JOURNAL OF HORTICULTURE AND POSTHARVEST RESEARCH
2021, VOL. 4(1), 25-36

Journal of Horticulture and Postharvest Research

Journal homepage: www.jhpr.birjand.ac.ir

University of Birjand

PCR-RFLP analyses of chloroplast DNA in some cultivated tea (*Camellia* sp.) genotypes

Shahin Jahangirzadeh Khiavi[1*], Koorosh Falakro[1] and Sanam Safaei Chaeikar[1]

1, *Tea Research Center, Horticultural Sciences Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Lahijan, Iran*

ARTICLE INFO

Original Article

Article history:
Received 19 January 2020
Revised 15 March 2020
Accepted 9 April 2020
Available online 26 June 2020

Keywords:
cpDNA
CAPs method
Genetic diversity
Morphological characters

DOI: 10.22077/jhpr.2020.3116.1121
P-ISSN: 2588-4883
E-ISSN: 2588-6169

***Corresponding author:**
Tea Research Center, Horticultural Sciences Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Lahijan, Iran.
Email: shjahangirzadeh@gmail.com

ABSTRACT

Purpose: A significant number of genetic resources of *Camellia sinensis* and its allied genotypes have been collected and preserved in Iran TRC. Information about them is mostly based on morphological data. **Research method:** PCR-RFLP technique and morphological characters were used for the identification of organelle DNA (cpDNA) diversity in 25 tea genotypes. Twenty-one qualitative and quantitative characteristics were evaluated. **Findings:** A pair-wise similarity among the samples ranged from 0.14 to 0.66 based on morphological data. The dendrogram was designed, and samples were grouped into three main clusters at 0.38 similarity. Using three universal primer pairs which introduced for chloroplast amplified about 4070bp of cpDNA, following the digestion of fragments with three restriction endonucleases (*HinfI*, *AluI* and *PstI*) and the result of this method was introduced six haplotypes. The most significant and widespread haplotype was H2 (frequency ≈ 28%). All of the detected mutations were insertion-deletions and they ranged from 30 to 60 bp. The calculated total cpDNA diversity in populations (h_T), a major portion of it was within populations were (h_S) and genetic differentiation among populations (G_{ST}) were 0.43, 0.17 and 0.61, respectively. It should have been noted that the calculated G_{ST} was low and no structure could be identified. **Limitations:** Applying allied species and using more potent markers such as cpSSR and sequencing can lead to more accurate results. **Originality/Value:** The results of this study indicate that the PCR-RFLP method and morphological characters are applicable in the identification of tea genotypes and cultivars. In studying *Camellia* genus phylogeny, the polymorphism in cpDNA has to be considered carefully.

INTRODUCTION

Tea is the most popular non-alcoholic soft and healthy beverage across the world (Chen et al., 2005a). Tea plants were originated from southwestern China, Yunnan province (Hasimoto & Simura, 1978; Fulian, 1986). In the genus of *Camellia*, tea (*C. sinensis*) has the most important commercial and ecological role. 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. Presently, tea genetic pool is one of the most essential materials for breeding plans, biotechnology investigation and in the future, it has precious potential for the whole tea industry. A significant number of genetic resources of tea, including the *Camellia sinensis* and its allied species and varieties, have been collected and preserved in China (Chen & Yamaguchi, 2002), Japan (Takeda, 2000), India, Kenya, etc. and newly it started in Iran. The success of tea genetic resource collection, preservation, exploitation, utilization, present, and long-term breeding programs depend primarily on the knowledge and understanding of the genetic background, diversity, relationship, and identification (Chen et al., 2005a).

Many studies were done on nuclear DNA in tea plants (Falakro & Jahangirzadeh Khiavi, 2020; Khiavi et al., 2020; Beris et al., 2005; Kafkas et al., 2009; Fang et al., 2012; Yang et al., 2016), but organelle DNA was not well investigated.

Because of the non-Mendelian mode of inheritance of the chloroplast genome, available information about it is one of the significant interests in phylogeny and population genetics study (Sugiura, 2005). It has been identified that chloroplast was inherited from the mother with approximately one-third having some degree of biparental inheritance (Whatley, 1982; Ishikawa et al., 1992) spatially in many plants (Mariac et al., 2000) and most angiosperms. The chloroplast DNA of angiosperm is a single circular molecule, mostly ranged from 120 to 150 kb in length. This circular genome is made up of large single-copy regions and small single-copy regions which separated from each other by two inverted repeats (IRa and IRb). Most of the difference in genome size between species is due to changes in the size of the IR regions. The entire structure of the chloroplast, along with the gene content, is well conserved among the higher plants, despite the loss of inverted repeat in some species and existence of some variations in gene content (Downie & Palmer, 1992). Sequencing data showed that the substitutions rate of nucleotide in the chloroplast genome is conservative relative to plant nuclear genes (Wolfe et al., 1987). A small amount of genetic diversity observed in the genome of chloroplast compared to the nuclear genome led the researchers to suggest that restriction fragment length polymorphism of amplified fragment (PCR-RFLP) of cpDNA could be useful in constructing molecular phylogenies (Palmer & Zamir, 1982; Palmer et al., 1985; Sytsma & Gottlieb, 1986). This method is a helpful technique for studying inter and intra-specific phylogenetic relationships in plants (Palmer, 1985; Liston, 1992; Amane et al., 2000).

