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Assessment of genetic diversity and relationships among tea genotypes in Iran based on RAPD and ISSR markers

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ABSTRACT

Purpose: Tea plant (*Camellia sinensis* L., O.Kuntze) is one of the most popular non-alcoholic beverage crops worldwide. Although tea is important in Iran's economy, little is known about the pattern of genetic variation among the various tea genotypes grown in Iran. **Research method:** The relationship and the genetic diversity of 20 genotypes of the tea germplasm belonging to three regions were analyzed to provide guidance for the breeding of tea tree using 10 RAPD and 8 ISSR markers. **Main findings:** Polymorphism percent was 78.6 in RAPD and 68.06 in ISSR fingerprinting. The results of the PIC analysis were in the range of 2 0.44 to 0.49 and 0.34 to 0.50 for RAPD and ISSR respectively. From these results, it can be seen that these primers can detect genetic differences very well. The pairwise similarity coefficient between the genotypes varied from 0.37 to 0.68 for RAPD and from 0.59 to 0.96 for ISSR. The 20 tea genotypes from genetic resources were grouped into three main groups by UPGMA cluster analysis based on RAPD data, and to five main groups by UPGMA cluster analysis based on ISSR data. Both molecular analyses showed a high degree of variation among the genotypes. **Limitations:** Application of others molecular markers such as AFLP, SSR and (cpDNA investigation can help to found the genetic relationships of samples better. **Originality/Value:** The present study revealed that RAPD and ISSR methods could be successfully utilized to identify genetic diversity and relationship of tea group and this will provide valuable information to assist parental selection in current and future tea breeding programs.

INTRODUCTION

Tea belongs to family Theaceace, genus *Camellia*, section *Thea* and usually involves one species (*Camellia sinensis*), including two or three botanical varieties: *C. sinensis* var. assamica, *C. sinensis* var. Pubilimba (Chen et al., 2000), and sometimes *C. sinensis* var. Kucha (Chang, 1984). Three seed varieties (Dhonjan, Rajghu and Betjan) made the foundation of tea genetic in Iran. By applying different molecular markers such as RAPD, ISSR, RFLP, AFLP and SSR, beneficial information has been achieved about tea genetics. However, in Iran these sorts of studies have been carried out in small cases and most assays were based upon morphological characters. The effects of environmental factor fewer concerns morphological parameters. Whilst molecular markers (DNA based and isozymes) are not affected by environmental factors and many kinds of them are available, their application for identifying and investigating the genetic relationship in plants is very common. Molecular markers were used for genetic relationship assessment in tea in numerous kinds of studies such as genetic diversity, cultivar identification, phylogenetic relationship, parentage identification and QTL (Balasaravanan et al., 2003; Beris et al., 2016; Jahangirzadeh et al., 2020; Kafkas et al., 2009; Lai et al., 2001; Liu et al., 2009; Ma et al., 2014; Paul et al., 1997; Rani et al., 2012; Roy & Chakraborty, 2009; Ueno & Tsumura, 2009). Paul et al. (1997) investigated genetic diversity and differentiation among populations of Indian and Kenyan tea by AFLP markers and reported that these markers were very useful and efficient for tea population identification (Paul et al., 1997). Kaundun et al. (2000) evaluated the genetic diversity among elite tea accessions from Korea, Japan and Taiwan with RAPD-PCR. Accessions from Korea showed higher level of diversity than accessions from Japan and Taiwan. This high level of diversity can be related to huge genetic pool in Korea (Kaundun et al., 2000). In another study, Chen and Yamaguchi (2002) assayed genetic diversity and phylogeny of tea plant and its related species and varieties in the section *Thea* genus *Camellia* by RAPD analysis, and their results showed that RAPD could reveal high level of diversity in tea and its related species. According to their result, it became known that cultivated species and wild related species had narrow genetic relationship (Chen & Yamaguchi, 2002). Beris et al. (2016) used ISSR markers for investigation of genetic diversity in cultivated tea clones (*Camellia sinensis* (L.) kuntze) in Turkey and found similarity range between 0.456-0.743 and clustered samples into two groups. Totally markers (like RAPD and ISSR) were widely used, mainly at the beginning of studies which no information was available about the amount of diversity; because of low DNA requirement, low cost, being fast in analyzing and other advantages. Jahangirzadeh et al. (2020) used SRAP marker to identification of genetic diversity and relationships of some Iranian tea genotypes. Reported similarity ranges were 0.393 to 0.933 and classified samples in five groups. Their results showed that SRAP marker could be useful in identifying polymorphic regions and estimating genetic distances and germplasm management in tea plants.

In the present study, RAPD and ISSR were used to determine genetic relationships among 20 tea genotypes from Iran to provide valuable information about tea genetic diversity.

MATERIALS AND METHODS

Plant material and DNA extraction

Fresh young leaf samples were taken from 20 tea genotypes collected from three locations on the north of Iran (Table 1). The leaves were stored at -80 ºC until being used for DNA extraction.

