UDC 575
DOI: 10.2298/GENSR1601087B
Original scientific paper

EVALUATION OF GENETIC DIVERSITY OF CULTIVATED TEA CLONES (*Camellia sinensis* (L.) KUNTZE) IN THE EASTERN BLACK SEA COAST BY INTER-SIMPLE SEQUENCE REPEATS (ISSRS)

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Beris S. F., N. Pehlivan, M. Kac, A. Haznedar, F. Coşkun, C.Sandalli (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, Vol 48, No. 1, 87-96.

Tea is the most globally consumed drink after spring water and an important breeding plant with high economical value in Turkey. In half a century, various kinds of tea cultivars have been bred in Turkey to improve the quality and yield of tea plants. Since tea reproduces sexually, tea fields vary in quality. Thus, determining the genetic diversity and relationship of the plants to support breeding and cultivation is important. In this study we aimed to determine the genetic diversity of tea cultivars breeding in the Eastern Black Sea coast of Turkey and the genetic relationship between them, to verify whether the qualitative morphological designations of the clones are genetically true by the ISSR markers. Herein, the genetic diversity and relationships of 18 Turkish tea cultivars were determined using 15 ISSR markers with sizes ranging from 250 to 3000 base pairs. The similarity indices among these cultivars were between 0.456 and 0.743. Based on cluster analysis using UPGMA, some of tea cultivars originating from the same geographical position were found to be clustered closely. Our data provide valuable information and a useful basis to assist selection and cloning experiments of tea cultivars and also help farmers to find elite parental clones for tea breeding in the Eastern Black Sea coast of Turkey.

Key words: Genetic polymorphism, PCR based markers, Tea breeding, Turkish tea cultivars, UPGMA clustering

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INTRODUCTION

Tea, made from the leaves of the tea plant, *Camellia sinensis* (L.) Kuntze, Theaceae, is enjoyed by many people across the globe, and is second only to water in popularity as a non-alcoholic caffeine-containing beverage (LI *et al*., 2011). The cultivated taxa of tea consist three natural hybrids, *C. sinensis* or China type, *C. assamica* (Masters) or Assam type and *C. assamica ssp. lasiocalyx* (Planchon ex Watt.) or Cambodian or Southern type (MONDAL *et al.*, 2004). Two types, (*C. sinensis* (China) and *C. assamica* (Assam), are well known and commercially planted around the world (MA *et al.*, 2012). The tea plant called "çay" in Turkish (pronounced Chai) is native to China, Tibet and northern India, but also grows well in the moist and mild climate of the eastern Black Sea coast in Turkey, which has plenty of rainfall and fertile soil. The first tea plantation was started in 1924 in Rize, by seeds transferred from neighboring Georgia. The first large scale cultivation carried out in 1937 with 20 tons of seeds brought from Batum, Georgian Republic, and plantation was conducted in Rize, yielding 30 kilos of tea (AYLANGAN, 2009).

The most important characteristic of tea plants grown in Turkey is that there are no residual pesticides (which are used to fight against insects, fungi, etc.) because of the lack of chemical control and additives used in their cultivation. On the other hand, Turkish tea is especially suited to hilly regions and no other country has snow on this kind of tea cultivation land in winter. These factors limit bacterial growth on tea plants and allow sustainable organic farming. Thus, Turkey is an important tea-producing country, ranked fifth in the world with an annual production of 221,600 metric tonnes (m/t) (Food and Agricultural Organization of the United Nations 2011). In tea-producing countries, the classification of breeding plants by morphological, physiological, biochemical, and molecular techniques is very important. In Turkey, tea plantation areas were established from seeds and the first clonal selection studies based on morphological and biochemical characterization were started 27 years after their establishment (BERIS *et al*., 2005). Traditional clonal identification methods are inadequate to determine the genetic relationships of tea as with other outcrossing crops because the morphological and biochemical traits are influenced by environmental conditions and evolutionary changes within genomic regions. However, molecular techniques, especially those based on DNA markers, provide phylogenetic resolving capacity to determine genetic diversity for plant breeding. There are many such techniques available for tea breeding. Among PCR-based DNA marker systems, the high cost and time demands of microsatellites and AFLP, and the low reproducibility of RAPD are major disadvantages (OLENA *et al*., 2013). However, the ISSR-PCR technique overcomes most of these disadvantages (ZIETKIEWICZ *et al*., 1994, MONDAL 2002, NYBOM, 2004, DAN, 2006, THOMAS *et al*., 2006, ANDERSON *et al*., 2010, KALPANA *et al*., 2012). The ISSR technique is a very simple and quick method to determine genetic diversity (ZIETKIEWICZ *et al*., 1994, MONDAL, 2002, DAN, 2006, CHEN *et al*., 2015). ISSR markers have been widely used to investigate the genetic relationships within and among populations of the genus Camellia in India, China, and Taiwan, as in other plants (MONDAL, 2002, DAN, 2006, THOMAS *et al*., 2006, CHEN *et al*., 2007, YAO *et al*., 2008, ROY and CHAKRABORTY, 2009, BEN-YING *et al*., 2010).

