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Study of Genetic Diversity between Some Tea Genotypes from Foman-Iran

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Abstract

One of the most important products in the northern region of Iran especially Guilan province is the tea plant (*Camellia sinensis* (L.) O. Kuntze) and plays an important role in the region's economy. Since today many tea plants in the region are being destroyed for various reasons, so having information about the genetics of those trees is helpful in designing breeding programs to reach appropriate plants for specific purposes and conservation of tea germplasm. RAPD markers, using 15 primers, were used to study the genetic relationships of 16 tea plant samples from west of Guilan province (Foman). In total, these 15 primers produced 135 scorable bands, 71.85 percent of which were polymorphic (97 bands) and 38 bands were shown monomorphic pattern (28.15 percent). The calculated PIC for all combinations was from 0.38 to 0.50 at an average of 0.49. Data analysis was performed by NTSYS software using Jaccard's similarity coefficient to determine the amount of similarity and the dendrogram was drawn based on UPGMA. Based on molecular data, the range of similarity between samples varied from 0.484 to 0.867. Samples were divided into three groups at a similarity level of 0.44. The second group (B) was divided into two subgroups at a similarity level of 0.50. Based on released data can be concluded that there is high variability between samples of tea. In general, the study of genetic diversity showed that the RAPD marker could be useful in identifying polymorphic regions and estimating genetic distances and germplasm management in tea plants.

1. Introduction

Tea (*Camellia sinensis*) from Theaceace family, is widely consumed by hundreds of millions of people in a perpetual manner, the possible effects of tea on human health is of particular importance in the field of medical, agricultural, and food research. Tea plants were originated from southwestern China, Yunnan province (Hasimoto and Simura 1978; Fulian 1986). Tea cultivation and industry is directly linked to people's economic life in several Asian and African countries, including China, India, Sri Lanka, Kenya, Iran, etc. Genetic base of this plant in Iran are from three jat, Betjan, Dhonjan and Rajghur (Ahmadishad *et al.*, 2009). Outcrossing behavior and self-incompatibility has led to the origin of numerous hybrid morphotypes. The success of tea genetic resource collection, preservation, exploitation, utilization, present, and long-term breeding programs depend primarily on the

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knowledge and understanding of the genetic background, diversity, relationship, and identification (Chen *et al*., 2005). Much different useful information has been achieved about genetics of tea by using many molecular markers such as RAPD, ISSR, RFLP, AFLP and SSR; but, in Iran, these kinds of investigations have been done in small cases and most studies were based on morphological parameters. Traditional methods of examining genetic differences based on morphological variation are less relevant because they are influenced by environmental factors. But these factors did not affect molecular markers (DNA based and isozymes) so many types of them are using in this type of study to identify genetic diversity and relationships (Falakro and Khiavi, 2020). The use of isozyme markers in identifying apple cultivars has been successful (Weeden and Lamb, 1985), but DNA-dependent markers are more abundant than morphological and isozyme markers, and the whole genome content can be verified by them.

The dominant molecular markers, such as RAPD, ISSR and AFLP, have shown that they can be used as instruments for investigation of genetic diversity and relationships (Pang *et al*., 2007; Simmons *et al*., 2007). As these kind of markers do not require genome sequencing information for primer design, they are used to evaluate genetic diversity in many different plants (Aggarwal *et al*., 1999; Culley and Wolfe, 2001; Fang *et al*., 1997; Khadivi-Khub *et al*., 2014; Khaivi and Ashourpour, 2017; Khiavi *et al*., 2015, Khiavi *et al*., 2016; Koopman *et al*., 2008 and Sedaghatfar *et al*., 2012). Recently, different molecular markers such as RFLP (Kaundun and Matsumoto 2003), RAPD (Ahmadishad *et al*., 2009; Chen *et al*., 2005; Roy and Chakraborty 2009; Falakro and Khiavi 2020), SRAP (Khiavi *et al*., 2020) AFLP (Raina *et al*., 2011; Kafkas *et al*., 2009), SSR (Bali *et al*., 2013) and ISSR (Ben-Ying, *et al*., 2010; Roy and Chakraborty 2009; Khiavi *et al*., 2020; Falakro and Khiavi. 2020) have been used for genetic relationship assessment in tea in numerous kinds of studies such as genetic diversity, cultivar identification, phylogenetic relationship, parentage identification and QTL.