Total DNA was isolated using the procedure described by the Diversity Arrays Technology Pty Ltd (DArT P/L) company (Diversityarrays, 2007). Approximately 0.2 g of plant material was ground to a fine powder by mortar and pestle under liquid nitrogen. They were transferred to a 2 ml tube and 1 ml of fresh buffer (Table 2 and 3) was added and incubated at 65°C for 1 hour. It was then allowed to cool down for 5 minutes on ice and 1 ml of chloroform: isoamyl alcohol (24:1) mixture was added. This suspension was well mixed for 30 minutes. After centrifugation at 10000 g for 20 minutes, the water phase was transferred to the fresh tube and the same volume of ice-cold isopropanol was added. Then tubes were inverted until nucleic acids became visible. Following centrifugation at 10000 g for 30 minutes, the supernatant was discarded and the pellet of DNA washed with 2 ml 70% ethanol. Ethanol was discarded, and pellet dried and dissolved in 250 µl of 1 X TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0).

Molecular Analysis

For RAPD amplification, thirty RAPD primers and for ISSR 20 primers were screened using four DNA samples. both markers (RAPD and ISSR) 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 $MgCl_2$ and 1X PCR buffer. The PCR was carried out with a Bio-Rad thermocycler in a condition that initial denaturation at 94°C for 4 min, 35 cycles of 1 minute at 94°C, 45 seconds at 38°C for RAPD and 54°C for ISSR, 2 minutes at 72°C and a final extension for 7 minutes at 72°C then brought down to 4°C. The amplification of the screened primers was repeated two to three times independently with the same procedure in order to verify the reproducibility of the RAPD and ISSR marker.

Gel scoring and Data Analysis

The amplification fragments were fractionated by 1.5% agarose gel electrophoresis in 1X TBE buffer at a constant voltage (70V), for 4 hours. Each mixture that loaded in agarose gel contained 10µl of PCR mixture, 3µl of loading buffer and 2µl safe stain. After it, fragments visualized under the UV light. The DNA size marker of 1 kb was used for analyzing the size of polymorphic bands.

Table 1. Tea samples used in RAPD and ISSR analysis

Row	Plant code	Sampling location	Row	Plant code	Sampling location
1	G1	Shahid Eslami Tea Research Station	11	G11	Shahid Eslami Tea Research Station
2	G2	Shahid Eslami Tea Research Station	12	G12	Kobijar village
3	G3	Sheykhzahed region	13	G13	Shahid Eslami Tea Research Station
4	G4	Shahid Eslami Tea Research Station	14	G14	Tonekabon, Garmaposhteh village
5	G5	Sheykhzahed region	15	G15	Kobijar village
6	G6	Sheykhzahed region	16	G16	Kobijar village
7	G7	Shahid Eslami Tea Research Station	17	G17	Shahid Eslami Tea Research Station
8	G8	Shahid Eslami Tea Research Station	18	G18	Shahid Eslami Tea Research Station
9	G9	Shahid Eslami Tea Research Station	19	G19	Shahid Eslami Tea Research Station
10	G10	Shahid Eslami Tea Research Station	20	G20	Sheykhzahed region

Table 2. Extraction buffers and lysis buffer stocks

Extraction buffer stock		Lysis buffer stock	
Sorbitol	0.35 M	TrisHCl pH 8.0	0.2 M
TrisHCl pH 8.0	0.1 M	EDTA pH 8.0	0.05 M
EDTA pH 8.0	5 mM	NaCl	2M
		CTAB	2% (w/v)

Both buffers should be volumed to 500 ml with distilled water.

Table 3. Preparation of fresh buffer

Chemical materials	Required amount	Example for 30 ml
Extraction buffer	The necessary amount based on the number of samples	12.5 ml
Lysis buffer	The necessary amount based on the number of samples	12.5 ml
Sodium dodecyl sulfate	0.5% (v/w)	0.15 g
Polyvinylpyrrolidone	2% (v/w)	0.6 g
Sarcosyl (5%)	0.4 (v/w)	5 ml

The buffer may be divided into two separate phases; therefore it should be heated till 65 °C and shaken before use.

Polymorphic bands were scored as either presence (1) or absence (0). These data were used to calculate the genetic similarity matrix with the Pearson correlation using Jaccard's coefficient for RAPD marker and Dice's coefficient for ISSR marker with the unweighted pair group method using the arithmetic average (UPGMA) as a clustering algorithm. The dendrogram was drawn using the SAHN module in NTSYSpc software (Rohlf, 1998). Polymorphic Information Content (PIC) was calculated for each primer according to PICi = 1-[fi^2+ $(1-fi)^2$]; where PICi is the PIC of primer ith, fi the frequency of ith primer fragment when present and 1 - fi is the frequency of *ith* primer when absent (Roldain-Ruiz et al., 2000).

RESULTS AND DISCUSSION

RAPD analyses

Ten RAPD primers were finally selected according to their diversity and reproducibility (Table 4), and were used for the amplification of the 20 Iranian tea genotypes. To ensure reproducibility, the primers generating no, weak, or complex patterns were discarded. The size of amplified fragments ranged from 100 to 3500 bp with the scorable region were from 250 to 2500 bp. Figure 1 showed some samples that amplified by RAPD primer. The number of fragments per primer ranged from 7 (P6: ACTGAACGGC) to 15 (P7: CACAGACACC).) as shown in Table 4. The number of scored polymorphic fragments ranged from 7 with (P8: GGTGAGGTCA and P6: ACTGAACGGC) to 12 with (P7: CACAGACACC) with an average of 8.8 per primer (Table 4). Of the total 112 scorable fragments, 78.60 percent were polymorphic among the samples. Primer P2 (TGAGCCTCAC) and primer P6 (ACTGAACGGC) showed minimum and maximum percentage of polymorphism (66.7 and 100 respectively) (Table 4).