The Ataturk Tea and Horticulture Research Institute was set up in 1924 in Rize, Turkey for agricultural research and development studies, especially on tea, kiwi, citrus, and hazelnut. After 1967, the institute was dedicated to breeding and selection studies of tea plants, and also the improvement of tea yield quality. Currently, the Fener-3, Derepazari-7, Muradiye-10, Tuglali-10, Gundogdu-3, and Pazar-20 clones are cultivated for their morphological, biochemical and phenological characteristics. However, because variations among tea clones exist due to their

sexual reproduction, plantations include mixtures of tea plants of different quality and yield (BERIS *et al*., 2005) and studies of the genetic relatedness of the cultivars grown in Turkey have not been completed magnificently up to date. In present study, ISSR markers were used to (i) determine the genetic diversity of tea cultivars to provide basic genetic information for tea breeding in Turkey, (ii) determine the genetic relationships between tea cultivars from the Institute and other samples spread across tea plantation areas in Turkey, and (iii) verify whether the morphological designations of the clones by the Institute are genetically true.

MATERIALS AND METHODS

Plant Materials

In total, 19 tea genotypes originating from the Rize and Trabzon provinces of Turkey were used in the present study. Ten tea clones were provided by the Rize Ataturk Tea and Horticulture Research Institute in the eastern Black Sea region of Turkey. These ten clones were previously selected for their biochemical and phenologycal characteristics and have been vegetatively propagated at the Ataturk Tea and Horticulture Research Institute for approximately 80 years (Table 1).

Table 1. Description of tea cultivars characterized by ISSR analysis and codes used in the present investigation, their province of origin in the Black Sea region of Turkey The tea cultivars studied and the in the present study

No	Tea genotypes	Province of origin	Varieties
1 (D7)	Derepazari 7	Rize	AssamxChina
2 (P20)	Pazar 20	Rize	AssamxChina
3 (M)	Muradiye	Rize	AssamxChina
4 (G)	Gundogdu	Rize	AssamxChina
5 (F3)	Fener 3	Rize	AssamxChina
6 (T10)	Tuglali 10	Rize	AssamxChina
7 (E1)	Enstitu 1	Rize	AssamxChina
8 (E2)	Enstitu 2	Rize	AssamxChina
9 (HB)	Hamzabey	Rize	AssamxChina
10 (H)	Hayrat	Trabzon	AssamxChina
11 (C)	Cayeli	Rize	AssamxChina
12 (A)	Ardesen	Rize	AssamxChina
13 (Fı)	Findikli	Rize	AssamxChina
14 (P)	Pazar	Rize	AssamxChina
15 (I)	Iyidere	Rize	AssamxChina
16 (D)	Derepazari	Rize	AssamxChina
17 (U)	Universite	Rize	AssamxChina
18 (U2)	Universite 2	Rize	AssamxChina
19	*C. olifera*	Rize	-

Young leaves of the other eight genotypes were randomly sampled in January 2013 (Fig. 1). All samples were immediately frozen at −20°C. These samples were used to address the generic delimitation and systematic relationships of Turkish tea clones within a broader framework. The samples were stored at −80°C until extraction of DNA. Additionally, *Camellia oleifera* was selected as an outgroup.

