Plant Genetic Resources: Characterization and Utilization

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Genetic fingerprinting of *Ziziphus jujuba* by using SCoT and REMAP molecular markers

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Research Article

Cite this article: Mohajer Kaboli Z, Sheidai M, Koohdar F (2023). Genetic fingerprinting of *Ziziphus jujuba* by using SCoT and REMAP molecular markers. *Plant Genetic Resources: Characterization and Utilization* **21**, 248–253. https://doi.org/10.1017/S147926212300031X

Received: 4 May 2022
Revised: 29 April 2023
Accepted: 2 May 2023
First published online: 11 September 2023

Keywords:
Genetic diversity; genetic fingerprinting; jujube; REMAP; SCoT

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Abstract

Jujube is both consumed as a food source and medicinal plant in local markets. It is expected that different geographical populations of *Ziziphus jujuba*, differ in their genetic content as they grow in different ecological conditions. It is important to have detailed information on population genetic structure and the available genetic variability to make a proper germplasm collection of jujube. We have no data on jujube populations of Iran based on SCoT and REMAP molecular markers, and therefore we planned a population genetic study of these trees in 10 geographical areas. We used SCoT and REMAP molecular markers for our genetic investigation. We found the loci with a high value of Gst (1.00) in SCoT and REMAP markers that can be used in fingerprinting of jujube.

Introduction

The genus *Ziziphus* Mill. (Family Rhamnaceae) is composed of 40 species that are widely distributed in the tropical and subtropical regions of the world, with South and Southeast Asia as their centre of evolution and distribution (Singh *et al.*, 2007). *Ziziphus* species are deciduous evergreen trees or shrubs and are well known as a source of medicine plant (Asatryan and Tel-Zur, 2013). The fruit is eaten fresh or dried and made into candy, tea or syrup (Gupta *et al.*, 2004; Jiang *et al.*, 2007). Moreover, some specific saponins, as well as ethyl acetate and water extracts of the fruit and bark, have explored the potential cytotoxicity of jujube. These extracts bring about apoptosis and differential cell cycle arrest, moreover, activity against certain human cancer cell lines has been demonstrated in vitro (Lee *et al.*, 2004; Huang *et al.*, 2007; Vahedi *et al.*, 2008).

Ziziphus jujuba (jujube) is one of the well-known medicinal species of the genus (Asatryan and Tel-Zur, 2013). It is mainly distributed in southwestern Asia. It has been traditionally used as medicine since 2500 years ago, in China. The fruit, seed and bark of this plant species are used to alleviate stress and insomnia and as appetite stimulants, digestive aids, antiarrhythmics and contraceptives (Vahedi *et al.*, 2008). *Ziziphus jujuba* is an important plant species to the mankind, due to which its cultivation and conservation gained high importance within recent years. Moreover, as jujube has wide geographical distribution and forms many local populations, it is important to be studied from population genetic point of view.

Population genetic study is an important step for genetic evaluation of medicinally important species as it gives insight on the genetic structure, genetic diversity and gene flow *vs* genetic fragmentation of these plant species. It also produces data on the number of potential gene pools for conservation and breeding strategies (Sheidai *et al.*, 2016). Various molecular markers have been used to perform the above tasks such as, amplified fragment length polymorphism (AFLP), simple sequence repeats (SSRs), inter-simple sequence repeats (ISSRs), start codon targeted (SCoTs), retrotransposon-microsatellite amplified polymorphism (REMAPs) etc. (e.g. Saboori *et al.*, 2019).

Different molecular markers have been used for population genetic investigation and phylogenetic studies in *Z. jujuba*. For example, Farahani *et al.* (2019) and Nabavi *et al.* (2019) revealed good level of genetic diversity among wild/ uncultivated populations of jujube by using ISSR, SRAP and IRAP molecular markers which can be used in conservation and breeding of this important horticultural crop plant within Iran. As well as Reche *et al.* (2019) revealed ISSR markers was able to differentiate the cultivars jujube, so they are useful for genetic studies of *Z. jujuba* specie. Similarly, the genetic relationships between different *Z. jujuba* cultivars and/ or wild jujuba individuals was studied by using random amplified polymorphic DNA (RAPD), AFLP, sequence-related amplified polymorphisms (SRAP), SSR, ISSR and chloroplast microsatellite (Cp-SSR) markers (see for example, Peng *et al.*, 2000; Liu *et al.*, 2005; Singh *et al.*, 2007; Wang *et al.*, 2007; Wang *et al.*, 2014; Zhang *et al.*, 2014; Huang *et al.*, 2015).