Chen et al. (2005) investigated genetic diversity, relationship and molecular identification of Chinese elite tea genetic resources [*Camellia sinensis* (L.) O. Kuntze] preserved in a tea germplasm repository, calculated these items and report 100% as maximum and 66% as the minimum of polymorphic percent. Roy and Cakraborty (2009) studied genetic diversity and relationships among tea (*Camellia sinensis*) cultivars by RAPD markers, and they reported the similar result for the polymorphic percentage (Max= 90% and Min=80%). Other studies also reported same result (Devarumath et al., 2002; Mishra & Sen-Mandi, 2004).

Table 4. Details of amplified bands generated in 20 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	13	9	69.2	0.48
2	P2	TGAGCCTCAC	12	8	66.7	0.48
3	P3	CTCACGTTGG	14	10	71.4	0.44
4	P4	ACTCCTGCGA	9	8	88.9	0.45
5	P5	GGTACTCCCC	11	8	72.7	0.49
6	P6	ACTGAACGGC	7	7	100	0.49
7	P7	CACAGACACC	15	12	80	0.47
8	P8	GGTGAGGTCA	10	7	70	0.48
9	P9	TCGCACAGTC	11	10	90.9	0.48
10	P10	GTGCTCCCTC	10	9	90	0.47
Total	-	-	112	88	-	0.48
Average	-	-	11.2	8.8	78.6	

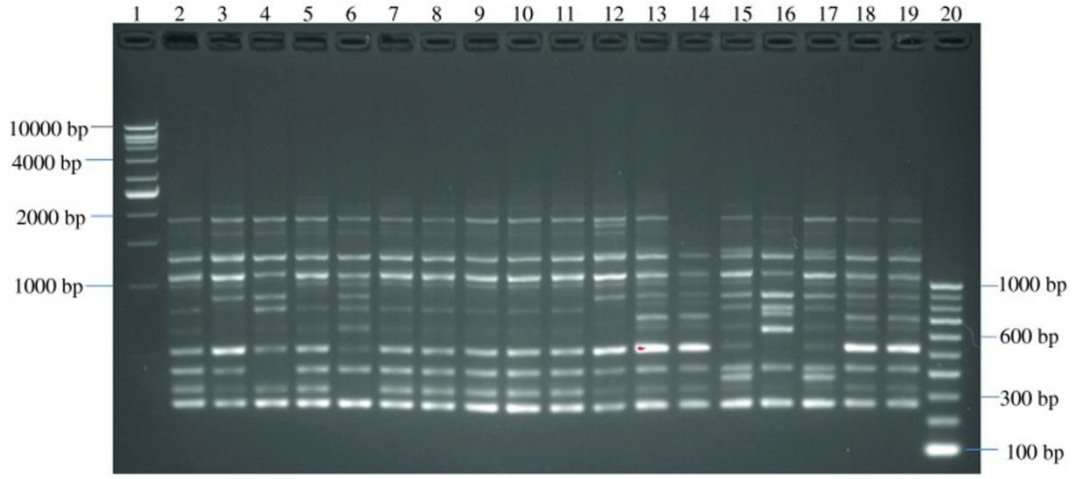

Fig. 1. An example of the polymorphisms detected among some test samples using RAPD primer (P5)

To find the potential of RAPD markers, used for our study, to distinguish our samples in a correct way, polymorphic information content (PIC) was calculated by using formula introduced by Roldain-Ruiz et al. (2000) regarding Table 4, all the PICs were high (minimum was 0.44 and maximum was 0.49), this range of PIC showed the efficiency of molecular marker used to detect polymorphism within the tea plant.

For all Jaccard's coefficient in three methods of clustering (UPGMA, complete and single), the correlation coefficient (r), based on Mantel Z-statistics (Mantel, 1967), was calculated. Mantel Z-statistics showed that Jaccard coefficient had the highest correlation coefficient with UPGMA (0.81). The amount of similarity between the similarity matrix and the cluster were shown with this coefficient.

Pairwise Jaccard similarity coefficient between 20 samples was calculated and ranged from 0.37 to 0.68. The highest and lowest similarities were observed between G14-G5 and G3-G20, with an average of 0.59 based on RAPD data. With a comparison of similarities in our study and other studies, it was found the same results were reported.

Lai et al. (2001) reported maximum and minimum similarities1.00, 0.238, respectively, this wide range of similarity found in wild genotypes of tea, could confirm our results, because Iranian tea germplasm was result of three jat (Rajghur, Betjan and Dhonjan) that used in origin for cultivation and for produce crop. Therefore, it can be stated that Iranian germplasm is more restricted than wild germplasm. On the other hand, comparing our results by papers reported genetic distances (Chen et al., 2005; Mewan et al., 2005), it was found that

our result was correct either. These similar results confirm that RAPD marker in Tea could be applied with high power in distinguishing genetic relationships.