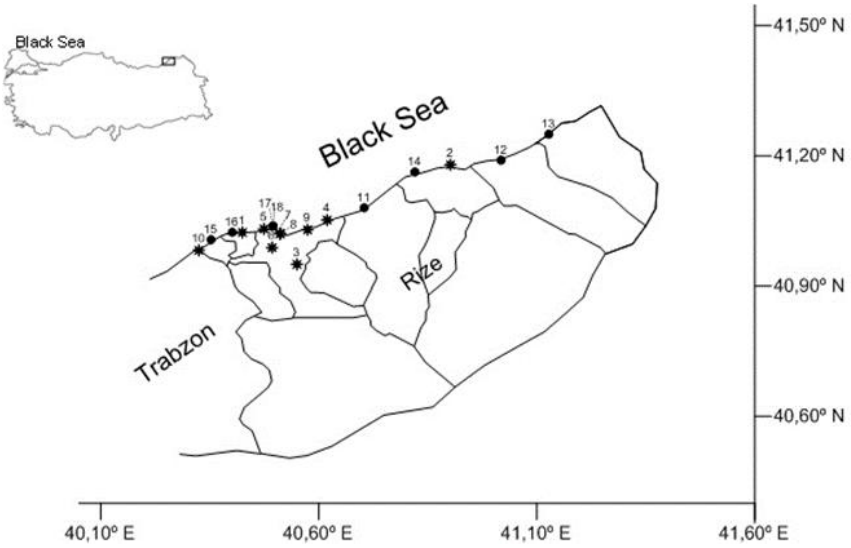

Fig. 1. Distribution and locations of sampling points. ● Eight randomly collected test samples. *****Ten test samples provided by the Rize Ataturk Tea and Horticulture Research Institute.

DNA Extraction

Before DNA isolation, all samples were sterilized to prevent contamination according to Beris et al. (2005). Genomic DNA from stored young and healthy leaf samples was isolated with the FavorPrep™ Plant Genomic DNA Extraction Kit (Favorgen Biotech Corp., Taiwan). The DNA integrity were checked on 0.7% agarose gels, and the concentration or purity of extracted DNA were determined by using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc. USA). Then, gDNA samples were adjusted to 1 ng·μL^{-1} in different aliquots for ISSR analysis and stored at −20 °C for further studies in polymerase chain reaction amplification (PCR).

ISSR Analysis

A preliminary experiment was performed to evaluate ISSR primer appropriateness. Screening out of 21 primers, 15 of those (Table 2) were selected according to the repeatability of their fragment patterns. After this first elimination, 15 primers applied for ISSR amplification across all 18 cultivars. The choice of these nucleotides was based on the probability of their manifoldness in the tea genome (MONDAL, 2002). One primer was used per PCR amplification, which was calculated in a total volume of 50 μL which contains 15 ng genomic DNA, 1.5 μL of 10 μM primer, 4 μL of 2.5 mM each deoxynucleotide triphosphates, 4 μL of 25 mM $MgC1_2$, 10

µL of 5X buffer, 1.5 U GoTaq® Flexi DNA Polymerase (Promega, Madison, WI, USA) and DNA-free double distilled water. The PCR was performed on a Bio-Rad T100™ gradient thermal cycler system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) coded for an initial melting step at 94°C for 2 min, followed by 38 cycles each of 94°C for 1 min, annealing at 35–59°C (depending on the primers, Table 2) for 1 min, and 72°C for 2 min, and ending with an extension period at 72°C for 7 min. Same reaction volume without DNA served as a negative control. We used Tween20 concentration of 1% as an adjuvant and it dramatically enhanced the clarity of banding patterns for most primers in PCR. The amplicons along with a 1 kb DNA ladder (New England Biolabs, Evry, France) were size fractionated in a 1.4% agarose gel at a constant 50 V for 2 h using 1XTAE (4.84 g TRIS base, 1.14 mL glacial acetic acid and 2.0 ml EDTA (0.5 M, pH 8.0) in 1000 mL of distilled water) running buffer and stained with ethidium bromide. After visualizing the DNA bands on a UV-transilluminator, the banding patterns were captured using a gel image system (UVP Bioimaging Systems, Upland, CA, USA).

Table 2. Details of the ISSR primers used

No.	**Primer sequence (5'–3')**	**Tm ^{0}C**
1	(AG)8C	48
2	$(TC)_8G$	50
3	$(CT)_8GG$	52
4	$(AC)_8T$	45
5	$(AC)_8G$	48
6	$(AC)_8C$	51
7	$(AC)_8TG$	50
8	$(AC)_8CG$	52
9	$G\_(AC)_8$	48
10	$(GT)_8C$	48
11	(GT)8T	48
12	$(CTC)_6$	58.1
13	$(GAA)_6$	44
14	$(CAG)_6$	58
15	$(CAG)_6GT$	59
16	$(CAA)_6G$	48
17	$(CAA)_6$	44
18	$(GATA)_4$	35
19	$(CTGA)_4$	45
20	$(GACA)_4T$	47
21	$(CTGA)_4G$	50

Statistical Data Analysis

Amplified bands were visually scored as present or absent (data not shown) and a similarity index was generated (SNEATH and SOKAL, 1973). Related dendrogram was created based on the similarity index data by UPGMA cluster analysis using the NTSYS-pc 2.02i computer program (ROHLF, 1988). The relationships between all tea samples were described graphically in the dendrogram.