In one study on lite tea accessions from Korea, Japan and Taiwan with RAPD-PCR f evaluation of genetic diversity, researchers report that Accessions from Korea had the highest level of diversity than other investigated accessions. They have reported that theses level of diversity can be related to massive genetic pool of tea in Korea (Kaundun *et al*., 2000). In the other investigation, Chen and Yamaguchi (2002) by RAPD analysis, assayed genetic diversity and phylogeny of tea and its related species and varieties in the section *Thea* genus *Camellia*, and they reported that RAPD markers could reveal high level of diversity in tea and its related species. According to the results, the molecular marker of RAPD showed a great diversity in the tea and its related species, also, the precise evidence of the high affinity of the tea species and its wild species achieved (Chen and Yamaguchi 2002).

In general, markers such as the RAPD marker, due to the need for small amounts of DNA, no need for basic DNA sequences to design and construct primers, low cost and high production and execution speed, the ability to simultaneously examine multiple loci in the genome, not needs probe (radioactive or fluorescent) and other benefits are widely used to investigate genetic diversity, especially in the early stages of studies where little is known about the extent of variation and how it is available. The overall objective of the present study is to investigate the genetic diversity of some tea plants that collected from Foman region and cultivated in Tea Research Center (TRC) of Iran collection by using RAPD markers to provide valuable information about tea genetic diversity and help the management of this collection.

2. Materials and methods

2.1. Plant material and DNA extraction

In this study, 16 accession of tea plant that cultivated in Shahid Eftekhari Fashalam Experimental Station, Tea Research Center, Guilan Province were tested. Table 1 shows the samples used, sampling place and their coding. Young and full expanded leaves were selected. Genomic DNA was extracted by

using the Dellaporta method (Dellaporta *et al*., 1983) with minor modifications. The quantity and quality of DNA were assayed by the Nano Drop device and agarose gel electrophoresis. Figure 1 showed extracted DNA in a 0.8% agarose gel.

Table 1. Tea samples used in RAPD and ISSR analysis.

Plant code	Sampling Location	Plant code	Sampling Location
SELfom1	Shahid Eftekhari Fashalam Experimental Station	SELfom9	Shahid Eftekhari Fashalam Experimental Station
SELfom2	Shahid Eftekhari Fashalam Experimental Station	SELfom10	Shahid Eftekhari Fashalam Experimental Station
SELfom3	Shahid Eftekhari Fashalam Experimental Station	SELfom11	Shahid Eftekhari Fashalam Experimental Station
SELfom4	Shahid Eftekhari Fashalam Experimental Station	SELfom12	Shahid Eftekhari Fashalam Experimental Station
SELfom5	Shahid Eftekhari Fashalam Experimental Station	SELfom13	Shahid Eftekhari Fashalam Experimental Station
SELfom6	Shahid Eftekhari Fashalam Experimental Station	SELfom14	Shahid Eftekhari Fashalam Experimental Station
SELfom7	Shahid Eftekhari Fashalam Experimental Station	SELfom15	Shahid Eftekhari Fashalam Experimental Station
SELfom8	Shahid Eftekhari Fashalam Experimental Station	SELfom16	Shahid Eftekhari Fashalam Experimental Station