Thus, universal primer pairs are designed to amplify some regions of this molecule (Taberlet et al., 1991; Demesure et al., 1995; Dumolin-Lapegue et al., 1997; Fofana et al., 1997; Heinze, 1998; Weising & Gardner, 1999; Grivet et al., 2001). Digesting of these amplified genome regions in many investigations on chloroplast DNA variation has been performed to understand phylogeny and population relationship (Cros et al., 1998; Panda et al., 2003; Turkec et al., 2006; Tanikawa et al., 2008; Kaundun & Matsumoto, 2011; Golein et al., 2012; Chen et al., 2012; Khiavi et al., 2013; Khadivi-khub et al., 2014).

In this study, we used PCR-RFLP markers to characterize the chloroplast genome in tea genotypes from different regions of Iran, for both commercial and selected clones from the breeding program. Molecular markers were used to study the genetic variation and phylogenetic relationships among Iranian tea genotypes.

MATERIALS AND METHODS

Plant material and DNA extraction

Fourteen germplasm samples of Iranian tea from the Tea research center, and 11 imported samples were used in this study (Table 1). These samples belonged to four different regions (Japan, Sri Lanka, Georgia and Iran). Accessions from Japan and Sri Lanka were selected from clones, but other samples were chosen from genotypes which there are not any information about them.

Morphological Analysis

Twenty-one qualitative and quantitative characteristics were evaluated using shrubs and leaves (Table 2). The selection of morphological characters was made based on the descriptors developed by the International Plant Genetic Resources Institute (IPGRI, 2000). The YBAR option of the Stand program from the NTSYS-pc 2.1 software was used for morphological data standardization (Rohlf, 1993). For each sample, duplicate measurements were averaged, and the data matrix of pair-wise similarities between genotypes was designed. The similarity was measured by a simple matching coefficient (SMC), as it was the coefficient with the best results following a cophenetic test (Mantel, 1967).

DNA Isolation and PCR-RFLP Analysis

Young and full expanded leaves were selected and stored at -80°C until they were used to study. Total DNA (nuclear and organelle) was isolated using the procedure described by the Dellaporta method (Dellaporta et al., 1983) with minor modifications. The quantity and quality of DNA were investigated by the spectrophotometric method and agarose gel electrophoresis.

Table 1. Studied genotypes and clones, their origins, places of collection, and determined haplotypes

NO.	Sample name	Origin	Place of collection	Haplotype
G1	183	Iran	Tea research Center	H1
G2	277	Iran	"	H1
G3	399	Iran	"	H1
G4	100	Iran	"	H2
G5	121	Iran	"	H2
G6	160	Iran	"	H2
G7	256	Iran	"	H2
G8	262	Iran	"	H2
G9	269	Iran	"	H2
G10	444	Iran	"	H2
G11	25	Iran	"	H3
G12	114	Iran	"	H3
G13	270	Iran	"	H3
G14	591	Iran	"	H3
G15	Sayama Kaori	Japan	"	H4
G16	Yabokita	Japan	"	H4
G17	3015	Sri Lanka	"	H1
G18	3020	Sri Lanka	"	H5
G19	DG39	Sri Lanka	"	H5
G20	DG2.5	Sri Lanka	"	H5
G21	KEN	Sri Lanka	"	H5
G22	DN	Sri Lanka	"	H3
G23	101 large leaf	Georgia	"	H6
G24	101 small leaf	Georgia	"	H6
G25	102a	Georgia	"	H6

Table 2. Investigated morphological characters

Row	characters	Row	characters
1	Internode length	12	Length/ Width of mature leaf
2	Pigmentation in young leaves (In growth season)	13	Length of mature leaf
3	Pigmentation in young leaves (In dormant season)	14	Width of mature leaf
4	Immature leaf color	15	Leaf angle
5	Mature leaf color	16	Leaf venation
6	Leaf shape	17	Leaf pose (angle)
7	Leaf upper surface	18	Leaf waxiness
8	Leaf apex shape	19	Petiole color
9	Leaf apex habit	20	Length of mature leaf petiole
10	Leaf base shape	21	Young shoot colour
11	Leaf margin		

Table 3. Chloroplast universal primers, their sequence, PCR conditions, size of amplified fragment, and quality of amplification, applied on tea accession in this study

Abbrev. of cpDNA primers*	Sequence (5'--3')	PCR conditions		Reported amplified fragment size in tobacco** (bp)	Amplified fragment size (bp)	Degree of amplification***
		Annealing temperature (°C)	Extension time (min.)			
DT	F:ACCAATTGAACTACAATCCC R:CTACCACTGAGTTAAAAGGG	55	2	1213	1160	++
LF	F:CGAAATCGGTAGACGCTACG R:ATTTGAACTGGTGACACGAG	56	2	1050	1030	++
HK	F:ACGGGAATTGAACCCGCGCA R:CCGACTAGTTCCGGGTTCGA	55	2	1831	1880	++
B1B2	F:TGCCTTGGTATCGTGTTCATAC R:CYTGTCTTYTTGTAGTTGGAT	54	3	1512	-	NA
SfM	F:GAGAGAGAGGGATTCGAACC R:CATAACCTTGAGGTCACGGG	62	2	1254	-	NA

*Abbreviations are the same as in Dumolin-Lapegue et al. (1997) and Grivet et al. (2001).
** The Amplified fragments reported size in tobacco was from Grivet et al. (2001).
*** NA: no amplification; ++: good amplification.

