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Full Length Research Paper

Molecular diversity in persimmon (*Diospyros kaki* L.) cultivars growing around Hatay province in Turkey

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Genetic relationships among *Diospyros kaki* L. growing around Hatay province in Turkey were assessed by randomly amplified polymorphic DNA (RAPD) assay. Ten decamer primers were selected from 50 primers. These primers yielded a total of 155 bands and 126 of them were recorded as polymorphic. Pairwise genetic distances of the samples were used to construct dendograms using Unweighted Pair-Group Method of Arithmetic Average (UPGMA). The study suggests that the morphological differences among cultivars of persimmon might be the result of genetic differences rather than the ecological or growing conditions. The study forms a basic contribution to the characterization of *D. kaki* population in Turkey.

Key words: DNA fingerprinting, *Diospyros kaki* L., genetic variability, RAPD.

INTRODUCTION

Persimmon (*Diospyros kaki* L.) is one of the important species that is native to China. Growth area of the fruit includes some of the Far East countries as well as some parts of United States. The vitamin (A and C) and mineral content of the fruit makes it a valuable fruit and growth area becomes wider throughout the world (Tuzcu and Yıldırım, 2000). Persimmon has 4 species (*Diospyros kaki* L., *Diospryros lotus* L., *Diospyros virginiana* L., *Diospryros oleifera* Cheng). *D. kaki* is widely grown in many parts of Turkey. Although the exact date of introduction of persimmon to Anatolia is not known, it is clear that dates back to rather old times (Onur, 1990). According to the available records, persimmon was introduced to Turkey from Russia via the route of Black Sea. Agricultural ministry of Turkey pioneered the introduction of different varieties after 1967 and with the aid of selection studies, the number of varieties reached to 14. After 1989 with the introduction of new varieties especially from Italy, and also from Israel, Japan, France and Pakistan the known number of varieties reached 74 (Tuzcu and Şeker, 1996).

All persimmon varieties growing in Turkey are not wild populations; they are all cultured plants. Although they are not provided by breeding they manifest high qualities suitable for selection studies. The culture plants selected in this study from Hatay region are totally unknown varieties, unique to this region, that display different and higher qualities most probably because of the selection by the local growers or the somatic mutations of the buds used for the propagation of the genotypes.

As in many fruit and vegetable species, Turkey is one of the leading producer countries of persimmon. However, genotype identification is required in order to produce them efficiently. In this regard, selection studies become important. Selection studies are quite difficult for perennial species especially for the ones adapted to large areas. Like many other horticultural fruits, especially in selection and improvement studies, discrimination of the cultivars on the basis of morphological characters is limited. In combination with morphological studies, polymerase chain reaction (PCR) based molecular markers are widely used to distinguish among cultivars of many species (Grassi et al., 2006; Aras, 2003; Ergül et al., 2002). Genetic polymorphisms revealed by molecular markers like RAPD analysis provide valuable and efficient information.

RAPD technique provides a simple and convenient method for the detection of polymorphism in the absence of sequence information with a relatively low cost.