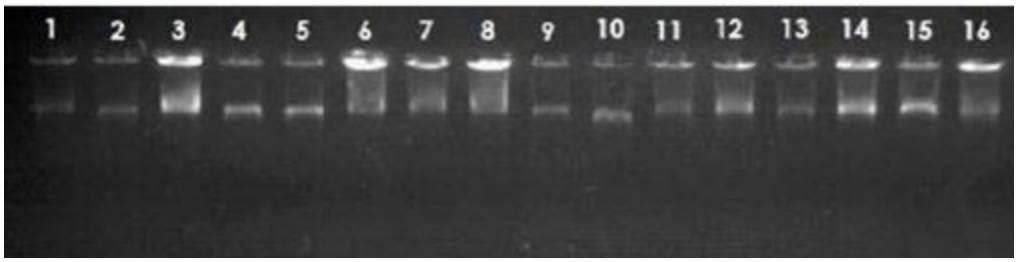

Figure 1. Some ectracted DNA of tea plant by using the Dellaporta method

2.2. Molecular Analysis

For RAPD amplification, 25 RAPD primers were screened by using four DNA samples which were not inserted in this investigation and 15 of them that had the best, strongest, and most repeatable propagating were selected to continue the investigation. The sequence of the used RAPD primers is presented in Table 2.

PCR reaction were performed and programed according to Falakro and Khiavi (2020). Amplifications were performed in 20µl of reaction mixture containing 50ng of total DNA, 10 ng of primer pair, 200µM of each four dNTPs, one-unit Taq DNA polymerase, 2mM of MgCl2 and 1X PCR buffer. The PCR was carried out with a Bio-Rad thermocycler in a condition that initial denaturation at 94^{o}C for 4 min, 35 cycles of 1 minute at 94^{o}C, 45 seconds at 38^{o}C, 2 minutes at 72^{o}C and a final extension for 7 minutes at 72^{o}C.

Table 2. Details of amplified bands generated in 16 tea genotypes based on ten RAPD primers

No.	Primer	Sequences	Total no. of amplified bands	No. of polymorphic bands	% of polymorphism	PIC value
1	P1	TCTCCGCTTG	11	8	72.73	0.42
2	P2	TGAGCCTCAC	9	5	55.56	0.45
3	P3	CTCACGTTGG	8	6	75.00	0.49
4	P4	ACTCCTGCGA	8	5	62.50	0.44
5	P5	GGTACTCCCC	9	6	66.67	0.49
6	P6	ACTGAACGGC	8	8	100.00	0.46
7	P7	CACAGACACC	8	6	75.00	0.39
8	P8	GGTGAGGTCA	9	4	44.44	0.50
9	P9	TCGCACAGTC	7	6	85.71	0.50
10	P10	GTGCTCCCTC	11	9	81.82	0.46
11	P11	CACCATCCGT	12	11	91.67	0.49
12	P12	AGGTGACCGT	7	7	100.00	0.46
13	P13	CAAACGTCGG	8	6	75.00	0.47
14	P14	TCTGGCGCAC	9	5	55.56	0.38
15	P15	AGGGTCGTTC	11	5	45.45	0.48
total	-	-	135	97		
average	-	-	9	6.47	71.85	0.49

2.3. Gel scoring and Data Analysis

The amplified products were separated on 2% agarose gels electrophoresis in a TBE buffer (1×), by running at 100 V for 120 minutes after electrophoresis, the gel was stained with ethidium bromide and the fragments were observed by UV light. To calculate genetic similarity based on Jaccard's similarity coefficient the NTSYS software was used, and cluster was designed according to the UPGMA algorithm by it. The polymorphic information content (PIC) of the combinations was calculated according to method that described by Roldain-Ruiz *et al*. (2000). Principal components analysis (PCA) was used to depict non-hierarchical relationships among the samples. Eigenvalues and eigenvectors were calculated by Past3 software.

3. Results

RAPD molecular marker was used to evaluate the genetic diversity of 16 accession of tea plant that cultivated in Shahid Eftekhari Fashalam Experimental Station, Tea Research Center, Guilan Province. Of the 25 RAPD markers after evaluating the efficacy and usefulness of markers on four tea plant DNA samples that were not included in the study, 15 markers that had higher replication and replicability than other markers were selected and used to investigate the diversity of the samples. The primers generating no, weak, or complex patterns were discarded to ensure reproducibility. The size of amplified fragments and scorable region ranged from 100 to 3500 bp and 250 to 2500 bp respectively. Polymorphic fragments that were clearly resolved on gels were scored, one for present or zero for absent (0). To ensure reproducibility, the primers generating no, weak, or complex patterns were discarded. Figure 2 showed some samples that amplified by RAPD primer. The number of amplified bands, the number of polymorphic bands, the polymorphism percentage, and the polymorphic information content (PIC) were showed in table 2.