Five sets of universal primer pairs for chloroplast DNA (DT, LF, HK, B1B2, and SfM) were tested to amplify chloroplast DNA (Dumolin-Lapegue et al., 1997; Grivet et al., 2001). Some sets showed no or faint amplification, so the three sets of chloroplast primers were chosen for this research. Primer sequences are listed in Table 3. The amplification of cpDNA regions were performed in 30µl of reaction mixture containing 30ng of total DNA, ten ng of each primer pair, 200µM of each four dNTPs, one unit Taq DNA polymerase (recombinant, Fermentas, Canada), 2mM of MgCl2 and 1X PCR buffer with KCl. The thermocycler system used was the Bio-Rad PCR system. The PCR was carried out in condition that described by Khiavi et al. (2013) (Table 3), using an initial cycle of 3 min at 94°C, followed by 35 cycles of 45 s at 94°C, 1 min at 54.5°C to 58.5°C and 2 or 3 min at 72°C (annealing temperature and extension time depending on the primer and the length of the fragment to be amplified; Table 3), and finally a 10 min extension at 72°C.

The PCR-amplified DNA fragments were digested with the restriction endonucleases *Hinf*I, *Alu*I, and *Pst*I (Fermentas, Canada) at 37ºC for 8h. The mixture of digestion consisted of 5µl PCR products, 5 units of restriction enzyme (0.5µl), 23.5µl H2O, and 2µl digestion buffer (10X). The digested DNA fragments were separated by electrophoresis on 3% agarose gels in TBE buffer at 70V for 4 hours and stained with ethidium bromide. After it, fragments were visualized in UV light. The sizes of markers used for analyzing the size of polymorphic bands were 1kb and 50bp ladders (Fermentas, Canada). Figure 1 showed amplified fragment

by primer DT (a) and HK (b) and the cutting pattern of two Primer-Restriction enzyme combinations HK/*Hinf*I (c) and SC/*Alu*I (d).

NTSYS pc (Rohlf, 1993) and POPGENE (Weir, 1996) were used, and a dendrogram was drawn by UPGMA clustering analysis using Jaccard's coefficient for the statistical analysis of the results. Haplotypes were identified using Khiavi et al. (2013) method.

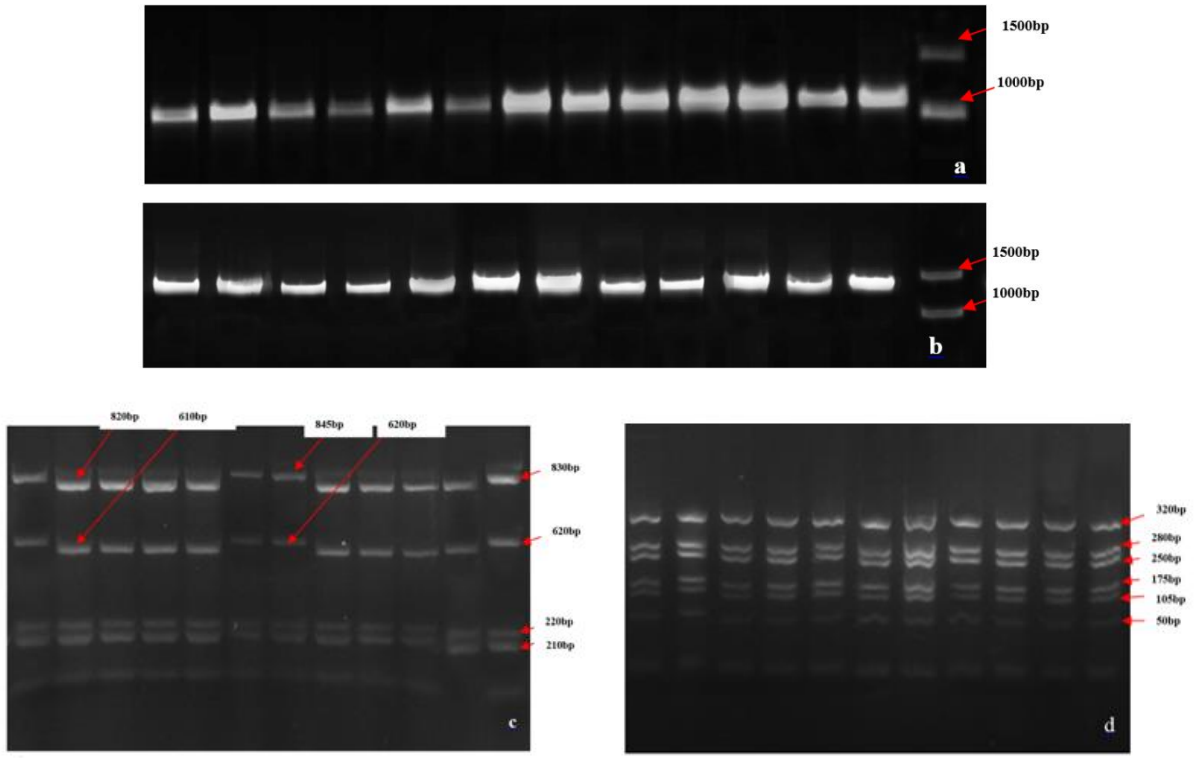

Fig. 1. Amplified fragment by primer DT (a) and HK (b) and the digested pattern of two Primer-Restriction enzyme combinations HK/*Hinf*I (c) and SC/*Alu*I (d) were shown in agarose gel.

RESULTS AND DISCUSSION

Morphological Analysis

Comparative analysis of 21 morphological characteristics in *Camellia* genotypes and clones showed moderate variations. A pair-wise similarity among the samples ranged from 0.14 to 0.67, with an average of 0.45 based on morphological data. The highest similarity (0.66) was observed between "genotype 269 (G9) and clone Sayama Kaori from Japan (G15), genotype 270 (G13) and clone DG39 from Sri Lanka (G19) and two Japanese clones (Sayama Kaori and Yabokita)". At the same time, the lowest (0.14) was found among an imported clone from Georgia, 101 large leaf, (G23) and selected genotypes "G3 or 399. In the previous study (not published), it was clarified that Iranian cultivated tea was imported from India, so this difference was predictable. According to these results, it could be understood that morphological characters can distinguish samples.