Due to medicinal importance of *Z. jujuba*, the present study was performed with the following aims:1- To produce data on genetic diversity in *Z. jujube* cultivars, 2- To investigate

discriminating power of the SCoT and REMAP markers by Gst and NM analysis for fingerprinting of *Z. jujube*.

Materials and methods

Plant materials

We randomly selected 100 trees of 10 geographical populations (10 trees per population) and used for population genetic investigation (Table 1 and online Supplementary Fig. S1).

Scot and REMAP assay

Fresh leaves were put to dry in silica gel powder. Cetyltrimethyl-ammonium bromide -activated charcoal protocol (CTAB) was applied to extract the genomic DNA (Križman *et al.*, 2006). The extraction was done by activating charcoal and poly vinyl pyrrolidone for binding of polyphenolics during extraction; for mild extraction and precipitation conditions, the high-molecular weight DNA isolation was boosted without the interference of impurities. The extracted DNA was examined in terms of quality and quantity by running on 0.8% agarose.

3 REMAP primer combinations, derived from one single IRAP primer (NIKITA) with 3 ISSR primers ((CA)7GT, (GA)9 T, (GA) 9C) were tested on plants samples. Using a 25 µl volume containing 20 ng genomic DNA and 3 U of Taq DNA polymerase (Bioron, Germany); 50 mM KCl; 10 mM Tris-HCl buffer at pH;8 1.5 mM MgCl2; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of each primer, polymerase chain reaction (PCR) was implemented.

The following programme was used for amplification of nuclear region in a PCR reaction: 5 min initial denaturation step 94°C, followed by 40 cycles of 1 min at 94°C; 1 min at 53.5°C and 2 min at 72°C. The reaction was completed by a final extension step of 7 min at 72°C. The amplification products were observed by running on 1% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Four primers (SCOT1, SCOT2, SCOT36 and SCOT41) based on Collard and Mackill (2009) for monocotyledons plants were selected. These primer sequences are: SCoT1: CAACAATGGCTACCACCA, SCoT2: CAACAATGGCTACCACCC, SCoT36: GCAACAATGGC TACCACC and SCoT41: CAATGGCTACCACTGACA.

PCR reaction mixture with total volume of 25 µl contained 10 mM Tris-HCl buffer (pH = 8), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM of dNTP (Bioron, Germany), 0.2 µM of primer, 20 ng genomic DNA and 1U of *Taq* DNA polymerase (Bioron, Germany).

The amplification reactions were performed in Techne thermocycler (Germany) with the following programme: 5 min at 94°C, 40 cycles of 1 min at 94°C, 1 min at 49–58°C (SCOT1 50°C, SCOT2 49°C, SCOT36 50°C, SCOT41 58°C) and 1 min at 72°C and a final cycle of 7 min at 72°C. The amplification products were visualised by running on 2% agarose gel, stained with sybergreen (Powerload, Kosar Co. Iran). The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Data analyses

The SCoT and REMAP bands obtained were treated as binary characters and coded accordingly (presence = 1, absence = 0). Detrended correspondence analysis (DCA) was used to evaluate suitability of SCoT bands obtained for population genetic analysis (Hill MO, Gauch, 1980). The number of private bands *vs* common bands and genetic diversity parameters like: The percentage of allelic polymorphism, allele diversity (Weising *et al.*, 2005), Nei' gene diversity (He) and Shannon information index (I) (Weising *et al.*, 2005), were determined. We used GenAlex 6.4 for these analyses (Peakall and Smouse, 2006). Discriminating power of REMAP and SCoT markers investigated by Gst and NM analysis as implemented in POPGENE32. The Neis' genetic distance was also being determined among these populations. Mantel test was also determined to illustrate if genetic distance is associated with geographical distance in the studied populations (Mantel, 1967).

Grouping of the species was done by different clustering and ordination methods such as NJ and Multidimensional scaling (MDS) (Podani, 2000). PAST version 2.17 (Hammer *et al.*, 2012) was used for multivariate analysis.

SCOT data was also analysed by TCS Networking as implemented in Pop ART (Population Analysis with Reticulate Trees) program (http://popart.otago.ac.nz). In order to investigate the province's separation of the studied cultivars, SCOT data were also analysed by the MDS plot in the PAST ver. 32 program.

