Indian Journal of Biotechnology
Vol 8, October 2009, pp 370-376

Genetic diversity and relationships among tea (*Camellia sinensis*) cultivars as revealed by RAPD and ISSR based fingerprinting

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Received 29 September 2008; revised 18 March 2009; accepted 15 May 2009

Genomic fingerprinting in 21 tea genotypes was carried out using 7 ISSR and 12 RAPD primers. Polymorphism was 88.54% in ISSR fingerprinting, but was 77.77% in case of RAPD based fingerprinting. Dendrogram was constructed on the basis of genetic similarity matrix using the UPGMA algorithm, which showed three clusters (China type, Assam type and Cambod type). The genetic diversity over all groups (H_T) on an average was 0.38, diversity within populations (H_S) was 0.27, and genetic differentiation (G_{ST}) between populations over all loci was 0.25. The China variety had shown the largest within-group diversity (H_S = 0.285-0.291), while the Cambod tea had the least diversity (H_S = 0.193-0.207) and moderate diversity existed in Assam tea (H_S = 0.223-0.241). Interpopulation gene flow [$N_m = 0.5(1 - G_{ST})/G_{ST}$] was 0.76; $N_m < 1.0$ shows the limited genetic exchange among populations.

Keywords: Dendrogram, genetic diversity, gene flow, RAPD-ISSR fingerprinting, tea

Introduction

Tea is the oldest, non-alcoholic, caffeine-containing beverage produced from the young shoot tips (two and a bud) of different tea varieties, *viz.*, *Camellia sinensis* var. *sinensis* (L.) O. Kuntze (China type), *C. sinensis* var. *assamica* (Masters) Wight (Assam type) and *C. sinensis* subspecies *lasiocalyx* (Planch.) Wight (Cambod type). All the three varieties of tea (*C. sinensis*) are highly cross-pollinated and intercrossable without any reproduction barrier. Thus, the existing population is a mixture of three categories of tea[1,2]. Plant breeders need genetic markers of the varieties, which can be used for the development of new improved varieties. DNA-based molecular markers are effective at evaluating genetic diversity among the species and cultivars, and the data are easier to obtain than classical morpho-anatomical descriptors. Previously, several types of molecular markers have been employed to quantify the genetic diversity within tea germplasm collections of various countries. These include AFLP based marker analysis[3], RFLP based fingerprinting[4], RAPD profiles[5-11] and ISSR based analysis[4,10]. In the present investigation, authors report the use of RAPD and ISSR markers for assessing the genetic diversity and relationships among 21 cultivated clonal genotypes from three tea varieties.

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Materials and Methods

Plant Material

Clonal genotypes (21) from three different varieties of tea were used for the present study (Table 1). These genotypes were collected from the different tea germplasm centre of India, such as, Darjeeling Tea Research Centre (DTRC), Kurseong, West Bengal; The United Planter's Association of Southern India (UPASI), Valparai, Tamil Nadu; and Tocklai Experimental Station (TES), Jorhat, Assam, and being maintained under similar cultural practices at Tea Germplasm Bank, Department of Botany, North Bengal University, West Bengal, India by propagating through single stem node cuttings to maintain genetic identity[12].

Genomic DNA Isolation and Purification

Tender unfolded leaf samples from 21 tea genotypes were collected from the clonally propagated plants and stored immediately at –80ºC for DNA extraction. The total genomic DNA was extracted from the stored leaf samples using the modified CTAB method[13] and purifide according to standard method[14]. Concentration of the purified genomic DNA in each case was adjusted to 10 ng/µL

Table 1—Tea genotypes from three categories of tea population used in the present investigation

Varieties	Tea genotypes
i) *Camellia sinensis* var. *sinensis* (China type)	T383, T78, T135, HV39, TeenAli17,AV2, BS/7A/76
ii) *C. sinensis* var. *assamica* (Assam type)	UPASI-3, UPASI-9, UPASI-26, TV20, TV21, TV27, TV28
iii) *C. sinensis* sub sp. *lasiocalyx* (Cambod type)	TV18, TV19, TV22, TV23, TV25, TV26, TV30

in different aliquots and stored at −20°C for use in PCR amplification.