RESULTS AND DISCUSSION

Sampling and DNA Extraction

In this study, we used ten cultivated tea genotypes, eight selected tea clones from planted areas, and one outgroup sample from the same family, *C. olifera*. Genomic DNA was extracted from all samples. We obtained different concentrations of isolated DNA (from 34 to 380 $ng \cdot \mu L^{-1}$) because of the different quality of the samples in various conditions.

PCR Conditions for ISSR Amplification

To determine the genetic similarity of 18 tea genotypes grown in Turkey, we tested 21 ISSR primers. From these, 15 primers were selected according to those that gave the most polymorphic bands and produced scorable PCR fragments. Primer pairs were designed according to a previous study on tea plants (MONDAL, 2002). The polymorphic bands that were scored ranged from 300 to 3000 bp in size, and were characterized by their melting temperatures (Tm) between 40 and 60°C. Weak and unclear bands were not scored for data analysis. We used seven di-, five tri-, and three four-nucleotide repeats in our primers. According to REDDY *et al*. (2002) and YAO *et al*. (2008), the di-nucleotides repeats (AG), (GA), (CT), (TC), (AC), and (CA) showed higher polymorphism in plants than other repeats. Herein, we used the ISSR primer combinations (AC)8G, (AC)8C, (AC)8TG, (AC)8CG, (AC)8GG, and G_(AC)8 as (AC) based primers, while (CT)8GG was a (CT) based primer. The size of the products ranged from 300 to 3000 bp (Fig. 2). The variation in the total number of fragments produced by each primer pair was similar to that in former studies (MONDAL, 2002, DAN, 2006).

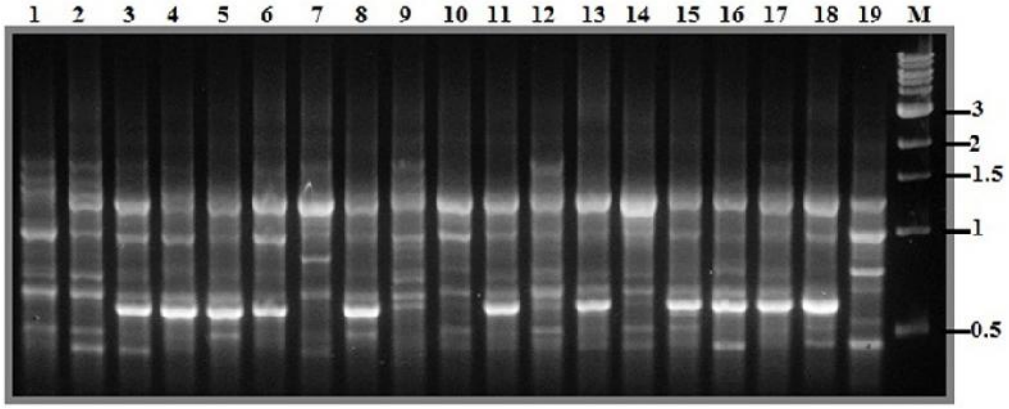

Fig. 2. Agarose gel electrophoresis pattern of the amplified DNA using primer ISSR-(GACA)4T (left to right: lanes 1–19, tea samples; lane 20, 1 kb DNA marker).

Genetic Similarity Matrix and Cluster Analysis

The results of the DNA fingerprinting of 18 tea genotypes are shown in Fig. 3. A similarity coefficient matrix (data not given) showed that the highest similarity was determined between Cayeli and Iyidere (0.743) while the lowest similarity index was between Derepazari-7 and Tuglali-10 (0.456). The dendrogram seperated all 18 genotypes into two groups (Fig 3). The first group had only two cultivated tea plants, Derepazari-7 and Hayrat. The similarity was 0.595 in this group. Group two was gradually branched and included the other 16 tea genotypes. In this group, the highest similarity index (0.743) was found between Cayeli and Iyidere. Hamzabey was 58% similar to all of the tea genotypes and formed a separate branch in this group. Pazar-20 and Muradiye had 0.649 similarity. Among the rest of the samples, Enstitu-1 and Enstitu-2 were separated from other samples in the group with a similarity index of 0.716. Since these genotypes are cultivated by the Rize Ataturk Tea and Horticulture Research Institute for their biochemical and phenological characteristics, the similarity values were high among all genotypes. Fener-3 had 63% similarity to the other genotypes in group two. Like the Institutional cultivars Enstitu-1 and -2, Universite-1 and Universite-2 were divided from the others with a 0.662 similarity index. These genotypes are grown in the university planting area. However, their similarity was not high because of sexual reproduction.