Based on clustering analysis, the 20 samples could be classified into three major groups at 46% similarity. In the first group (A) separated from other groups at 0.39 of similarity just had one member, sample from west of Mazandaran (Tonekabon, Garmaposhteh village) (G14). This separation, according to the geographical distance, was acceptable because all other samples were collected from Guilan province. The second group (B) was separated from other samples at 43% similarity. This group had one member like the first group; this member (G4) belonged to Shahid Eslami Tea Research Station in Lahijan, Guilan province, Iran. Due to, cross-pollination and propagation method of tea in the past (sexual propagation; seed was used in propagating tea at first in Iran) separation of G4 from other samples was not strange.
In the third group (C), the biggest group with 18 members, all samples belongs to Guilan province. The placement of samples from one area showed a close genetic relationship between them. This group can be divided into three sub-groups at 0.51 similarities. The first subgroup showed high level of differentiation from the other subgroups (C2 and C3) and divided from them at % 47.5 similarities.

The first divided sub-group (C1) covered four samples (G2, G12, G15 and G16). Three of the covered samples belong to Kobijar village (G12, G15 and G16) and other one was from Shahid Eslami Tea Research Station (G2). The situation of three samples from Kobijar village near each other with high level of similarity (these samples were not separated from each other until 62% similarity coefficient) could be the result of germplasm conservation in this area. In another hand, placement of G2 (sample of Shahid EslamiTea Research Station) next to the samples of Kobijar village are acceptable because plants in this station were selected in the past from other areas that tea had been cultivated so it was possible that sample G2 was selected from Kobijar village in the past and cultivated in this station. However, it should be noted that G2 was taken in clustering at 0.53 similarities (approximately). This amount of similarity did not validate nor reject this theory.

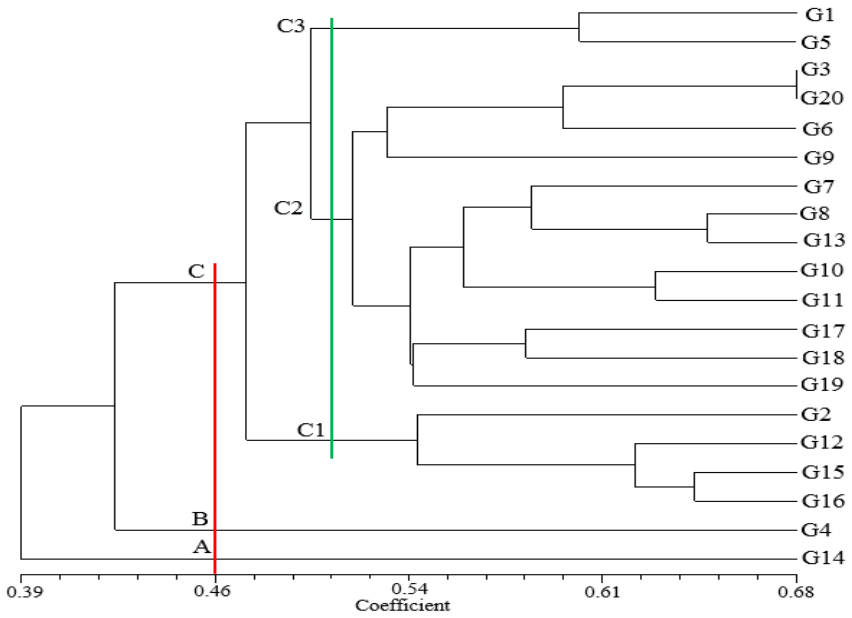

Fig. 2. The phylogenetic dendrogram of 20 tea genotypes (*Camellia sinensis*) constructed from RAPD data using Jaccard similarity coefficient and UPGMA algorithm

Subgroup C2 consists of samples from Shahid Eslami Tea Research Station and Sheykhzahed region (G3, G6, G7, G8, G9, G10, G11, G13, G17, G18, G19 and G20). This sub-group was the biggest subgroup with 12 members. It is noteworthy that in this sub-group, all samples from Sheykhzahed region were separated from Shahid Eslami Tea Research Station samples in %51 similarities (approximately). The maximum similarity was observed between two samples of Sheykhzahed region (G3 and G20).

Moreover, the last sub-group just had two members (G1 and G5). One of these members was from Shahid Eslami Tea Research Station and the other one from Sheykhzahed region.

In this study, samples from Shahid Eslami Tea Research Station showed a high level of conservation and made the special group; however, it should be noted that some samples from this place have been located in others groups (G4: group B, G2: sub-groupC1, G1: sub-group C3). Considering the history of Shahid Eslami Tea Research Station, it is well-known that the planted tea shrubs were collected from different area in the past. Therefore, this deployment in different groups while clustering was acceptable. Lai et al. (2001) also found that samples from different places revealed different grouping in cluster analyses. Mewan et al. (2005) reported the same result by studying tea in Sri Lanka Using RAPD Markers and their clustering divided tea plants from different areas into different groups with some disorder.