PCR Amplification for RAPD and ISSR Fingerprinting

Twelve RAPD primers L5, OPA-1, OPA-4, OPA-9, OPA-11, OPA-12, OPA-13, OPA-16, OPA-18, OPA-19, OPB-10 and OPB-20 were used for PCR amplification of the genomic DNA of 21 tea clones[15]. RAPD reactions were performed in a 25 µL reaction mixture containing 10 µL template DNA (30 ng), 2.5 µL 10× PCR buffer [500 m*M* KCl, 100 m*M* Tris-HCl (*p*H 8.0), 15 m*M* $MgCl_2$, 0.1% Gelatin], 2 µL dNTPs mixture (100 n*M*), 5 µL of single 10-mer random primer (50 n*M*), 1 µL of 1 Unit Taq DNA polymerase and final volume was adjusted by adding sterile distilled water. A negative control with no DNA was included in each PCR run. The PCR amplification reactions were performed in a Gene Amp PCR System 2400 (Perkin-Elmer) programmed for an initial denaturation step of 94ºC for 4 min, followed by 35 cycles of 1 min at 94ºC, 1 min at 36ºC and 2 min at 72ºC. After the last cycle, a final step of 7 min at 72ºC was added to allow complete extension of all amplified fragments and reaction was ended at 4ºC forever.

Seven ISSR primers ISSR13, ISSR14, ISSR17, ISSR18, ISSR814.1, UBC830 and UBC825 were used in this ISSR based profiling[16]. Thermal Cycler was programmed for an initial denaturation step at 94ºC for 4 min, followed by 35 cycles of 1 min at 94ºC for denaturation, 1 min at 50ºC for primer annealing and 2 min at 72ºC for primer extension. A final step of 7 min at 72ºC was carried out for polishing the ends of PCR products and reaction was ended at 4ºC forever. Both the PCR amplified products (RAPD and ISSR) were size fractionated in 1% agarose gel in 1× TAE buffer and visualized the DNA bands on UV-transilluminator after staining with ethidium bromide and photographed.

Statistical Data Analysis

Each polymorphic band was considered as a binary character and was scored as 1 for their presence or 0 for their absence across the clones for both RAPD and ISSR markers to generate a binary data matrix. The binary data was used to calculate genetic similarity matrix among samples using the standard coefficient method[17]—$Sij = 2Nij/(Ni + Nj)$, where, Nij is the number of bands that are shared by genotypes i and j, Ni is the number of bands present in genotypes i, and Nj is the number of bands present in genotype j. The dendrograms were constructed using the UPGMA[18] (unweighted pair group method with arithmetic average) algorithm in SAHN clustering module from NTSYS-pc software version 1.5[19]. The Pearson's correlation coefficients between the similarity matrices based on different marker system (RAPD & ISSR data) were calculated using the standardized Mantel coefficient[20]. The significance level for the correlation coefficient was calculated following standard method[21]. The POPGENE freeware software[22] was used to partition genetic diversity into within and between populations according to Nei's formula[23]. The diversity within-population (H_S), total gene diversity (H_T) and coefficient of gene differentiation (G_{ST}) are calculated on the basis of Nei's method[23] in POPGENE software. The H_S was defined in terms of gene diversities. The estimate of gene flow from G_{ST} was calculated as $N_m = 0.5(1 - G_{ST})/G_{ST}$. The same software was also used to calculate the Shannon's gene diversity index. Significance of the genetic differences ($P < 0.001$) was measured by using a third approach, Analysis of Molecular Variance (AMOVA) with a software program WINAMOVA 1.55[24]. Analysis of Molecular Variance (AMOVA) is a method of estimating population differentiation directly from molecular data and testing hypotheses about such differentiation.