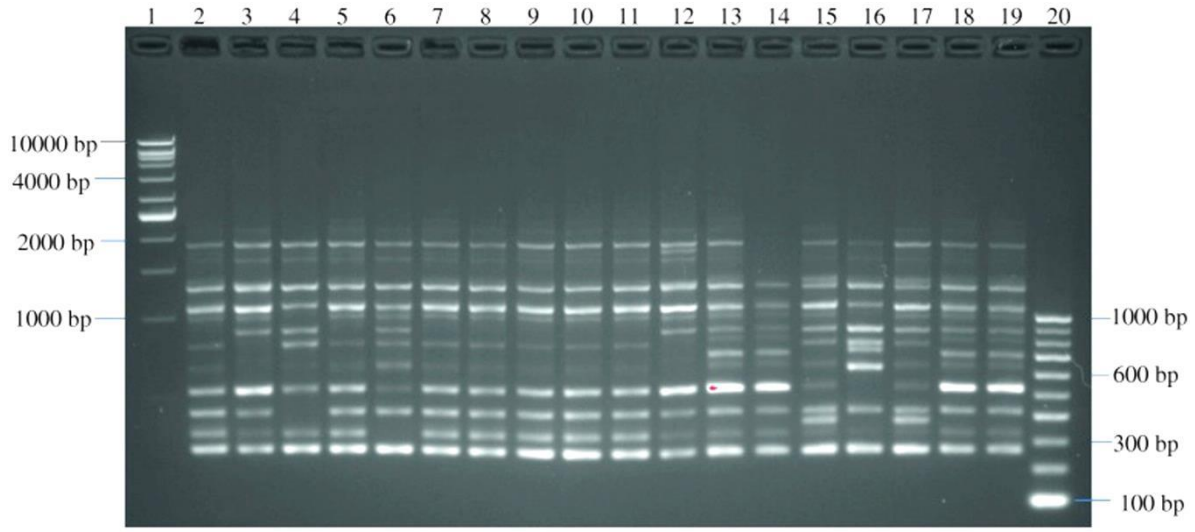

Figure 2. An example of the polymorphisms detected among some test samples using RAPD primer (P5)

Fifteen primers that used in this investigation generated 135 band whit good qualities for this study, with an average 9 bands per primer. The number of amplified fragments per primer ranged from 7 (P9: TCGCACAGTC and P12: AGGTGACCGT) to 12 (P11: CACCATCCGT). The scorable polymorphic bands ranged from 4 (P8: GGTGAGGTCA) to 11 (P11: CACCATCCGT) with an average of 6.47 per primer (Table 2). Of the total 135 scorable fragments, 71.85 percent were showed polymorphic pattern among the samples. Primer P8 (by 44.44%) and primers P6 and P12 (by 100%) showed minimum and maximum percentage of polymorphism. According to the results of other similar studies on tea plant (Chen *et al*., 2005; Devarumath *et al*., 2002; Roy and Cakraborty, 2008 and Falakro and Khiavi, 2020), this calculated percentage is acceptable.

Regarding the polymorphic information content (PIC), the used markers also showed favorable conditions. The calculated polymorphic information content (PIC) in all used markers ranged from 0.38 (P14) to 0.50 (P8 and P9). The polymorphic information content (PIC) for all primers was calculated 0.49; these amounts of PIC indicates that the used marker were sufficiently appropriate to identification of genetic diversity and relationship in tea plant.

The cophenetic coefficient was calculated for the three different similarity (Jaccard, simple matching (SM) and Dice) and UPGMA algorithm in order to find the best way to analyzing and calculating the similarity between the selected samples for assay, and it was found that if used Jaccard's similarity coefficient and the UPGMA algorithm, 89% of the similarity matrix information was transmitted to the designed cluster.