ISSR analyses

From 20 ISSR primers tested on four samples, not included in this research, eight primers that showed best amplification and reproducibility were selected and used for ISSR analyses of 20 tea genotypes from north of Iran. Fragments amplified by the usage of ISSR markers were arranged from 100bp to 3000bp, but scorable and acceptable fragments for scoring were between 250bp and 2500bp. The same size range was reported by Beris et al. (2016). Figure 3 showed some samples that amplified by ISSR primer.

Applying ISSR primers have been amplified 72 bands totally which only 49 fragments showed the polymorphism amplified fragment in each primer included 7 for primer I1 ((AG)8G) until 12 bands for primer number 2 (I2 (GA)8T) averages of amplified bands in all of primers were calculated as 9 bands for each used primer for ISSR analyses of 20 tea genotypes from north of Iran. Polymorphic fragments numbers in ISSR primers were from five fragments for primer I1: ((AG)8G), I4 ((AG)8C) and I8 ((ATC)6T) until eight fragments for primers I2 ((GA)8T) and I6 (BDB(TCC)5). The average of polymorphic fragments for all primers was 6.1. According to all amplified fragments and those showed polymorphic pattern, percentages of polymorphism was calculated. The range of this item was from 62.50% for I4 ((AG)8C) and I8 ((ATC)6T) to 80% for I6 (BDB(TCC)5). Previous studies (Chen et al., 2005; Roy & Chakraborty, 2009) reported the same result. Roy and Chakraborty (2009) used ISSR marker for clarifying the genetic diversity and relationships among tea cultivars and reported 88.54% for the average polymorphic percentage. Ji et al. (2011) studied ISSR diversity and genetic differentiation of ancient tea (*Camellia sinensis* var. *assamica*) plantations from China and reported 62.5% as minimum and 89.9% as maximum percent of polymorphic bands Thomas et al. (2006) used 15 ISSR markers for investigating genetic integrity of somaclonal variants in tea (*Camellia sinensis* (L.) O Kuntze) and proclaimed low percentage of polymorphism (min=30.5 and max=78.0) the acquired present of polymorphism was acceptable according to their research and thus, this vindicates our result that percentage of obtained polymorphism was not low for fingerprinting and genetic diversity studies.

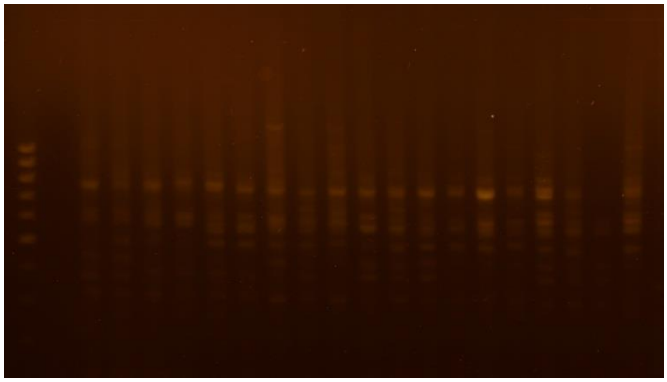

Fig. 3. An example of the polymorphisms detected among some test samples using ISSR primer (I5)

Table 5. Details of amplified bands generated in 20 tea genotypes based on ten ISSR primers

No.	Primer	Sequences	Total no. of amplified bands	No. of polymorphic bands	% of polymorphism	PIC value
1	I1	AGAGAGAGAGAGAGAGG	7	5	71.43	0.35
2	I2	GAGAGAGAGAGAGAGAT	12	8	66.67	0.34
3	I3	CGAGAGAGAGAGAGAGA	9	6	66.67	0.46
4	I4	AGAGAGAGAGAGAGAGC	8	5	62.50	0.50
5	I5	GAGAGAGAGAGAGAGAC	9	6	66.67	0.46
6	I6	BDBTCCTCCTCCTCCTCC	10	8	80.00	0.50
7	I7	ACACACACACACACACC	9	6	66.67	0.39
8	I8	ATCATCATCATCATCATCT	8	5	62.50	0.43
Total	-	-	72	49		
Average	-	-	9	6.1	68.06	0.45

Regarding Table 5, all the PICs were high, and it was ranged from 0.35 (minimum) to 0.50 (maximum), (the range of PIC in dominant markers was zero to 0.50, and if it was high, showed the marker's power in distinguishing polymorphism, genetic diversity, and differences between samples. The calculated range of PIC, showed the efficiency of molecular marker, used to detect polymorphism within the *Camellia* genus and especially in tea genotypes.

Based on ISSR data, pairwise DICE coefficient between 20 samples was calculated and ranged from 0.59 to 0.96. The highest and lowest similarities were observed between G14-G17 and G12-G13, with an average of 0.73 (data not shown). Comparing similarities in our study with others represented the same results. Lai et al. (2001) announced maximum and minimum similarity via assessing genetic relationships in cultivated tea clones and native wild tea in Taiwan using ISSR markers as 0.270 and 0.923, respectively. Yao et al. (2008) analyzed genetic diversity among tea cultivars from three different countries (China, Japan and Kenya) and estimated similarity was 0.538 (maximum) and 0.162 (minimum). In other research, Ben-Ying et al. (2010) in Yunnan used ISSR markers to detect diversity of and relationship of Tea germplasm, the estimates of similarity among tested germplasms passed from 0.445 to 0.819 with an average of 0.512 These similar results confirm that ISSR marker in *Camellia* and related genera could be applied with high power in order to distinguish genetic relationships. In a study conducted in Turkey, which the history of tea cultivation, is not old, like Iran, (it was established in 1940s.), Kafkas et al. (2009) and Beris et al. (2016) reported narrow range of genetic similarity (0.68 to 0.92 by average of 0.76 by kafKas et al. (2009) and 0.456 to 0.743 by Beris et al. (2016).