Results and Discussion

Genetic Similarity Matrix and Cluster Analyses

The results of the DNA fingerprinting of 21 tea clones are shown in Figs 1 (ISSR14 primer), 2 (RAPD OPA-19 primer), 3 (ISSR13 primer), 4 (ISSR primer UBC830) and 5 (ISSR primer UBC825). The polymorphisms obtained from the DNA fingerprinting data were summarized in Tables 2 (RAPD markers) and 3 (ISSR markers). The similarity matrixes obtained from the Tables 2 and 3 were used in an UPGMA algorithm for cluster analysis. The dendrogram obtained from RAPD matrix was represented in Fig. 6 and from ISSR matrix in Fig. 7.

Both the dendrograms show three clusters of the tea genotypes, namely China, Assam and Cambod type at a similarity index of 0.5. The China type consisted of seven tea clones T383, T78, T135, HV39, TeenAli17, AV2 and BS/7A/76; Assam type included UPASI-3, UPASI-9, UPASI-26, TV20, TV21, TV27 and TV28 clones; and lastly Cambod type consisted of TV18, TV19, TV22, TV23, TV25, TV26 and TV30 clones.

Genetic and gene diversity measures were calculated according to Nei's index[23] in POPGENE software and results were depicted in Table 4. The genetic heterozygosity (H) ranged from 0.129 to 0.435. The Cambod variety was found to be least diverse (0.25). The China variety displayed the highest level of variability (0.33), while Assam variety revealed intermediate diversity (0.30) when all the data were analyzed. Nei's total genetic heterozygosity varied from 0.139 to 0.316 and on an average 0.25 in Cambod variety, which showed least variability, while Assam variety displayed heterozygosity from 0.177 to 0.376 with an average 0.30. The heterozygosity ranged from 0.129 to 0.435 in China variety with an average 0.33. The average gene diversity within populations (H_S) was 0.27 and the total diversity (H_T) amounted to 0.38 (Table 4).

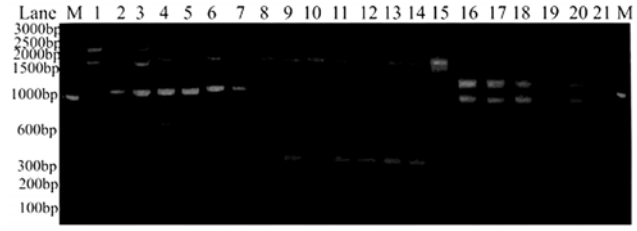

Fig.1—DNA fingerprinting of 21 tea genotypes based on ISSR14 primer. Lane M: Low range DNA ruler marker; Lanes 1-21: Tea clones T383, T78, T135, Hv39, TeenAli7, Av2, BS/7A/76, UPASI-3, UPASI-9, UPASI-26, Tv20, Tv21, Tv27, Tv28, Tv18, Tv19, Tv22, Tv23, Tv25, Tv26 and Tv,30, respectively.

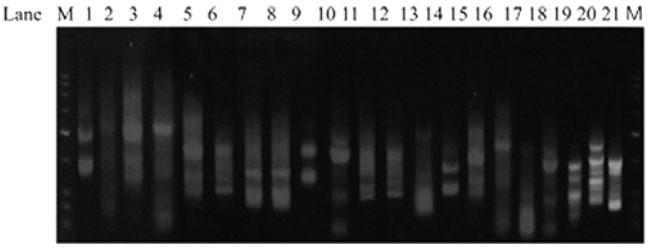

Fig. 2—DNA fingerprinting of 21 tea genotypes based on RAPD marker using OPA-19. Lane M: Low range DNA ruler marker; Lanes 1-21: Tea clones T383, T78, T135, Hv39, TeenAli7, Av2, BS/7A/76, UPASI-3, UPASI-9, UPASI-26, Tv20, Tv21, Tv27, Tv28, Tv18, Tv19, Tv22, Tv23, Tv25, Tv26 and Tv,30, respectively.