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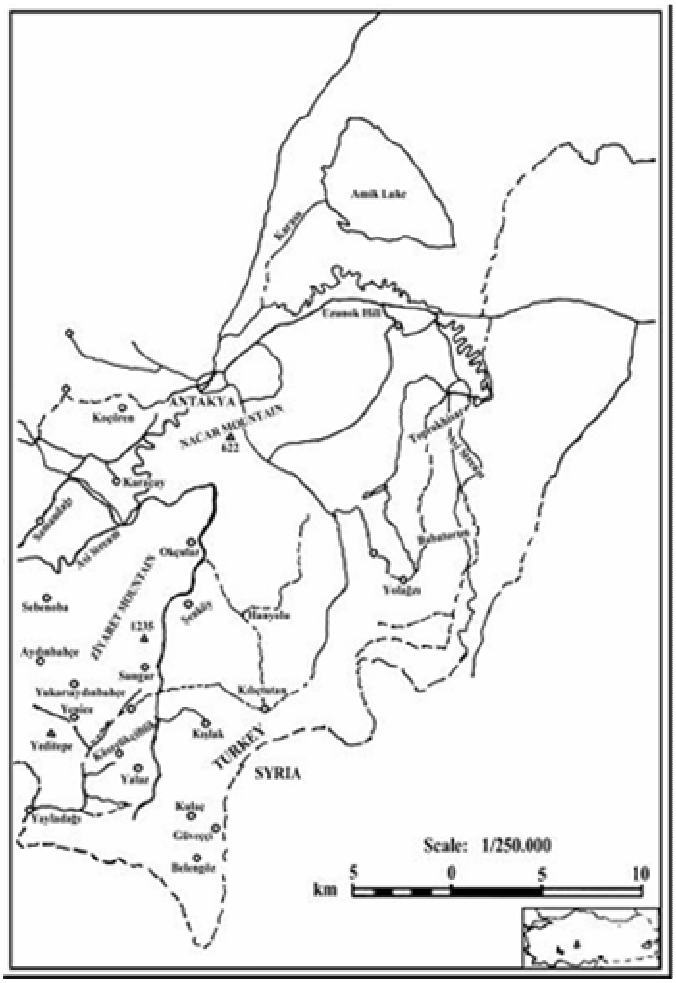

Figure 1. Regional map of Hatay province located at South Anatolia.

Although, questions regarding the reliability and reproducibility of the technique have been argued in many studies (Williams and Clair, 1993; Ellsworth et al., 1993), advantages of the technique has led it to be used in a wide range of plant species in determining genetic diversity including tomato (Williams and Clair, 1993), maize (Peije et al., 1998), grape (Aras et al., 2005; Ergül et al., 2002), chickpea (Sudupak et al., 2002), dogrose (Aras, 2003), bean (Grassi et al., 2006) and genetic mapping in spruce sp. (Arcade et al., 2000), pine (Costa et al., 2000). RAPD markers have also been used as DNA fingerprints for persimmon cultivars (Badanes et al., 2003; Yamagashi et al., 2005). In addition to cultivar identification, Tamura et al. (1998) assessed interspesific somatic hybrid calluses yielded between *D. glandulosa* and *D. kaki* cv. Jiro by RAPD analysis.

The aim of the present study is to determine the genetic relationships among persimmon cultivars from Hatay region that will be utilized for future selection studies. Besides, the study also presents an improved DNA isolation protocol suitable for the species.

MATERIALS AND METHODS

Plant Materials

Persimmon (*Diospyros kaki* L.) leaf materials used in the study for RAPD analysis were collected from Hatay, a south Anatolian province of Turkey. 18 samples were selected for the study from different regions around Hatay province. Some general characteristics of the persimmon grown around Hatay and Adana province are given in Table 1. The specific locations and elevations of the samples used in the study are displayed in Table 2. The locations were also displayed on a regional map (Figure 1)

DNA isolation

The methods examined for DNA isolation were; the protocol by Doyle and Doyle (1987), the procedure described by Dellaporta (1983) and the method by Kim et al. (1997). None of the methods yielded DNA of enough purity for polymerase chain reaction (PCR) amplification. An improved protocol was used to isolate persimmon DNA by modifying several existing ones. The new extraction procedure was as follows; leaf material (300 - 400 mg) was ground twice in liquid nitrogen into a fine powder. 800 µl prewarmed extraction buffer (100 mM Tris-HCl (pH 8), 50 mM EDTA, 1.4 M NaCl, 2% CTAB, 1% PVPP) was added to the samples. They were taken to the 1.5 mL eppendorf tubes and 1.5 µl of Rnase A (10 mg mL^{-1}) was added to each sample. The samples were incubated in 37°C for 15 min and then in 65°C for another 60 min. Following the incubation periods, samples were cooled to room temperature and 250 µl, 5 M potassium acetate was added and incubated on ice for 30 min. Samples were centrifuged at 17,000 *g* (14,000 rpm) for 15 min, and the supernatant was transferred to a fresh tube. 500 µl phenol : chloroform : isoamylalcohol (25:24:1) was added to the mixture and extraction was carried on by gently shaking for 40 - 50 times. It was observed that at this stage incubating the samples on ice for at least 15 min increases the efficiency of DNA yield and could be prolonged until several hours. After centrifugation for 10 min at 17,000 *g*, 250 µl 0.3 M sodium acetate and 500 µl absolute ethanol were added and stored at –20°C for at least 40 min to precipitate DNA. The DNA pellet was precipitated by spinning at 17,000 *g* for 10 min followed 70% ethanol wash. The pellet was air dried until all ethanol was removed. If the samples did not have enough purity the procedure could be repeated starting from the extraction step. The DNA pellet obtained was dissolved in appropriate volume of TE buffer (10 mM Tris-HCl (pH 8), 0.1 mM EDTA) and stored at –20°C until use. Concentrations of DNA samples were determined spectrophotometrically (260/280 nm) (Table 3) and also by running on 1% agarose gel with 100 bp DNA ladder molecular weight marker.

