{ij})^2,$$

where P_{ij} is the frequency of the ith pattern revealed by the jth primer summed across all patterns revealed by the primers.[12] Similarity matrices based on RAPD and ISSR markers were compared using the Mantel-correspondence test.[13] All procedures were computed with the computer package NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System, Biostatistics, New York, USA, Software Version 2.02 Package).

Results

Maximum leaf area (30.07 cm²) per plant and maximum leaf length (15.61 cm) were recorded for the Assam hybrid BSS-449 and the minimum was recorded for the China hybrid T-383. Maximum average leaf width (2.38 cm) was recorded for BSS-379 and minimum (1.58 cm) for T-383. In the case of plant height no clear differences was observed (Table 2).

The cluster analyses (Fig. 3) based on leaf area per plant showed two major groups. The first group contained T-383, T-78, P-312, RR-17, AV-2 and UPASI-9, and the second group consisted of BSS-379 and BSS-449. The latter two clones showed maximum dis-

Table 2: Morphological parameters of tea clones (n = 10)

Leaf characteristics	Tea clones							
	BSS-449	BSS-379	P-312	UPASI-9	T-383	RR-17	AV-2	T-78
Leaf area (cm²)	30.07±2.68	23.29± 1.98	16.06±0.88	17.61±1.41	13.95±0.67	15.72±1.28	15.04±1.19	16.22±2.17
Leaf length (cm)	15.61±0.53	9.55±0.47	9.01±0.44	8.72±0.40	8.08±0.42	8.95±0.45	8.43±0.43	8.51±0.53
Avg. leaf width (cm)	1.72±0.07	2.38±0.11	1.6±0.10	1.96±0.11	1.58±0.03	1.71±0.06	1.66±0.07	1.67±0.17
Plant height (cm)	83.77±3.43	89.77±2.48	92.11±2.20	88.66±3.10	82.22±1.40	86.22±1.00	90.55±1.40	85.44±3.7

tance with the other six clones. Minimum distance was observed between P-312 and RR-17 while the maximum was observed between BSS-449 and T-383.

In the present study, 60 RAPD primers were screened, out of which, 6 produced clear and score able amplification products in the 8 different tea clones. The amplification profiles from eight tea clones with six random primers produced total 56 fragments ranging in size 0.2–2.9 kb; out of which 46 (82.14%) were polymorphic (Table 3). The number of fragments produced by a primer ranged from 4 (OPA12) to 14 (OPA16). Similarity coefficient ranged 0.33–0.91 in the clones examined in the present study. These similarity coefficients were used to generate a tree for cluster analysis using UPGMA method (Fig. 4). Data of RAPD analyses indicate that clone P-312 and AV-2 showed maximum (0.91) similarity while minimum similarity (0.33) was observed between clone UPASI-9 and RR-17 (Table 4). The UPGMA dendrogram showed two clusters at a similarity index of 0.5. Cluster I consisted of clones BSS-379, T-78, P-312, AV-2, BSS-449 and RR-17 while Cluster II consisted of clones UPASI-9 and T-383. Clone BSS-449 is an Assam hybrid and clustered together with China hybrids. Clone RR-17 showed minimum similarity while P-312 and AV-2 showed maximum similarity (Fig. 4).

Out of 33 ISSR primers screened, 6 produced non-ambiguous and reproducible bands. A total of 41 bands were obtained, out of which 33 were polymorphic revealing 80.48% polymorphism and the fragment size varied in range 0.2–1.5 kb (Table 3). In the present investigation similarity index of the tea clones ranged 0.15–0.85. The data also indicates that maximum similarity (0.85) was recorded between clone P-312 and AV-2, while the lowest (0.15) was between BSS-449 and RR-17 (Table 5). The UPGMA dendrogram generated for eight tea clones showed two main clusters at the similarity index of 0.5. The cluster I consisted of all China hybrids except BSS-379, whereas the second cluster consisted of one Assam hybrid (BSS-449) and one China hybrid (BSS-379; Fig. 5).

A comparative account of RAPD and ISSR analysis is presented in Table 6. It is important to mention here that different markers have different properties and will reflect different aspects of genetic diversity.[14] In this work the RAPD and ISSR surveys among eight tea clones revealed 80.14% and 80.48% of polymorphic bands, respectively (nearly same; Table 6). Mantel test revealed the different distribution patterns of the polymorphism between RAPD and ISSR markers and the correlation coefficient (r) was found as r = 0.1892.

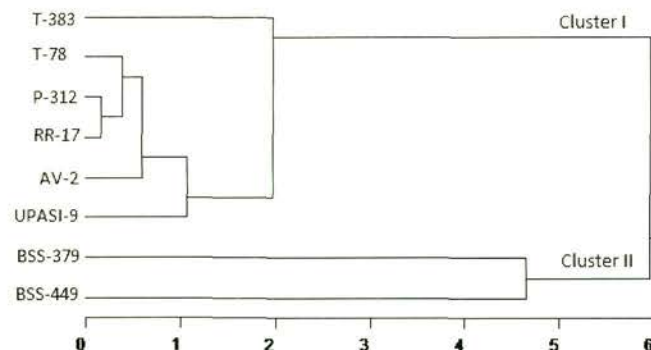

Figure 3. Cluster analysis based on leaf area per plant.