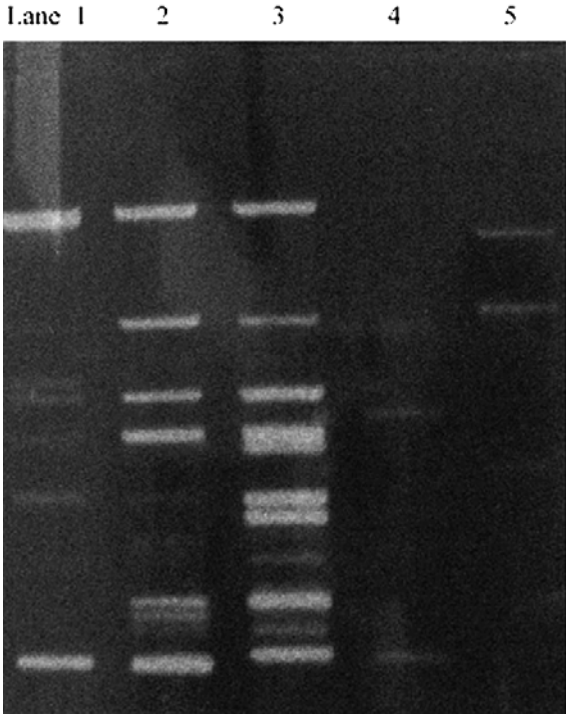

Fig. 3—DNA fingerprinting of 5 tea genotypes based on ISSR13 primer. Lanes 1-5: Tea clones BS/7A/76, Av2, TeenAli17, Tv20 and Hv39, respectively.

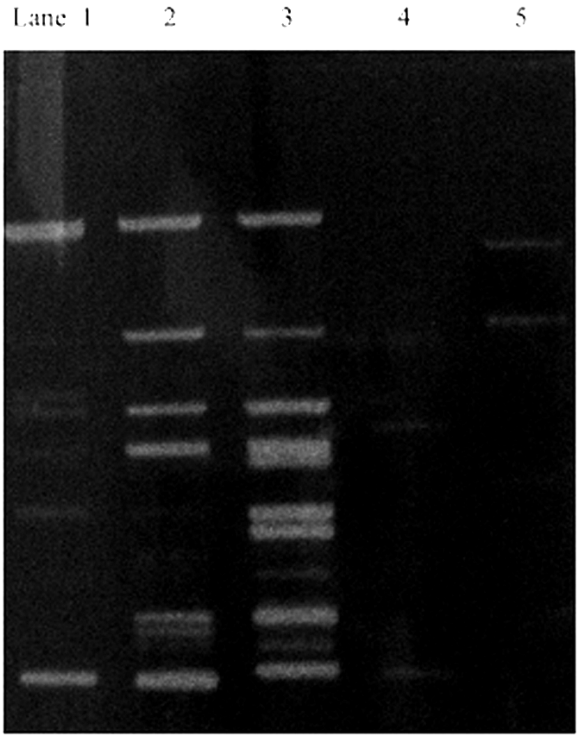

Fig. 4—DNA fingerprinting of 8 tea genotypes generated using ISSR primer UBC830. Lanes 1-8: Tea clones TV28, Tv18, Tv22, Tv23, Tv25, Tv26, and Tv30, respectively.

The value of H_S ranges from 0.178 to 0.364 and value of H_T ranges from 0.254 to 0.497. The mean level of genetic differentiation (G_{ST}) between populations over all loci was 0.25. This indicated that only a low (25%) proportion of diversity is observed between populations as compared with diversity within-populations (75%). The estimated gene flow from one variety to the other over generations (N_m) on an average was 0.769, while the lowest (0.673) was between China and Cambod varieties and highest (0.878) between Assam and Cambod variety. Interpopulation gene flow [$N_m = 0.5(1 - G_{ST})/G_{ST}$] was 0.76; $N_m < 1.0$ shows the limited genetic exchange among populations, i.e., fewer than one migrant per generation into a population.