RAPD analysis

RAPD analysis was carried out with an initial screening of 50 decamer primers (Operon Technologies). 10 primers yielded clear and reproducible bands were used to identify genetic variation among persimmon specimens used in the study. The 10 primers used in the experiments were: OPA 01, OPA 03, OPA-18, OPA 19, OPC 11, OPC 12, OPC 15, OPC 20, OPO 07, and OPO 10 which the sequences are listed in Table 4. PCR amplification reaction volume was 25 µl each containing 100 ng genomic DNA, 2.5 µl 10X reaction buffer, 2 mM $MgCl_2$, 50 µM dNTPs, 20 nM primer, 0.5 unit Taq DNA polymerase. The reactions were heated to an initial step of 95°C for 5 min, and then subjected to 35 cycles of the following program: 94°C for 1 min, 34°C for 1 min, and 72°C for 2 min. After the last cycle the temperature was held at 72°C for 5 min as a final extension step. Amplification products were electrophoresed in a

Table 1. Some general characteristics of persimmon (*Diospryros kaki* L.) grown around Hatay and Adana provinces.

Flowering	Crop maturity	Annual yield/ tree	Average annual yield/tree	Fruit weight	Fruit meat potency	Average seeds/fruit	Average fruit juice pH	Soluble dry matter	Blooming of the leaf buds	Fruit sheddin
Beginning or 2nd week of April	September or October	15 -70 kg	50 kg	80 – 170 g. Average = 100g	17 lb (during tree formation) 4 lb (edible form)	Min: 1.5 Max: 4	5	12%	Middle of March	May (small fruits) June (excess temperature and drought)

(Source: Onur, 1990)

Table 2. Locations and elevations of the *D. kaki* samples used in the study.

S/N	Location (Antakya county, Hatay province)	Elevation from sea level
1	Yayladağı central region.	408 m
2	Kocaman village. Village of Yayladağı county. 30 km to Yayladağı.	350 m
3	Yayladağı central region.	408 m
4	Çöğürlü village. 30 km to Antakya. Village of Samandağı county.	350 m
5	Karaköse village. 40 km to Antakya from Samandağı road.	372 m
6	Hisarcık village. 40 km to Antakya from Harbiye road.	703 m
7	Hisarcık village. 40 km to Antakya from Harbiye road.	703 m
8	Hisarcık village. 40 km to Antakya from Harbiye road.	703 m
9	Karaköse village. 34 km to Antakya from Samandağı road.	372 m
10	Karaköse village. 34 km to Antakya from Samandağı road.	372 m
11	Hisarcık village. 40 km to Antakya from Harbiye road.	703 m
12	Aslanyazı village. 42 km to Antakya from Harbiye road. Village of Yayladağı.	630 m
13	Güney söğüt. 6 km to Antakya. Village of Samandağı.	69 m
14	Harbiye Şeyh Davut Road. 10 km to Antakya.	142 m
15	Güney söğüt. 6 km to Antakya. Village of Samandağ.	69 m
16	Güney söğüt. 6 km to Antakya. Village of Samandağı.	69 m
17	Uzunbağ. Village of Samandağı. 22 km to Antakya.	20 m
18	Aslanyazı village. 42 km to Antakya from Harbiye road. Village of Yayladağı.	630 m

Table 3. Mean DNA yield obtained from spectrophotometric analysis (A_{260}) with different DNA isolation methods.