Table 3. Genetic similarity matrix based on RAPD data between studied tea accessions estimated according to Jaccard's similarity cophiceint

	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13	G14	G15	G16
G1	1.000															
G2	0.602	1.000														
G3	0.667	0.631	1.000													
G4	0.765	0.629	0.673	1.000												
G5	0.718	0.623	0.667	0.610	1.000											
G6	0.621	0.706	0.726	0.610	0.857	1.000										
G7	0.654	0.673	0.678	0.587	0.673	0.748	1.000									
G8	0.598	0.520	0.590	0.606	0.720	0.637	0.577	1.000								
G9	0.538	0.563	0.634	0.484	0.625	0.661	0.560	0.689	1.000							
G10	0.661	0.714	0.667	0.613	0.696	0.704	0.690	0.623	0.549	1.000						
G11	0.691	0.637	0.661	0.643	0.867	0.778	0.632	0.804	0.602	0.689	1.000					
G12	0.713	0.654	0.734	0.777	0.615	0.632	0.648	0.673	0.596	0.673	0.649	1.000				
G13	0.654	0.729	0.661	0.679	0.579	0.617	0.631	0.594	0.557	0.708	0.579	0.800	1.000			
G14	0.655	0.655	0.729	0.679	0.832	0.857	0.632	0.636	0.602	0.639	0.750	0.685	0.667	1.000		
G15	0.568	0.592	0.680	0.515	0.735	0.811	0.667	0.500	0.614	0.577	0.629	0.521	0.485	0.705	1.000	
G16	0.700	0.680	0.667	0.647	0.680	0.724	0.673	0.639	0.645	0.642	0.691	0.634	0.596	0.636	0.653	1.000

G1, G2, G3 until G16 were used instead of SELfom1, SELfom2, SELfom3 until SELfom16

According to Jaccard's similarity coefficient, similarity coefficients ranged from 0.494 to 0.867 with a mean of 0.656, indicating a significant variation among the studied samples. The maximum and minimum similarities were observed between SELfom5-SELfom11 and SELfom4-SELfom9. Based on previous studies on the tea plant using RAPD markers (Liu *et al*., 2009; Ben-Ying *et al*., 2010 and Falakro and Khiavi, 2020), it is found that calculated similarity was acceptable.

Of course, the similarity range reported by the present study was broader than the mentioned reports, which could be attributed to the number of additional markers used and the number of samples used in the present project. Comparing the present study with previous studies, the similarity range of 0.484 to 0.867 shows a high genetic diversity likes Yang *et al*., 2009, Yao *et al*., 2007 and Mishra and Sen-Mandi., 2004 which the results of it can return to outcrossing behavior, self-incompatibility (Bali *et al*., 2013) and sexual propagation of tea in Iran. Table 3 showed calculated similarity by used of RAPD markers. In general, in cluster analysis by molecular data, the high level of cophenetic coefficient is showed to the efficiency of the designed cluster in expressing the calculated similarities. According to the calculations, Jacquard's coefficient and UPGMA algorithm were the most suitable method for calculating similarity and designing cluster (mentioned up).

Based on clustering analysis, the 16 selected samples could be classified into three major groups at 48% similarity (figure 3). In the first group (A) separated from other samles at 0.44 of similarity just had two member (SELfom8 and SELfom9). The second group (B) was the biggest group with 10 members (62.5% of samples). This group at 0.50 of similarity divided to two sub-groups, the first sub-group (B1) containing 7 members and the other sub-group (B2) containing 3 members. The last main group (C) had 4 members.

According to unregistered studies, these samples were collected from different regions, so separation of samples and placement in different groups is acceptable. Similar results have been reported by studies of Khiavi *et al*., 2020 and Falakro and Khiavi, 2020, which have worked on Iranian tea samples and they also reported that the distribution of samples does not follow geographical pattern.