Table 1. *Ziziphus jujuba* population in REMAP and SCoT studies

Number of population	Locality	Longitude	Latitude
1	Iran, Qom , Kalaghneshin	50.2536°	34.4122°
2	Iran, Qom , Ghaziolia	50.2850°	34.3222°
3	Iran, Qom , Dolatabad	50.3032°	34.1258°
4	Iran, Qom , Jafarieh	50.3429°	34.4722°
5	Iran, Qom , Khalajestan	50.3844°	34.2852°
6	Iran, Markazi, Aveh	50.2523°	34.4732°
7	Iran, Markazi, Delijan	50.4102°	33.5926°
8	Iran, Markazi, Saveh	50.2124°	35.0117°
9	Iran, Esfahan, Kashan niasar	51.0856°	33.5822°
10	Iran, Esfahan, Koohpayeh	52.2623°	32.4249°

https://doi.org/10.1017/S147926212300031X Published online by Cambridge University Press

Results

SCoT results

DCA plot (Not figure), revealed that SCot molecular markers are scattered along the plot and therefore are distributed throughout the genome and are suitable for population genetic studies. The primary analysis of SCoT molecular data in the studied jujube cultivars revealed that these cultivars produced 70 loci or bands. In total, each cultivar studied contained 9–22 SCoT bands (online Supplementary Fig. S2). Six cultivars had 2–5 private/ specific bands due to their peculiar genetic structure. These cultivars are namely, No.1–2 and4, No. 7–8 and No. 10. Such specific bands may be formed due to specific genetic content either through local adaptation or cultivation practice and selection.

Genetic diversity parameters determined in the studied cultivars are provided in Table. The genetic polymorphism varied from 1.92% in cultivar No. 1 and 3 to 25.00% in the cultivar No. 7. The mean value of genetic polymorphism, is indicative of low genetic variability within each cultivar. The other genetic diversity parameters like Shanon information index (I) as well as gene diversity (He), were also very low in the studied jujube cultivars (online Supplementary Table S1).

The AMOVA test showed significant genetic differences among *Z. jujube* populations ($P = 0.001$). The results show that the populations of *Z. jujube* have been genetically distinguished from each other using SCoT marker (online Supplementary Fig. S3).

Nei genetic distance obtained among cultivars ranged from 0.11 to 0.66. We obtained a significant correlation between genetic distance and geographical distance of the studied populations (p. <0.01), by Mantel test.

Different ordination and clustering methods like MDS and NJ produced similar results; therefore, only MDS plot is presented here. MDS and NJ plots (Fig. 1 and online Supplementary Fig. S4) grouped the specimens of each population together, separated from each other. This means that these cultivars are genetically differentiated from each other. This is in agreement with AMOVA results which showed significant genetic difference ($P < 0.05$) among these cultivars.

The TCS plot (Fig. 2) also confirmed the results of MDS on the separation between cultivars. In this plot, the lines that are placed horizontally on each branch represent the number of the locus that varies between the cultivars. Although the cultivars are well separated from each other, the difference between the loci represents a variation within them.

REMAP results

DCA plot (Not figure), revealed that REMAP molecular markers are scattered along the plot and therefore are distributed throughout the genome and are suitable for population genetic studies. The primary analysis of REMAP molecular data in the studied jujube cultivars revealed that these cultivars produced 60 loci or bands (online Supplementary Fig. S5).

Six cultivars had 1–3 private/ specific bands due to their peculiar genetic structure. These cultivars are namely, No.2, No. 3, No. 6, No. 7, No. 9 and No. 10. Such specific bands may be formed due to specific genetic content either through local adaptation or cultivation practice and selection.

Genetic diversity parameters determined in the studied cultivars are provided in Table. The genetic polymorphism varied from 0.00% in cultivar No. 1 to 9.09% in the cultivar No. 7. The mean value of genetic polymorphism is indicative of low genetic variability within each cultivar. The other genetic diversity parameters like Shanon information index (I) as well as gene diversity (He), were also very low in the studied *Ziziphus* cultivars (online Supplementary Table S2).