Based on these polymorphic bands, the similarity coefficient was evaluated and similarity dendrogram (Fig. 4) constructed using UPGMA cluster analysis. Based on clustering analysis, the 20 samples could be classified into five major groups at 74% similarity (approximately).

Comparing the similarity range achieved by Kafkas et al. (2009) and results obtained by the research team of this paper, two cases should be considered: (1) potency and accuracy of used markers in both study, and (2) the way of tea introduction in these countries. To initiate tea plantation in turkey in 1938, the required seed was provided from Batumi (Georgia) (Beris et al., 2005); whereas it was introduced directly from India (one of the sources of tea) to Iran without any mediator, so these differences between similarities ranges were acceptable.

Cluster analysis for 20 tea genotypes was done based on the calculated DICE similarity coefficient and UPGMA algorithm. Five major groups were generated at 46% similarity. The first two groups (groups A and B), divided from other groups at 0.66 similarity coefficient, were separated from each other in 68%, and also generated the special group. However, the other three groups have a special way with each other until 0.72 level of similarity that third group (C) was separate from fourth and fifth groups (D and E). These last two groups (D and E), also were divided from each other at 0.74 similarity coefficient. As it is known, these separations and groupings are carried out in a narrow range, which indicates the acceptable genetic linkage of tea plants, because all tea plants in Iran were generated from a small population introduced in the past.

Group (A) contain just one genotype from Shahid Eslami Tea Research Station (G17). According to geographic distance, this separation was not acceptable due to existing geographic distance, though regarding the fact that self-incommutability and other pollination in tea plants is likely to occur, fragmentation of traits and heterozygosis is extremely high and given that tea propagation was done by seed in the past (sexual way), this division is acceptable. Beris et al. (2016) also, reported that the variations among tea clones exist in Turkey due to their sexual reproduction.

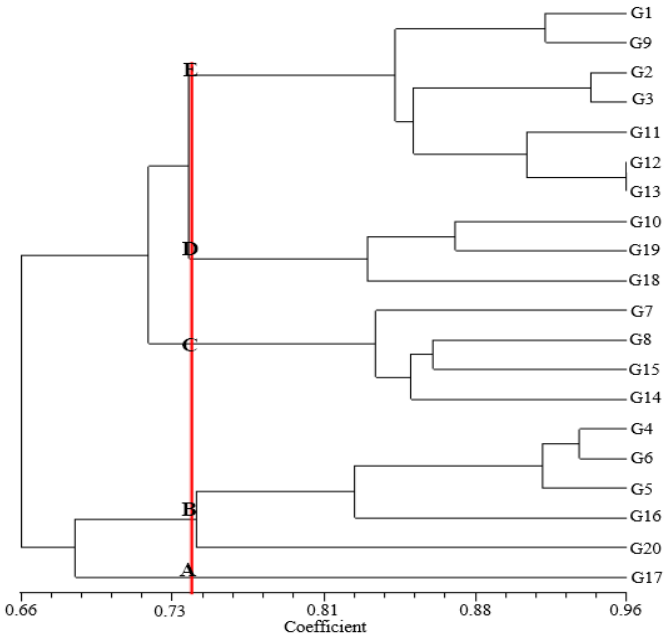

Fig. 4. The phylogenetic dendrogram of 20 tea genotypes (*Camellia sinensis*) constructed from ISSR data using Dice similarity coefficient and UPGMA algorithm

Three genotypes from Sheykhzahed region (G5, G6 and G20); one genotype from Shahid Eslami Tea Research Station (G4) and one genotype from Kobijar village were generated as the second group (B). The important point in this group is the high mixing of samples from three sampling areas of the north of Iran, which is acceptable due to the propagation method (using seed for propagation) in the past. Of course, it is necessary to point out that the sample of the Kobijar village (G20), which has geographical distance from the two other sampling regions, was divided faster from other four samples.

Third group (C) have four members, two members from Shahid Eslami Tea Research Station (G7 and G8), one members from Kobijar village (G15) and one member from Mazandaran province, Tonekabon, Garmaposhteh village (G14), locating locating samples with a large geographical distance in one group can be justified due to seed propagation and free pollination. On the other hand, all tea plants cultivated in different regions originated from first tea plants that Kashef alsaltaneh brought from India and cultivated in Lahijan, so the genetic similarities among them are natural. This sample (G14, genotype from Tonekabon, Garmaposhteh village) was inserted in this study for in Iran's tea trees have been cultivated on the long narrow region of the southern margins of Caspian Sea. Therefore, we intended to investigate whether there are any genetic relationships between plants grown in eastern and middle parts of tea cultivation regions (which includes larger areas of plantations). The fourth group (D), consist of three samples from Shahid Eslami Tea Research Station (G10, G18 and G19) were separated from each other at 87 percent of similarity.