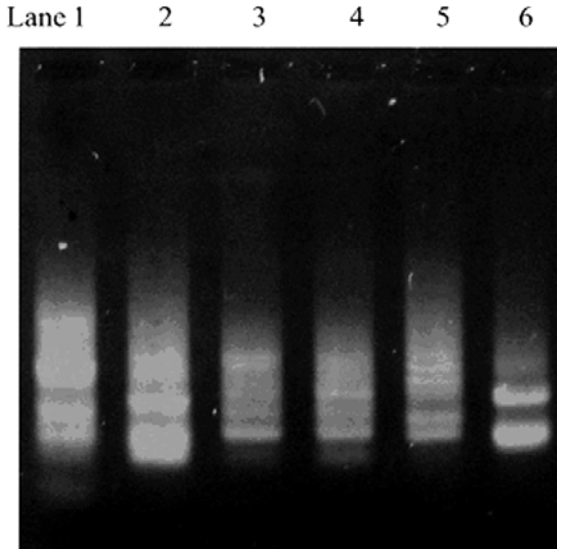

Fig. 5—DNA fingerprinting of 6 tea genotypes generated using ISSR primer UBC825. Lanes 1-6: Tea genotypes Hv39, UPASI-26, TeenAli17, Tv18, Tv20 and Tv22, respectively.

The Nei's[25] genetic identity and distance were shown in Table 5, indicating that the highest identity (0.67) was observed between Assam and Cambod type tea clones, while lowest identity (0.47) was between China and Cambod type tea clones. In order to clarify the correlation between populations, UPGMA algorithm was used to draw a dendrogram of the three tea populations (Fig. 8) on the basis of Nei's[25] genetic identity (Table 5). Dendrogram shows the close relationship between Assam and Cambod types as compared to China and Assam type. Similar observation has also been made earlier in case of *C. sinensis*[3], which was outcrossing/out breeding in nature.

Table 2—Analysis of polymorphisms obtained with RAPD primers in clonal tea cultivars [*C. sinensis* (L.) O. Kuntze]

Primer	Total no. of RAPD bands (a)	No. of polymorphic bands (b)	Polymorphism= b/a × 100 (%)	Approx. band size (bp) Min	Max
OPA-1	5	3	60.00%	250	2500
OPA-4	4	3	75.00%	250	2500
OPA-9	6	5	83.33%	250	2500
OPA-11	5	4	80.00%	250	2500
OPA-12	7	6	85.71%	250	2500
OPA-13	6	4	66.66%	250	2500
OPA-16	7	6	85.71%	250	2500
OPA-18	6	4	66.66%	250	2500
OPA-19	8	7	87.50%	250	2500
L5	4	3	75.00%	250	2500
OPB-10	9	7	77.77%	250	2500
OPB-20	5	4	80.00%	250	2500
Total: 12	Total: 72	Total: 56	Avg : 77.77%		

Table 3—Analysis of the polymorphisms obtained with ISSR markers in clonal tea cultivars [*Camellia sinensis* (L.) O. Kuntze] (Y=G/C)

Primer	Sequence 5′ to 3′	Total no. of ISSR bands (a)	No. of polymorphic bands (b)	Polymorphism = b/ax100 (%)	Approx. band size (bp) Min	Max
ISSR13	$(AC)_8C$	10	8	80.00%	300	2500
ISSR14	$(TG)_8G$	11	10	90.90%	300	2500
ISSR17	$(TC)_8C$	10	9	90.00%	300	2500
ISSR18	$(TG)_8G$	09	8	88.88%	300	2500
ISSR814.1	$(CT)_8TG$	10	9	90.00%	300	2500
UBC830	$(GA)_8C$	10	9	90.00%	300	2500
UBC825	$(AC)_8Y*T$	10	9	90.00%	300	2500
Total: 7		Total: 70	Total: 62	Avg : 88.54%		

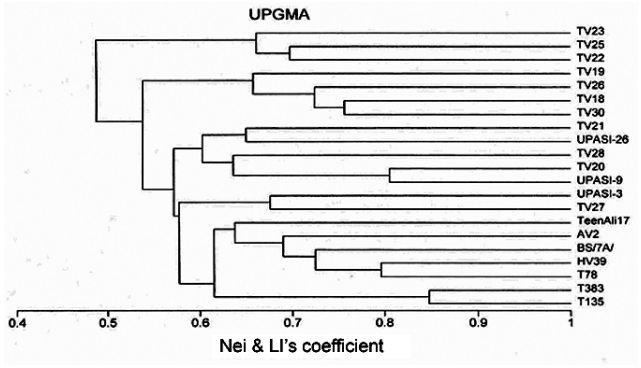

Fig. 6—Dendogram showing genetic relationship among the 21 tea genotypes based on RAPD markers using Nei & Li[17] coefficient