The cophenetic analyses comparing the UPGMA cluster analysis and the simple matching similarity matrix demonstrated that the correlation was 0.67, indicating that data in the matrix was well represented by the dendrogram. Table 4 shows the calculated cophenetic correlation. The most significant number comparing the coefficient matrix and cophenetic matrix indicate better fitting for the cluster and similarity matrix.

Dendrogram, which was generated based on morphological parameters, grouped samples into three main clusters at 0.38 similarity coefficient (Fig. 2). Cluster one (A) which, was separated from other groups by ≈0.36 coefficient similarity, has five individual members, G1, g5, G11, G14, and G23, that G23 is originated from Georgia and other members are natural hybrids from Iran tea germplasm, without any information about their origins and parents. Interestingly, genotypes 23 and 11 were separated from other members of the group (at similarity level 0.39). Cluster two (B) just has two individual members (G2 and G22), G22 is originated from Sri Lanka, and G2 is a natural hybrid from Iran tea germplasm.

Cluster 3 (C) was the largest group and cover 72 percent of all study samples; this group at the similarity of 0.44 formed three subgroups. Subgroup one (C-1) has five members, in this subgroup; clone KEN from Sri Lanka and four Iranian genotypes were located. Subgroup two (C-2) has six members; three of these members belong to Iran germplasm, two from Japan, and one belong Georgia (G25: 102a). Subgroup three (C-3) has five members; four of these members belong to Sri Lanka imported clone and one from Iran germplasm (G13: 270). Subgroup four (C-4) has two members, one of these members belongs to Iranian germplasm (G7: 256) and another one from Georgia (G24: 101 small leaf). Based on clustering results, the distribution of samples does not follow the geographical distribution, which returns to the origin of the tea plants (Chen et al., 2005b) and the influence of the environment on morphology.

Table 4. Calculated cophenetic correlation in morphological analyses

	Jaccard	DICE	simple matching
UPGMA	0.67	0.66	0.67.

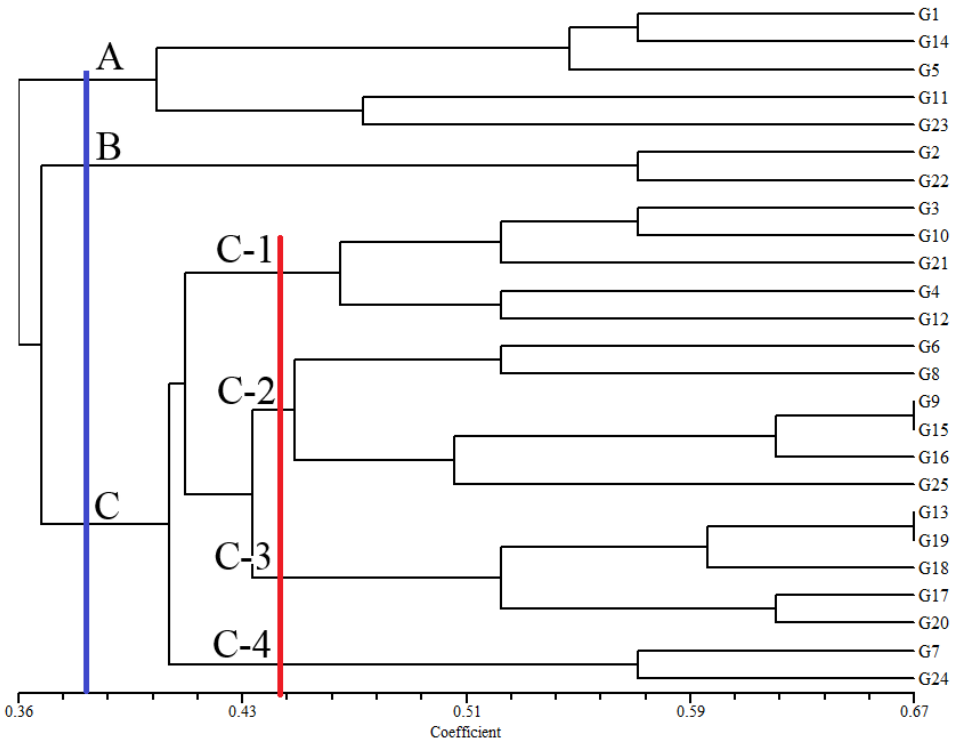

Fig. 2. Dendrogram generated from morphological characteristics of 25 of tea samples and cultivars based on SM coefficient using the UPGMA method.

PCR-RFLP Analyses

From five universal chloroplast primer pairs tested on *Camellia* spp. (DT, LF, HK, B1B2, and SfM), only primer pair B1B2 did not amplify any band in this work. Other primer pairs amplified the fragments, and primer pair SfM amplified several fragments in one PCR reaction. All used primers amplified 4600bp approximately in all PCR reactions.

From nine combinations of primer-restriction enzyme used in this study for CAP analysis of cpDNA, only four combinations (DT/*Hinf*I, DT/*Alu*I, LF/*Pst*I, and HK/*Hinf*I) showed polymorphic patterns and other combinations had a monomorphic pattern or did not have any restrict site. Three combinations (DT/*Hinf*I, DT/*Alu*I, and LF/*Pst*I) showed two polymorphic patterns, but in combination with HK/*Hinf*I, having two fragments that showed a polymorphic pattern, the first fragment had three polymorphic patterns and the second fragments showed two polymorphic patterns. All of these polymorphic patterns are because of insertion-deletion (indel) mutation between 30 to 60bp ranges, and the same range of mutation was reported by khiavi et al. (2013) in PCR-RFLP analyses of *Malus* spp in Iran.