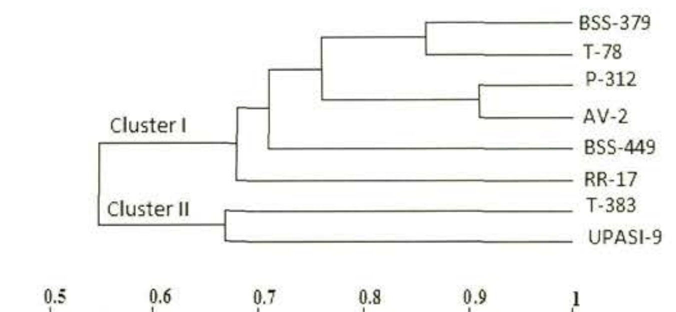

Figure 4. Cluster analysis of RAPD marker using UPGMA.

Table 3: Total number of amplified fragments and number of polymorphic fragments generated by PCR using RAPD and ISSR markers

Name of primer	Sequence of primer (5′-3′)	Total no. of amplified products	No. of polymorphic products	Size range (kb)	PIC value
OPA-3	AGTCAGCCAC	7	5	0.5–1.2	0.240
OPA-4	AATCGGGCTG	8	4	0.7–2.8	0.398
OPA-7	GAAACGGGTG	10	10	0.3–1.8	0.298
OPA-12	TCGGCGATAG	4	3	0.5–1.2	0.396
OPA-6	AGCCAGCGAA	14	12	0.2–2.4	0.233
OPC-2	GTGAGGCGTC	13	12	0.6–2.9	0.354
UBC-18	CACACACACACACACAG	5	3	0.2–1.5	0.332
UBC-27	ACACACACACACACACG	7	5	0.4–1.2	0.149
UBC-30	TGTGTGTGTGTGTGTGC	6	5	0.4–1.5	0.325
UBC-47	CACACACACACACACARC	3	1	0.7–1.1	0.200
UBC-50	GTGTGTGTGTGTGTGTYC	5	4	0.2–1.4	0.312
UBC-73	GACAGACAGACAGACA	15	15	0.2–1.4	0.296

R= (A,G) and Y= (C,T).

Discussion

Clonal identification has traditionally been based on morphological descriptors such as plant height, shape, leaf texture, young leaf type, fruit shape, etc. In the past these morpho-physiological markers, to a certain extent, helped in the selection of clones for desired agronomic traits, however, these are liable to be influenced by environmental factors.[15,16] These parameters are also valuable for identification of varietal groups and can reveal, to some extent, inter and intra varietal polymorphism but can not account for the total diversity in the species. Therefore, applications of DNA based markers are more reliable tools for molecular characterization.

The potential uses of different molecular markers such as RFLP,[17] RAPD[18] and AFLP[19] have been reported in tea. In recent years, DNA profiling through RAPD technique has been used for the analysis of genetic diversity and taxonomic relationship,[16] analysis of genetic fidelity[19,20,21,22,23] and management of genetic resources.[24] Drought tolerance associated DNA marker was developed by Mishra and Sen-Mandi[25] in tea clones growing in Darjeeling, India. These workers reported that the Darjeeling-grown tea plants selected under the investigation belonged to a narrow gene pool originating through intra- as well as inter-specific hybridization. RAPD analysis using 11 primers, revealed 180 PCR products of which 131 were polymorphic bands. The investigation indicated that RAPD method can reveal appreciable levels of polymorphism in tea clones.[25]

In the present investigation, morphological parameters and molecular markers (RAPD and ISSR) were used to characterize and identify the tea clones selected from different agroclimatic zones of India. The cluster analysis based on morphological parameters (*i.e*, leaf area) showed two different groups, clones BSS-449 and BSS-379 formed one group while the remaining clones

Table 4: Similarity matrix for Nei and Li's coefficient of eight tea clones based on RAPD analysis

	BSS-449	BSS-379	P-312	UPASI-9	T-383	RR-17	AV-2	T-78
BSS-449	1							
BSS-379	0.77	1						
P-312	0.73	0.85	1					
UPASI-9	0.46	0.75	0.71	1				
T-383	0.5	0.4	0.62	0.67	1			
RR-17	0.44	0.5	0.6	0.33	0.36	1		
AV-2	0.6	0.46	0.91	0.62	0.5	0.67	1	
T-78	0.6	0.77	0.73	0.62	0.5	0.67	0.8	1

Table 5: Similarity matrix for Nei and Li's coefficient of eight tea clones based on ISSR analysis