The AMOVA test showed significant genetic differences among *Z. jujube* populations ($P = 0.001$). The results show that the populations of *Z. jujube* have been genetically distinguished

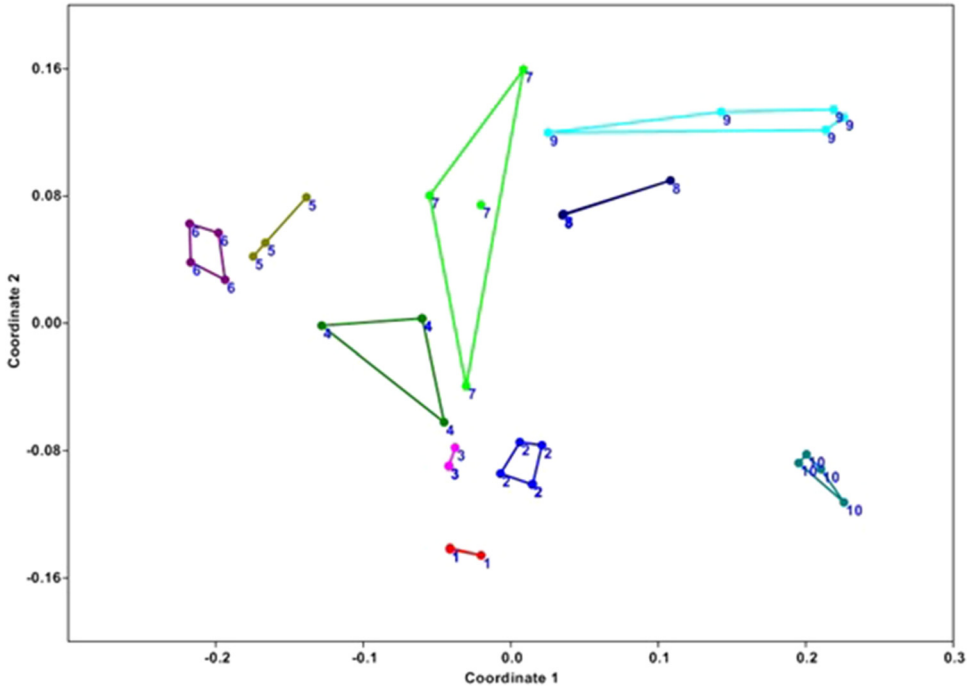

Figure 1. MDS plot of SCoT marker in *Ziziphus jujube*.

https://doi.org/10.1017/S147926212300031X Published online by Cambridge University Press