According to the polymorphic pattern, all genotypes were grouped into six haplotypes (Table 1). The members of haplotypes were from two to seven. From the six haplotypes that were identified, only haplotype H2 (the largest identified haplotype) was specific for Iran population, the important point which should be noted was H1 and H3 (each haplotype) had one member from Sri Lanka, and other members were from Iran (see Table 1). The remaining haplotypes, each of which was special in a particular region, Haplotype 2 (H2) was specific for Japan population, haplotype 5 (H5) was specific for the Sri Lanka population and haplotype 6 (H6) for Georgia population.

Total diversity (Ht), and a major portion of diversity is within populations (Hs), and the level of population subdivision (Gst) calculated by the POPGENE program was 0.43, 0.17, and 0.61, respectively (Table 5). The same results were reported by Khiavi et al. (2013) with an investigation of cpDNA of *Malus* spp (Ht= 0.467, Hs=0.445) that approve the conservation of cpDNA in plants. According to the genetic differentiation among populations in *Camellia*s pp (Gst=0.61), it could be determined that genetic differentiation among populations in present study (Gst) is low and is much smaller than forest species such as *Quercus petraea* (Gst =0.90; Petit et al., 1993), *Fagus sylvatica* (Gst =0.83; Demesure et al., 1996), *Argania spinosa* (Gst =0.60; EL Mousadik & Petit, 1996) and *Alnus glutinosa* (Gst =0.87; Andrew King & Ferris, 1998). From this value of Gst, it can be stated that four different populations (Iran, Georgia, Japan, and Sri Lanka) have different evolutionary paths.

Petit et al. (1993) reported that in some species, the Gstn (genetic differentiation among populations using nuclear markers) is much lower than the Gstc. It could be deduced that cytoplasmic genomes that have an inheritance from maternal parents are relatively much more structured than the nuclear genome because cytoplasmic gene flow is limited just by seed dispersal.

For cluster analyses of the investigated samples, the results of the cophenetic test (Mantel, 1967) revealed that the Jaccard coefficient method and UPGMA algorithm were the best tools to construct a dendrogram. The calculated cophenetic coefficient shows %96.6 of data with a similarity matrix (Table 6). In the similarity matrix of the Jaccard coefficient, the average of calculated similarities was 0.42. It seems that this amount of deliberate similarity was low, but it should be noted that we used just polymorphic patterns in the study. Therefore, differences become highlighted.

In cluster analysis, all haplotypes were separated at a similarity level of 0.67. First, two haplotypes H1 and H2 were separated from four other haplotypes at a similarity level of 0.18 and preceded with their evolutionary path. Haplotype 2 (H2) was specific for Iran population,

and haplotype 1 (H1) had one member from the population of Sri Lanka; then, it could note that it was Iranian specific haplotypes.

Table 5. Nei's analysis of gene diversity in subdivided populations

Combination	Digestion pattern	Sample size	na	ne	h	I	Ht	Hs	Gst
DT/*Hinf*I	A	25	2.00	1.97	0.49	0.69	0.41	0.24	0.43
DT/*Hinf*I	B	25	2.00	1.97	0.49	0.69	0.41	0.24	0.43
DT/*Alu*I	A	25	2.00	2.00	0.50	0.69	0.50	0.17	0.66
DT/*Alu*I	B	25	2.00	2.00	0.50	0.69	0.50	0.17	0.66
LF/*Pst*I	A	25	2.00	2.00	0.50	0.69	0.50	0.24	0.52
LF/*Pst*I	B	25	2.00	2.00	0.50	0.69	0.50	0.24	0.52
HK/*Hinf*I	A	25	2.00	1.85	0.46	0.65	0.44	0.11	0.75
HK/*Hinf*I	B	25	2.00	1.37	0.27	0.44	0.28	0.11	0.60
HK/*Hinf*I	C	25	2.00	1.47	0.32	0.50	0.50	0.00	1.00
HK/*Hinf*I	A	25	2.00	1.97	0.49	0.69	0.34	0.17	0.50
HK/*Hinf*I	B	25	2.00	1.97	0.49	0.69	0.34	0.17	0.50
Mean	-	25	2.00	1.87	0.46	0.65	0.43	0.17	0.61
St. Dev	-	-	0.00	0.23	0.08	0.09	0.01	0.01	-

Table 6. Calculated cophenetic correlation in PCR-RFLP analyses

	Jaccard	DICE	simple matching
UPGMA	0.966	0.924	0.877.