	BSS-449	BSS-379	P-312	UPASI-9	T-383	RR-17	AV-2	T-78
BSS-449	1							
BSS-379	0.46	1						
P-312	0.26	0.57	1					
UPASI-9	0.3	0.33	0.71	1				
T-383	0.33	0.36	0.61	0.72	1			
RR-17	0.15	0.5	0.57	0.66	0.72	1		
AV-2	0.16	0.5	0.85	0.66	0.72	0.5	1	
T-78	0.16	0.18	0.61	0.36	0.6	0.54	0.54	1

formed another group. It can be observed that BSS-449 showed maximum difference with other clones. The differences would be attributed to the origin of the BSS-449, as this clone is an Assam hybrid, and is known for its bigger leaves compared to other tea clones. Clone BSS-379, although a China hybrid is a biclonal seed stock and since it also possess bigger leaves, therefore showed closed proximity with BSS-449. The other remaining clones of China hybrids formed a separate group in the dendrogram (Fig. 3).

Based on the cluster analysis of RAPD data, two main groups could be recognized, *i.e.* clones UPASI-9 and T-383 formed one group while remaining ones another. The latter group consisted mainly of China hybrid clones where the similarity ranged from 0.33 to 0.91. A lower similarity was observed amongst the China hybrids. BSS-449, an Assam hybrid, grouped with China hybrids in dendrogram and showed comparatively higher similarity with China hybrids; this could be due to the interspecific hybridization. A study on AFLP analysis of tea genomes for determining the relationship between Indian tea clones (from different parts of India), including a large number of *C. assamica* types and Kenyan tea clones have revealed about 50% similarity, suggesting that those clones have originated mainly through interspecific hybridization.[18] These workers reported that Assam genotypes both from India and Kenya formed the same group supporting the fact that the Kenyan clones have been derived from collection made from India. Our findings, therefore, are in congruence with the earlier studies carried out in tea.

Genetic differentiation based on ISSR polymorphism was similar to those obtained using RAPD markers in the present study; two main groups were observed. Although, maximum similarity was observed between the clones P-312 and AV-2 by both the markers, however, ISSR markers were able to separate the Assam hybrid BSS-449 from the China hybrids; it formed one group with clone BSS-379 in the dendrogram while rest of the six clones formed another group. Lowest similarity was observed between BSS-449 and RR-17. BSS-449 also showed comparatively lower similarity with the remaining six clones. Cluster of morphological parameters also showed similar results.

Results obtained from RAPD and ISSR studies indicated that the percent of polymorphic fragments were 82.14 and 80.48, respectively. The reason behind the high level of polymorphism could be the agrocli-

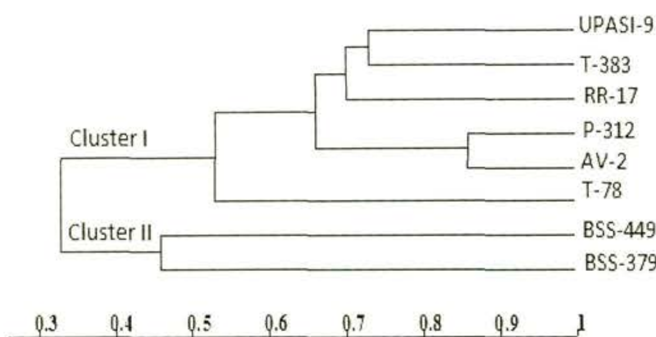

Figure 5. Cluster analysis of ISSR marker using UPGMA.

Table 6: Summary of RAPD and ISSR amplified products from 8 mixed samples of *C. sinensis*

Description	RAPD	ISSR
Total bands scored	56	41
Number of monomorphic bands	10	8
Number of polymorphic bands	46	33
Percentage of polymorphism	82.14	80.48
Number of primers used	6	6
Average polymorphism/ primer	7.66	5.5
Average no. of fragments/ primer	9.33	6.83
Size range of amplified fragments (kb)	0.2–2.9	0.2–1.5

matic zones of clones in the different region. High level of polymorphism has also been reported by AFLP markers.[26,27] The results obtained from cluster analysis based on RAPD and ISSR data sets were different. This was also reflected in the correlation coefficient calculated for the elements of RAPD and ISSR similarity matrices by Mantel test. Although the value correlation coefficient between RAPD and ISSR markers was significant as $r = 0.1892$, it shows a very poor fit between two markers, according to its interpretation ($0.7 \leq r > 0.8$ poor fit; $r > 0.7$ very poor fit). This inferred that the two sets of markers explore genetic variation differently. It is likely that RAPD and ISSR target different regions of genome which are subjected to different mechanisms generating genetic variation. Genomic regions sampled by the RAPD and ISSR markers maintain a different evolutionary process under selection.

In conclusion, RAPD and ISSR markers were powerful dominant DNA markers and efficient to detect polymorphism and changes in the DNA levels occurring in tea clones. These results may also serve as basic information for rapid characterization and clonal identification of tea clones.

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