Figure 2. TCS plot of SCoT marker in *Ziziphus jujube.*

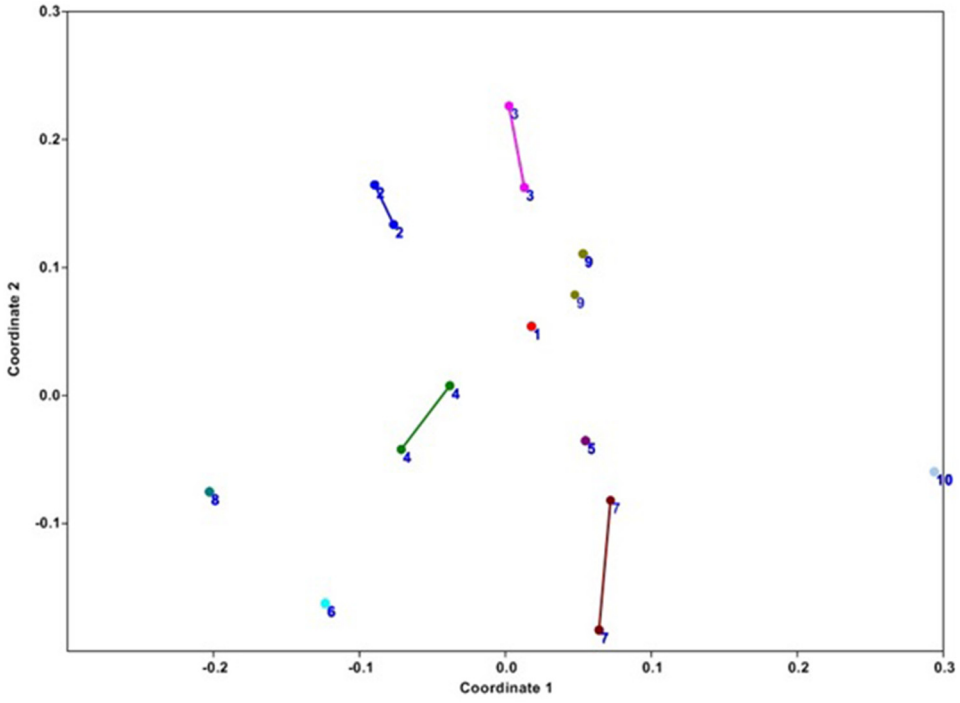

Figure 3. MDS plot of REMAP marker in *Ziziphus jujube.*

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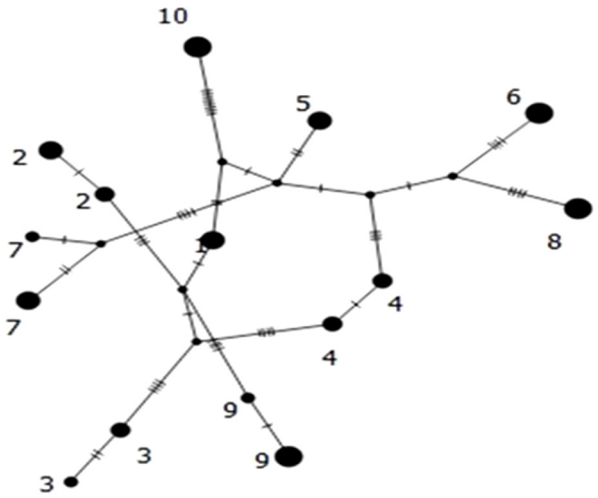

Figure 4. TCS plot of REMAP marker in *Ziziphus jujube*.

from each other using REMAP marker (online Supplementary Fig. S6).

Nei genetic distance obtained among cultivars ranged from 0.16 to 0.72. We obtained a significant correlation between genetic distance and geographical distance of the studied populations (p. <0.01), by Mantel test.

Different ordination and clustering methods like MDS and NJ produced similar results; therefore. MDS plot (Fig. 3) grouped the specimens of each population together, separated from each other. This means that these cultivars are genetically differentiated from each other. This is in agreement with AMOVA results which showed significant genetic difference ($P < 0.05$) among these cultivars.

The TCS plot (Fig. 4) also confirmed the results of MDS on the separation between cultivars. In this plot, the lines that are placed horizontally on each branch represent the number of the locus that varies between the cultivars. Although the cultivars are well separated from each other, the difference between the loci represents a variation within them.

Fingerprinting

Discriminating power analysis of molecular markers is important for fingerprinting. For this purpose, Gst and Nm parameters was measured by Popgene analysis. The value of Gst in 23 loci in SCoT and 27 loci in REMA marker in *Ziziphus jujube cultivars* was 1.00 while the mean Nm value was 0.00, which indicated these markers are suitable molecular tool to investigate genetic structure and genetic finger printing of *Ziziphus jujube cultivars*.

Discussion

Ziziphus jujuba with good medicinal and food importance (Vahedi *et al.*, 2008) should be studied from genetic and breeding points of view. This tree species grows in different geographical regions of our country and its population genetic study is of immediate importance. Data produced can be utilised in future conservation and breeding programmes.

Population genetic study produces insight on the genetic structure, the stratification *vs* gene flow, as well as genetic divergence of the populations (Freeland *et al.*, 2011).

The present study reports genetic variability of jujube cultivars in different localities of Iran. We revealed that multilocus molecular markers like SCoTs and REMAPs are powerful technique for genetic finger printing and discriminating jujuba cultivars and populations. The results obtained by DCA analyses revealed that these molecular markers can be used for genetic fingerprinting in jujube germ plasm.

This is in close agreement with the other reports on these cultivars. For example, Farahani *et al.* (2019) and Nabavi *et al.* (2019) used ISSRs, SRAPs and IRAP markers to investigate genetic diversity in many cultivars in IRAN. On the other hand, Singh *et al.* (2017) investigated genetic variation and relationships among cultivars of *Ziziphus mauritiana* (Lamk.) native of India by using SCoT, ISSR and ribosomal DNA (rDNA) markers. They reported high level of polymorphism among SCoT (61.6%) and ISSR (61%) markers. Difference in the results of these studies is probably due to difference in geographical isolation of the studied populations.

In present study, the distance between populations is great as they are located in different provinces ranging from south to north of the country with no intermediately plant populations among them.

Genetic differentiation of the studied populations may be attributed to a combination of adaptation to different environmental conditions and limited capacity for long-distance dispersal (Zhang *et al.*, 2014).

However, we also noticed good genetic differentiation within each province between wild and cultivated *Z. jujuba* plants; this is probably due to effects of cultivation practice and artificial selection made by jujube growers in the gardens. Such selection pressure is absent in wild plants.

Conclusion

In the present study, we found the loci with the high value of Gst (1.00) in SCoT and REMAP markers that can be used in fingerprinting of *Z. jujuba*.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S147926212300031X.

Ethical standards. On behalf of all the co-authors, the author testifies that this article has not been published in whole or in part elsewhere. Data (including images) provided in this paper are not fabricated or manipulated in any way to support our conclusions.

Competing interest. None.

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