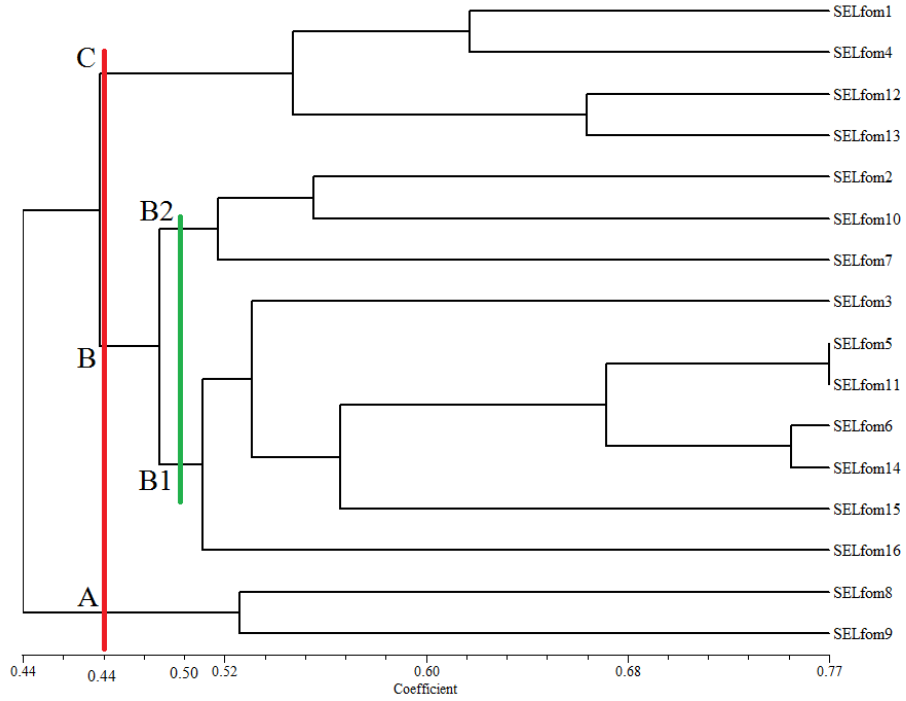

Figure 3. The phylogenetic dendrogram of 16 tea samples constructed from RAPD data using Jaccard similarity coefficient and UPGMA algorithm.

D-plot analysis (Fig. 4) showed that the samples did not follow a specific geographical pattern, which is a confirmation of the cluster analysis of the ISSR data that the grouping of the samples did not follow the geographical pattern.

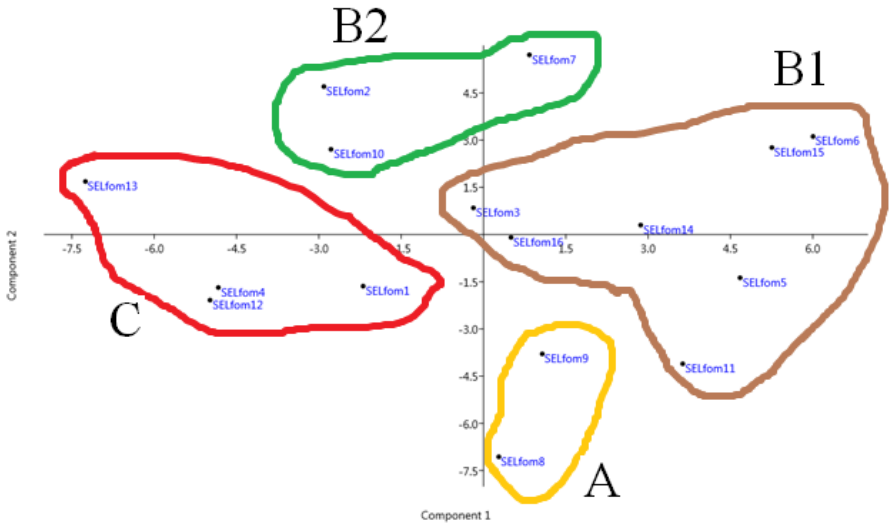

Figure 4. D-plot that generated based RAPD marker data of 16 of tea samples.

Principal component analysis revealed that the first five indices accounted for 57.56% of the variance. The first index with 15.87% had the highest effect, followed by the second index with

12.10% and the third with 10.56%. These results confirm the results of cluster analysis of ISSR markers.

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