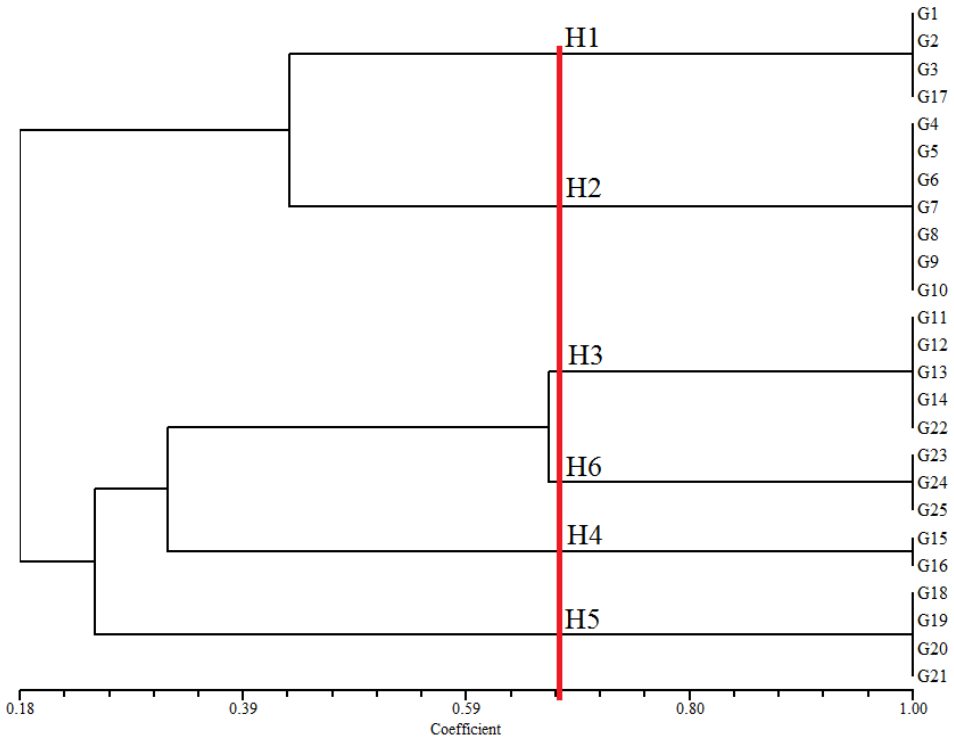

Fig. 3. Dendrogram generated from PCR-RFLP data of 25 tea samples and clones based on Jaccard coefficient using the UPGMA method.

In the case of the other four haplotypes, at 0.23 haplotype 1 (H1) (haplotype-specific Sri Lanka), the H4 haplotype (haplotype-specific Japan) was dissociated at a similarity of 0.28. The highest level of similarity was detected between two H3 and H6, which were separated from each other at 67% similarity. Figure 3 shows the dendrogram generated from PCR-RFLP data of 25 tea samples and clones based on the Jaccard coefficient using the UPGMA method.

Table 7 shows Nei's genetic identity and genetic distance. As can be seen from Table 7, the level of similarity between populations is moderate. The highest level of similarity was between two populations of Iran and Sri Lanka (0.698), and the most top difference was between two populations of Georgia and Iran (0.776). Figure 4 shows the dendrogram generated from PCR-RFLP data of 4 populations of tea samples and clones based on genetic di stance using the UPGMA method.

Table 7. Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

pop ID	Iran	Japan	Sri Lanka	Georgia
Iran	****	0.5554	0.6985	0.4602
Japan	0.588	****	0.561	0.6364
Sri Lanka	0.3588	0.5781	****	0.6358
Georgia	0.7761	0.452	0.4529	****

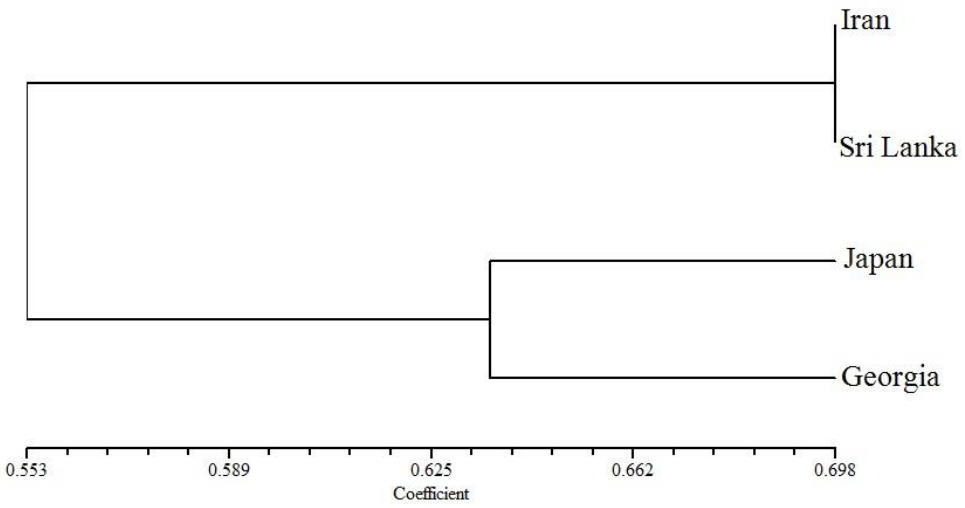

Fig. 4. Dendrogram generated from PCR-RFLP data of 4 populations of tea samples and clones based on genetic similarity using the UPGMA method.

CONCLUSIONS

The results of this study showed that there is no genetic structure among the samples of the study area. These results also confirmed that it is possible to use morphological traits and the PCR-RFLP method of chloroplast genome to investigate the variation between tea and its allied genotypes. Of course, to achieve accurate results, more markers and enzymes need to be used, and the morphological trait is investigated several times and years. By using these methods, genetic variation was observed in the tea genotypes, but this diversity was not such as to be able to distinguish the genotypes of different regions. The results of this study also showed that the genotypes of tea cultivated in Iran have high genetic diversity since most of

them were propagated sexually, and tea plants have self-incompatibility. Hence, the seed had to be results of cross-pollination.

Conflict of interest

The authors have no conflict of interest to report.