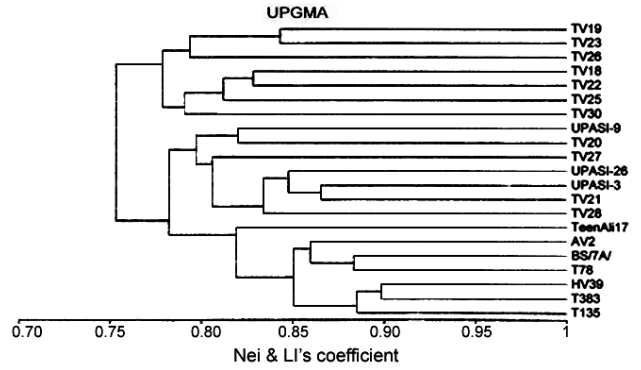

Fig. 7— Dendogram showing genetic relationship among the 21 tea genotypes based on ISSR markers using Nei & Li[17] coefficient

Highly significant differences ($P < 0.001$) were detected among populations when the data were submitted to an analysis of molecular variance using WINAMOVA software ver.1.55 (Table 6). AMOVA revealed that the variance components among groups and among individual samples within groups were

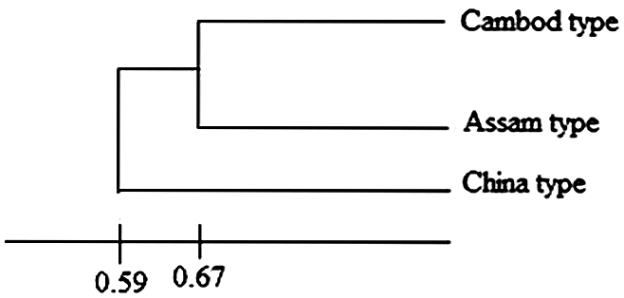

Fig. 8—Phenetic dendogram constructed on the basis of genetic identity shown in Table 4 based on ISSR markers among the three populations of tea varieties (China, Assam & Cambod types)

Table 5—Nei's genetic identity (above diagonal) and genetic distance (below diagonal) of three populations of Tea, *viz*., China type, Assam type and Cambod type (Nei, 1972)

Population	China tea	Assam tea	Cambod tea
China	***	0.59	0.47
Assam	0.42	***	0.67
Cambod	0.52	0.34	***

Table 4—Genetic and gene diversity within and between tea populations estimated according to Nei's (1973) diversity measure for RAPD and ISSR markers

Markers	China type	Cambod type	Assam type	*H_T	H_S	G_{ST}
OPA-1	0.129	0.189	0.177	0.278	0.227	0.186
OPA-4	0.209	0.177	0.276	0.381	0.245	0.189
OPA-9	0.309	0.211	0.286	0.309	0.283	0.211
OPA-11	0.254	0.267	0.294	0.254	0.298	0.199
OPA-12	0.367	0.315	0.303	0.367	0.211	0.251
OPA-13	0.421	0.139	0.288	0.379	0.178	0.254
OPA-16	0.361	0.305	0.346	0.361	0.267	0.217
OPA-18	0.272	0.293	0.376	0.483	0.293	0.232
OPA-19	0.349	0.288	0.322	0.497	0.367	0. 321
L5	0.312	0.271	0.342	0.367	0.364	0.308
OPB-10	0.267	0.316	0.331	0.338	0.297	0.271
OPB-20	0.377	0.289	0.344	0.388	0.237	0.254
ISSR13	0.405	0.275	0.287	0.304	0.286	0.211
ISSR14	0.352	0.286	0.324	0.453	0.286	0.233
ISSR17	0.347	0.247	0.303	0.377	0.247	0.318
ISSR18	0.435	0.231	0.321	0.342	0.271	0.277
ISSR814.1	0.385	0.247	0.297	0.476	0.247	0.305
UBC830	0.394	0.299	0.307	0.456	0.283	0.301
UBC825	0.387	0.243	0.302	0.423	0.266	0.299
Avg	0.33	0.25	0.30	0.38	0.27	0.25
SD	0.078	0.050	0.040	0.069	0.045	0.043