The larger group generated by cluster analyses was fifth group (E) which was included seven genotypes (G1, G2, G3, G9, G11, G12 and G13). This group covered 30 percent of all studied samples. Furthermore, in this group, mixing of this three studied area as (Shahid Eslami Tea Research Station, Sheykhzahed region and Kobijar village) was observed.

CONCLUSIONS

RAPD and ISSR analyses among 20 cultivated genotypes of tea (*camellia*) from three regions of Iran were successfully employed to compute genetic variability and to calculate genetic relationships. According to our data, it was confirmed that RAPD and ISSR markers in tea were successfully utilized for estimating genetic diversity and relationship, which were in agreement as the result of Chen et al. (2005); Thomas et al. (2006); Roy and Chakraborty, (2009); Ji et al. (2011) and Ben-Ying et al. (2010).

RAPD and ISSR data generated from 20 genotypes with then and eight primers, respectively, were sufficient to provide inferences on genetic differentiation and relationships among them. PIC values were also recorded high in both markers (RAPD=0.48 and ISSR=0.45), showing the efficiency of the molecular marker used to detect polymorphism within the tea group. In both markers (RAPD and ISSR), studied samples were slightly different in similar groupings. All 20teagenotypes were classified into three and five main groups by the usage of RAPD and ISSR (respectively) with some differences.

This study represented the first attempt to use two molecular markers, RAPD and ISSR, to study the genetic diversity of 20 Iranian tea genotypes from three different regions of Iran that their relationships were somewhat clarified. The results from this study also open a door to tackle the long-standing problem of tea classification and identification in this country. However, we suppose that this kind of researchers needs to be continued since Iran has a very large and various tea germplasm because of the propagation method, it is being propagated by seed, which gives researchers a chance to find new genotypes, which should be classified, investigated and introduced as a new cultivar.

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Conflict of Interest
The authors declare that they have no conflict of interest.

REFERENCES

Balasaravanan, T., Pius, P. K., Kumar, R. R., Muraleedharan, N., and Shasany, A. K. (2003). Genetic diversity among south Indian tea germplasm (*Camellia sinensis*, *C. assamica* and *C. assamica* spp. lasiocalyx) using AFLP markers. *Plant Science. 165*(2). 365-372. https://doi.org/10.1016/S0168-9452(03)00196-1

Ben-Ying, L. I. U., You-Yong, L. I., Yi-Chun, T. A. N. G., Li-Yuan, W. A. N. G., Cheng, H., & Ping-Sheng, W. A. N. G. (2010). Assessment of genetic diversity and relationship of tea germplasm in Yunnan as revealed by ISSR markers. *Acta Agronomica Sinica*, *36*(3), 391-400. https://doi.org/10.1016/S1875-2780(09)60037-7

Beris, F. S., Pehlivan, N., Kac, M., Haznedar, A., Coşkun, F., & Sandalli, C. (2016). Evaluation of genetic diversity of cultivated tea clones (*Camellia sinensis* (L.) Kuntze) in the eastern black sea coast by inter-simple sequence repeats (ISSRS). *Genetika*, *48*(1), 87-96. https://doi.org/10.2298/GENSR1601087B

Beris, F. S., Sandalli, C., Canakci, S., Demirbag, Z., & Belduz, A. O. (2005). Phylogenetic analysis of tea clones (*Camellia sinensis*) using RAPD markers. *Biologia*, *60*, 457-461.

Chang, H. T. (1984). A revision of the tea resource plants. *Acta Sientiarum Naturalium Universitatis Sunyatseni*, *106*, 1–12.

Chen, L., Gao, Q. K., Chen, D. M., & Xu, C. J. (2005). The use of RAPD markers for detecting genetic diversity, relationship and molecular identification of Chinese elite tea genetic resources [*Camellia sinensis* (L.) O. Kuntze] preserved in a tea germplasm repository. *Biodiversity and Conservation*, *14*(6), 1433-1444. https://doi.org/10.1007/s10531-004-9787-y

Chen, L., & Yamaguchi, S. (2002). Genetic diversity and phylogeny of tea plant (*Camellia sinensis*) and its related species and varieties in the section Thea genus *Camellia* determined by randomly amplified polymorphic DNA analysis. *The Journal of Horticultural Science and Biotechnology*, *77*(6), 729-732. https://doi.org/10.1080/14620316.2002.11511564

Chen, L., Yu, F., & Tong, Q. (2000). Discussions on phylogenetic classification and evolution of Sect. Thea. *Journal of Tea Science*, *20*(2), 89-94.

Devarumath, R., Nandy, S., Rani, V., Marimuthu, S., Muraleedharan, N., & Raina, S. (2002). RAPD, ISSR and RFLP fingerprints as useful markers to evaluate genetic integrity of micropropagated plants of three diploid and triploid elite tea clones representing *Camellia sinensis* (China type) and *C. assamica* ssp. assamica (Assam-India type). *Plant Cell Reports*, *21*(2), 166-173. https://doi.org/10.1007/s00299-002-0496-2

Diversityarrays. (2007). http://www.diversityarrays.com/sites/default/files/pub/DArT_DNA_isolation.pdf bfw. ac. at/ 200/ 1859. Html

Ji, P. Z., Li, H., Gao, L. Z., Zhang, J., Cheng, Z. Q., & Huang, X. Q. (2011). ISSR diversity and genetic differentiation of ancient tea (*Camellia sinensis* var. assamica) plantations from China: implications for precious tea germplasm conservation. *Pakistan Journal of Botany 43*(1):281-291.