REFERENCES

Amane, M., Ouazzani, N., Lumaret, R., & Debain, C. (2000). Chloroplast-DNA variation in the wild and cultivated olives (*Olea europaea* L.) of Morocco. *Euphytica*, *116*(1), 59-64. https://doi.org/10.1023/A:1004025431960

Andrew King, R., & Ferris, C. (1998). Chloroplast DNA phylogeography of *Alnus glutinosa* (L.) Gaertn. *Molecular Ecology*, *7*(9), 1151-1161. https://doi.org/10.1046/j.1365-294x.1998.00432.x

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

Chen, L., Gao, Q. K., Chen, D. M., & Xu, C. J. (2005a). 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, S. X., Qi, G. N., Li, H., Shan, H. L., & Zou, Y. (2012). Rapid establishment of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) system for chloroplast DNA in tea [*Camellia sinensis* (L.) O. Kuntze]. *African Journal of Biotechnology*, *11*(33), 8181-8188. http://dx.doi.org/10.5897/AJB11.4167

Chen, J., Wang, P., Xia, Y., Xu, M., & Pei, S. (2005b). Genetic diversity and differentiation of *Camellia sinensis* L. (cultivated tea) and its wild relatives in Yunnan province of China, revealed by morphology, biochemistry and allozyme studies. *Genetic Resources and Crop Evolution*, *52*(1), 41-52. https://doi.org/10.1007/s10722-005-0285-1

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

Cros, J., Combes, M. C., Trouslot, P., Anthony, F., Hamon, S., Charrier, A., & Lashermes, P. (1998). Phylogenetic analysis of chloroplast DNA variation in *Coffea* L.. *Molecular Phylogenetics and Evolution*, *9*(1), 109-117. http://dx.doi.org/10.1006/mpev.1997.0453

Dellaporta, S. L., Wood, J., & Hicks, J. B. (1983). A plant DNA minipreparation: version II. *Plant Molecular Biology Reporter*, *1*(4), 19-21. https://doi.org/10.1007/BF02712670

Demesure, B., Comps, B., & Petit, R. J. (1996). Chloroplast DNA phylogeography of the common beech (*Fagus sylvatica* L.) in Europe. *Evolution*, *50*(6), 2515-2520. https://doi.org/10.1111/j.1558-5646.1996.tb03638.x

Demesure, B., Sodzi, N., & Petit, R. J. (1995). A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. *Molecular Ecology*, *4*(1), 129-134. https://doi.org/10.1111/j.1365-294x.1995.tb00201.x

Downie, S. R., & Palmer, J. D. (1992). Use of chloroplast DNA rearrangements in reconstructing plant phylogeny. In *Molecular systematics of plants*. Springer, Boston, MA. pp. 14-35. https://doi.org/10.1007/978-1-4615-3276-7_2

Dumolin-Lapegue, S., Pemonge, M. H., & Petit, R. J. (1997). An enlarged set of consensus primers for the study of organelle DNA in plants. *Molecular Ecology*, *6*(4), 393-397. https://doi.org/10.1046/j.1365-294x.1997.00193.x

El Mousadik, A., & Petit, R. J. (1996). Chloroplast DNA phylogeography of the argan tree of Morocco. *Molecular Ecology*, *5*(4), 547-555. https://doi.org/10.1111/j.1365-294X.1996.tb00346.x

Falakro, K., & Jahangirzadeh Khiavi, S. (2020). Assessment of genetic diversity and relationships among tea genotypes in Iran based on RAPD and ISSR markers. *Journal of Horticulture and Postharvest Research*, 3(2), 209-220. https://doi.org/10.22077/jhpr.2019.2832.1094

Fang, W., Cheng, H., Duan, Y., Jiang, X., & Li, X. (2012). Genetic diversity and relationship of clonal tea (*Camellia sinensis*) cultivars in China as revealed by SSR markers. *Plant Systematics and Evolution*, *298*(2), 469-483. https://doi.org/10.1007/s00606-011-0559-3

Fofana, B., Harvengt, L., Baudoin, J. P., & Du Jardin, P. (1997). New primers for the polymerase chain amplification of cpDNA intergenic spacers in *Phaseolus phylogeny*. *Belgian Journal of Botany*, *129*(2), 118-122.

Fulian, Y. (1986). Discussion on the originating place and the originating center of tea plant. *Journal of Tea Science*, *6*(1): 1–8.

Golein, B., Bigonah, M., Azadvar, M., & Golmohammadi, M. (2012). Analysis of genetic relationship between 'Bakraee' (*Citrus* sp.) and some known Citrus genotypes through SSR and PCR-RFLP markers. *Scientia Horticulturae*, *148*, 147-153. https://doi.org/10.1016/j.scienta.2012.10.012

Grivet, D., Heinze, B., Vendramin, G. G., & Petit, R. J. (2001). Genome walking with consensus primers: application to the large single copy region of chloroplast DNA. *Molecular Ecology Notes*, *1*(4), 345-349. https://doi.org/10.1046/j.1471-8278.2001.00107.x

Hasimoto, M., & Simura, T. (1978). Morphological studies on the origin of the tea plant. *Japanese Journal of Tropical Agriculture*, *21*(2), 93-101. https://doi.org/10.11248/jsta1957.24.1

Heinze, B. (1998). PCR-based chloroplast DNA assays for the identification of native *Populus nigra* and introduced poplar hybrids in Europe. *Forest Genetics*, *5*(1), 31-38.

IPGRI (2000). Descriptors for tea. International Plant Genetic Resources Institute, Rome, Italy, Available at: http://www.cgiar.org/ipgri.