*H_T: Genetic diversity over all groups; H_S: Genetic diversity within-populations; G_{ST}: Coefficient of gene differentiation, *i.e,* proportion of genetic diversity between populations.

Table 6—Results of analysis of molecular variance (AMOVA) of tea genotypes sampled from three tea populations (Statistics include degree of freedom (df), mean squared deviations (MSDs), variance component estimates, the probability (p) of obtaining a more extreme component estimate by chance alone after 1,000 permutations and the percentage of total variance contributed by each component)

Sources of variation	RAPD					ISSR				
	df	MSD	Variance component	% of total variance	*p*-value	df	MSD	Variance component	% of total variance	*p*-value
Among groups	2	27.52	3.535	31.07	< 0.001	2	28.11	3.227	28.87	< 0.001
Among individuals within-groups	18	7.76	7.763	68.93	< 0.001	18	7.87	7.876	71.13	< 0.001

29% (28.87) and 71% (71.13), respectively. Nevertheless, and in agreement with the G_{ST} result, 71.13% of the total diversity was attributed to variation among individuals within the populations and only 28.87% to population differences. The Pearson's correlation coefficients between different matrices subjected to the Mantel test[20] were found to be highly significant ($r = 0.579 – 0.879$, $p < 0.001$).

Thus, the present study revealed considerable amount of genetic variation among the 21 tea genotypes on the basis of DNA polymorphism generated by RAPD and ISSR fingerprinting, which is very much consistent with the earlier report[5,8,9,26]. Using RAPD markers genetic diversity has been detected among the four tea population of China[11]. The genetic diversity and relationship among the 36 clonal tea cultivars of China using ISSR markers was also detected by other[10]. The diversity and genetic differentiation of India and Kenyan tea using AFLP markers has been studied[3]. They showed that most of the diversity was within population, with 79% of the variation being within and 21% being between populations of India and Kenyan tea.

In the present investigation, the AMOVA result (Table 6) revealed that the variance components among groups and among individual samples within groups were 3.535 (31.07%) and 7.763 (68.93%), respectively in case of RAPD markers, and 3.227 (28.87%) and 7.876 (71.13%) in case of ISSR markers, respectively. The average diversity within-populations (*Hs*) was 0.27 and the total diversity (H_T) accounted to 0.38 (Table 4). The mean level of genetic differentiation (G_{ST}) between populations over all loci was 0.25, which indicates that only a low (25%) proportion of diversity is observed between populations as compared with diversity within-populations (75%). The gene flow (N_m) between China and Assam varieties was 0.878 and between China and Cambod was 0.673, while between Cambod and Assam tea was 0.743. The highest population heterozygosity (H_T) was observed between China and Assam tea (0.245). Similarly, the second highest H_T was observed in cases where Assam and Cambod pairs (0.233).

In the light of present results, it is wise to conserve the whole diverse populations of the tea germplasms rather than selected individuals from different populations or only some elite tea clones. Individuals from populations containing rare alleles should nevertheless be secured so that important genotypes are saved. Genetic diversity was greatest within China type clones, followed by Assam and Cambod type. In this respect, it is noteworthy that China type tea clones should be conserved properly along with other varietal genotypes for the future improvement of commercial tea through breeding and genetic technological approaches. Therefore, all the existing population of tea should be protected *in situ* from anthropogenic and agricultural impacts. Furthermore, we should collect samples from all the natural populations and develop methods for *ex situ* conservation of the genetic resources for tea.

Acknowledgement

The financial assistance to S C Roy by University Grants Commission, New Delhi to carry out this research work is gratefully acknowledged.

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