Kafkas, S., Ercişli, S., Doğan, Y., Ertürk, Y., Haznedar, A., & Sekban, R. (2009). Polymorphism and genetic relationships among tea genotypes from turkey revealed by amplified fragment length polymorphism markers. *Journal of the American Society for Horticultural Science*, *134*(4), 428-434. DOI: 10.21273/JASHS.134.4.428

Kaundun, S. S., Zhyvoloup, A., & Park, Y. G. (2000). Evaluation of the genetic diversity among elite tea (*Camellia sinensis* var. sinensis) accessions using RAPD markers. *Euphytica*, *115*(1), 7-16. https://doi.org/10.1023/A:1003939120048

Jahangirzadeh S., Gonbad, R. A., & Falakro, K. (2020). Identification of genetic diversity and relationships of some Iranian tea genotypes using SRAP markers. *Journal of Horticulture and Postharvest Research*, *3*(1), 25-34. doi: 10.22077/JHPR.2019.2582.1067

Lai, J. A., Yang, W. C., & Hsiao, J. Y. (2001). An assessment of genetic relationships in cultivated tea clones and native wild tea in Taiwan using RAPD and ISSR markers. *Botanical Bulletin of Academia Sinica*, *42*, doi:10.7016/BBAS.200104.0093

Liu, B. Y., Wang, L. Y., Li, Y. Y., He, W., Zhou, J., Wang, P. S., & Cheng, H. (2009). Genetic diversity in tea (*Camellia sinensis*) germplasms as revealed by ISSR markers. *Indian Journal of Agricultural Sciences*, *79*(9), 715-721.

Ma, J. Q., Yao, M. Z., Ma, C. L., Wang, X. C., Jin, J. Q., Wang, X. M., & Chen, L. (2014). Construction of a SSR-based genetic map and identification of QTLs for catechins content in tea plant (*Camellia sinensis*). *PloS One*, *9*(3), e93131. https://doi.org/10.1371/journal.pone.0093131

Mantel, N. (1967). The detection of disease clustering and a generalized regression approach. *Cancer Research*, *27*(2 Part 1), 209-220.

Mishra, R. K., & Sen-Mandi, S. (2004). Genetic diversity estimates for Darjeeling tea clones based on amplified fragment length polymorphism markers. *Journal of Tea Science*, *24*(2), 86-92. doi: 10.13305/j.cnki.jts.2004.02.003

Paul, S., Wachira, F. N., Powell, W., & Waugh, R. (1997). Diversity and genetic differentiation among populations of Indian and Kenyan tea (*Camellia sinensis* (L.) O. Kuntze) revealed by AFLP markers. *Theoretical and Applied Genetics*, *94*(2), 255-263. https://doi.org/10.1007/s001220050408

Rani, A., Singh, K., Ahuja, P. S., & Kumar, S. (2012). Molecular regulation of catechins biosynthesis in tea [*Camellia sinensis* (L.) O. Kuntze]. *Gene*, *495*(2), 205-210. doi: 10.1016/j.gene.2011.12.029

Rohlf, F.J. (1998) *NTSYS-pc Numerical Taxonomy and Multivariate Analysis System, Exeter Software*. Setauket. New York.

Roldain-Ruiz, I., Calsyn, E., Gilliland, T. J., Coll, R., Van Eijk, M. J. T., & De Loose, M. (2000). Estimating genetic conformity between related ryegrass (*Lolium*) varieties, 2. AFLP characterization. *Molecular Breeding*, *6*, 593-602. https://doi.org/10.1023/A:1011398124933

Roy, S. C., & Chakraborty, B. N. (2009). Genetic diversity and relationships among tea (*Camellia sinensis*) cultivars as revealed by RAPD and ISSR based fingerprinting. *Indian Journal of Biotechnology*, *8(4)*, 370-376.

Thomas, J., Vijayan, D., Joshi, S. D., Lopez, S. J., & Kumar, R. R. (2006). Genetic integrity of somaclonal variants in tea (Camellia sinensis (L.) O Kuntze) as revealed by inter simple sequence repeats. *Journal of Biotechnology*, *123*(2), 149-154. DOI: 10.1016/j.jbiotec.2005.11.005

Ueno, S., & Tsumura, Y. (2009). Development of microsatellite and amplicon length polymorphism markers for *Camellia japonica* L. from tea plant (*Camellia sinensis*) expressed sequence tags. *Molecular Ecology Resources*, *9*(3), 814-816. doi:10.1111/j.1755-0998.2008.02316.x

Yao, M. Z., Chen, L., & Liang, Y. R. (2008). Genetic diversity among tea cultivars from China, Japan and Kenya revealed by ISSR markers and its implication for parental selection in tea breeding programmes. *Plant Breeding*, *127*(2), 166-172. https://doi.org/10.1111/j.1439-0523.2007.01448.x