Ishikawa, S., Kato, S., Imakawa, S., Mikami, T., & Shimamoto, Y. (1992). Organelle DNA polymorphism in apple cultivars and rootstocks. *Theoretical and Applied Genetics*, *83*(8), 963-967. https://doi.org/10.1007/BF00232957

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. https://doi.org/10.21273/JASHS.134.4.428

Kaundun, S. S., & Matsumoto, S. (2011). Molecular evidence for maternal inheritance of the chloroplast genome in tea, *Camellia sinensis* (L.) O. Kuntze. *Journal of the Science of Food and Agriculture*, *91*(14), 2660-2663. https://doi.org/10.1002/jsfa.4508

Khadivi-Khub, A., Jahangirzadeh, S., Ahadi, E., & Aliyoun, S. (2014). Nuclear and chloroplast DNA variability and phylogeny of Iranian apples (*Malus domestica*). *Plant Systematics and Evolution*, *300*(8), 1803-1817. https://doi.org/10.1007/s00606-014-1007-y

Khiavi, S. J., Azadi Gonbad, R., & 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. https://doi.org/10.22077/JHPR.2019.2582.1067

Khiavi, S. J., Zamani, Z., Mardi, M., & Moghdam, M. F. (2013). Evaluation of chloroplast relationship between some apple genotype from Azerbaijan of Iran and their comparison with other local genotypes, cultivars and rootstocks. *African Journal of Agricultural Research*, *8*(1), 106-112. https://doi.org/10.5897/AJAR11.2348

Liston, A. (1992). Variation in the chloroplast genes rpoC1 and rpoC2 of the genus *Astragalus* (Fabaceae): evidence from restriction site mapping of a PCR-amplified fragment. *American Journal of Botany*, *79*(8), 953-961. https://doi.org/10.1002/j.1537-2197.1992.tb13679.x

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

Mariac, C., Trouslot, P., Poteaux, C., Bezançon, G., & Renno, J. F. (2000). Chloroplast DNA extraction from herbaceous and woody plants for direct restriction fragment length polymorphism analysis. *Biotechniques*, *28*(1), 110-113. https://doi.org/10.2144/00281st07

Palmer, J. D. (1985). Comparative organization of chloroplast genomes. *Annual Review of Genetics*, *19*(1), 325-354. https://doi.org/10.1146/annurev.ge.19.120185.001545

Palmer, J. D., Jorgensen, R. A., & Thompson, W. F. (1985). Chloroplast DNA variation and evolution in *Pisum*: patterns of change and phylogenetic analysis. *Genetics*, *109*(1), 195-213.

Palmer, J. D., & Zamir, D. (1982). Chloroplast DNA evolution and phylogenetic relationships in *Lycopersicon*. *Proceedings of the National Academy of Sciences*, *79*(16), 5006-5010. https://doi.org/10.1073/pnas.79.16.5006

Panda, S., Martín, J. P., & Aguinagalde, I. (2003). Chloroplast DNA study in sweet cherry cultivars (*Prunus avium* L.) using PCR-RFLP method. *Genetic Resources and Crop Evolution*, *50*(5), 489-495. https://doi.org/10.1023/A:1023986416037

Petit, R. J., Kremer, A., & Wagner, D. B. (1993). Geographic structure of chloroplast DNA polymorphisms in European oaks. *Theoretical and Applied Genetics*, *87*(1-2), 122-128. https://doi.org/10.1007/BF00223755

Rohlf, F. J. (1993). *Numeric taxonomy and multivariate analysis system*. *NTSYS-pc*.

Sugiura, M. (2005). History of chloroplast genomics. In *Discoveries in Photosynthesis*. Springer, Dordrecht. pp. 1057-1063. https://doi.org/10.1023/A:1024913304263

Sytsma, K. J., & Gottlieb, L. D. (1986). Chloroplast DNA evolution and phylogenetic relationships in Clarkia sect. Peripetasma (Onagraceae). *Evolution*, *40*(6), 1248-1261. https://doi.org/10.2307/2408951

Taberlet, P., Gielly, L., Pautou, G., & Bouvet, J. (1991). Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology*, *17*(5), 1105-1109. https://doi.org/10.1007/BF00037152

Takeda, Y. (2000). *History and development in Japanese tea breeding*. Tea Culture, Tea Food Industry and Tea Breeding in Korea, China and Japan. The Korea Tea Society, Korea, pp. 139-158.

Tanikawa, N., Onozaki, T., Nakayama, M., & Shibata, M. (2008). PCR-RFLP analysis of chloroplast DNA variations in the atpI-atpH spacer region of the genus *Camellia*. *Journal of the Japanese Society for Horticultural Science*, 77(4), 408-417. https://doi.org/10.2503/jjshs1.77.408

Turkec, A., Sayar, M., & Heinze, B. (2006). Identification of sweet cherry cultivars (*Prunus avium* L.) and analysis of their genetic relationships by chloroplast sequence-characterised amplified regions (cpSCAR). *Genetic Resources and Crop Evolution*, 53(8), 1635-1641. https://doi.org/10.1007/s10722-005-2285-6

Weir, B. S. (1996). Interspecific differentiation. In *Molecular systematics. 2nd Edition*. Hillis, D. M. et al. (Eds). Sinauer Associates, Sunderland. pp. 385-403.

Weising, K., & Gardner, R. C. (1999). A set of conserved PCR primers for the analysis of simple sequence repeat polymorphisms in chloroplast genomes of dicotyledonous angiosperms. *Genome*, *42*(1), 9-19.

Whatley, J. M. (1982). Ultrastructure of plastid inheritance: green algae to angiosperms. *Biological Reviews*, *57*(4), 527-569. https://doi.org/10.1111/j.1469-185X.1982.tb00373.x

Wolfe, K. H., Li, W. H., & Sharp, P. M. (1987). Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. *Proceedings of the National Academy of Sciences*, *84*(24), 9054-9058. https://doi.org/10.1073/pnas.84.24.9054

Yang, H., Wei, C. L., Liu, H. W., Wu, J. L., Li, Z. G., Zhang, L., & Zhang, Z. Z. (2016). Genetic divergence between *Camellia sinensis* and its wild relatives revealed via genome-wide SNPs from RAD sequencing. *PLoS One*, *11*(3). e0151424. https://doi.org/10.1371/journal.pone.0151424