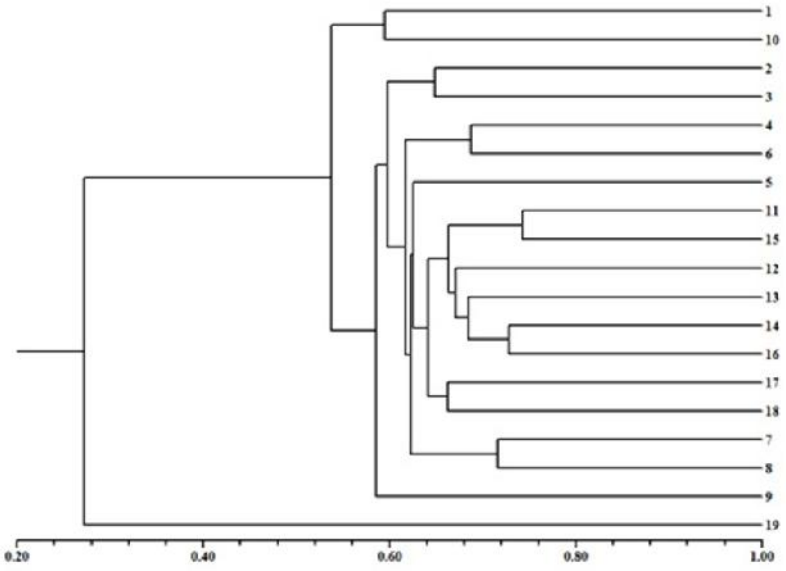

Fig. 3. Dendrogram of 19 tea genotypes. Tea sample numbers are given according to Table 1. Number 19 is the outgroup sample, *C. olifera*. In the figure, 1.00 indicates 100% similarity.

In group two, Pazar and Derepazari were 72.5% similar. This was the second highest similarity among all of genotypes. According to the similarity indices and dendrogram, some tea cultivars inherited from the same geographical position were found to be closely clustered. For

example, Enstitu-1 and -2 share the same location and had a 0.716 similarity index. At the same time, some tea cultivars and clones from the same geographical position were separated from each other because of sexual reproduction, a lack of tea gene resource conservation, and movement between tea gardens to set up new tea plantation areas by producers. Plants produced from seeds in the same plantation or geographic area may show different individual genotypes because of seed fertilization. As mentioned above, the tea plantation areas were created by tea farmers in this way in Turkey, and we see different branches in the dendrogram as a result of this. Additionally, the results obtained in previous studies deal with polymorphism detection among populations, support our findings (BERIS *et al*., 2005, KAFKAS *et al*., 2009). Our results indicated that the genotyping and breeding problems of Turkish tea can be overcome by using ISSRs that has a lot more polymorphic amplicon number and the highest fragment resolution power in compare with the other DNA-based markers.

In conclusion, our genetic diversity data of Turkish tea cultivars settled down Eastern Black Sea coast may help showing genetic relationships of elit tea lines to improve parental selection thus tea breeding and clone preservation for scientific utilization.

ACKNOWLEDGEMENTS

This work was supported by Recep Tayyip Erdogan University Research Fund Grants BAP_2013.102.03.4.

Received September 04th, 2015
Accepted January 25th, 2016

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EVALUACIJA GENETIČKE DIVERGENTNOSTI KLONOVA GAJENOG ČAJA (*Camellia sinensis* (L.) Kuntze) NA ISTOČNOJ OBALI CRNOG MORA TURSKE KORIŠĆENJEM MOLEKULARNIH MARKERA (ISSRs)

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Izvod

Determinacija genetičke različitosti čaja je značajna u kreiranju programa oplemenjivanja. Vršena su ispitivanja genetičke različitosti kultivara čaja na istočnoj obali Crnog mora u Turskoj i njihov genetički odnos u cilju uverifikacije da li je moguće koristiti ISSR markere u kvalitativnom morfološkom obeležavanju. U eksperimentima je vršena genetička determinacija i utvrđivanje odnosa 18 turslih kultivara čaja korišćenjem 15 ISSR markera čija veličina je bla u opsegu od 250 – 3000 baznih parova. Indikatori sličnosti među kultivarima su bili između 0.456 i – 0.743. Korišćenjem UPGM analize klastera (grupa) utvrđeno je da se neki kultivari čaja poreklom iz iste geografske zone grupišu vrlo blisko. Podaci obezbeđuju značajne informacije i korisnu osnovu za selekciju i razmnožavanje klonovima kao ipomoć farmerima da nađu elitne roditeljske klonove za oplemenjivanje čaja na istočnoj obali Crnog mora Turske.

Primljeno 04. IX. 2015.
Odobreno 25. I.2016.