Extraction method	Yield (mg g^{-1})	$A_{260/280}$
Doyle and Doyle, (1987) (method with CTAB)	< 1	< 1.5
Dellaporta et al. (1983) (method with SDS)	< 1	< 1.5
Kim et al. (1997) (method with PVP)	≤1	< 1.5
Improved CTAB method for persimmon	20 - 35	1.8 - 2.0

Table 4. Sequence of RAPD primers used to amplify *D. kaki* genomic DNA.

Primer	Sequence (5'-3')	Total amplified bands	Polymorphic bands	Polymorphic percentage (%)
OPA-01	CAGGCCGTTC	13	10	76.92
OPA-03	AGTCAGCCAC	23	20	86.95
OPA-18	AGGTGACCGT	14	13	92.85
OPA-19	CAAACGTCGG	22	16	72.72
OPC-11	AAAGCTGCGG	15	11	73.33
OPC-12	TGTCATCCCC	12	10	83.33
OPC-15	GACGGATCAG	12	11	91.66
OPC-20	ACTTCGCCAC	12	10	83.33
OPO-07	CAGCACTGAC	15	11	73.33
OPO-19	GGTGCACGTT	17	14	82.35
Total		155	126	81.29

1.2% agarose gels containing 0.5 µg mL^{-1} ethidium bromide and visualized on UV transilluminator (Figures 2 and 3).

Data analysis

RAPD assays were repeated twice for each primer and only clear, reproducible bands were scored with specific attention to evaluate the sharp bands, while the faint ones were ignored. Distance matrices were constructed using the Nei and Li (1979) similarity index (Table 5). The MVSP software package version 3.1 (Kovach 1999) was used to calculate similarity coefficients. The dendogram was constructed by Unweighted Pair-Group Method of Arithmetic Average (UPGMA) (Figure 4). Principal Coordinate Analysis (PCA) was also carried out to show multiple dimensions of the distribution of the genotypes in a scatter-plot (Figure 5).

RESULTS AND DISCUSSION

DNA fingerprinting of the *D. kaki* specimens collected from around Hatay province was conducted by RAPD analysis. Of the fifty different primers tested, 10 yielded clear and reproducible bands and were selected for the reactions. The RAPD profiles obtained with the primers OPA-01 and OPA-18 were shown in Figures 2 and 3, respectively. A total of 155 bands were obtained by RAPD analysis and 126 of these bands were recorded as polymorphic. Amplified fragments ranged from 150 bp to 2000 bp and the number of bands for each primer is from 12 to 22.

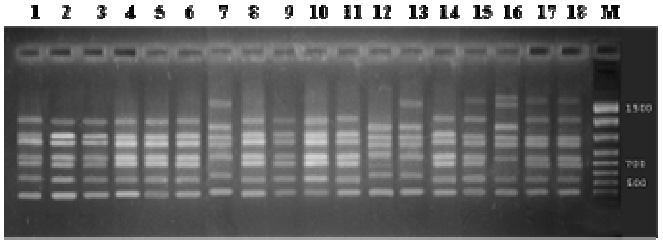

Figure 2. Displays the results of RAPD analysis by the primer OPA-01. Loading order is as given in Table 2. M is the 100 bp ladder DNA molecular weight marker.

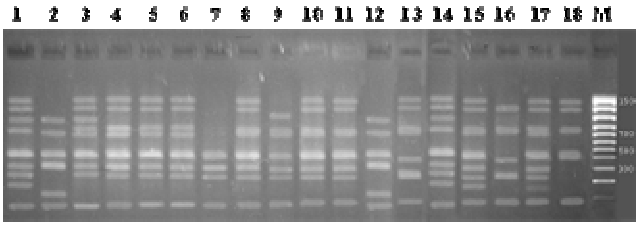

Figure 3. Shows the PCR assay conducted by the primer OPA-18. Loading order is as given in Table 2. M is the 100 bp ladder DNA molecular weight marker.

The RAPD data matrix was used to compute pairwise genetic distances of the samples according to Nei and

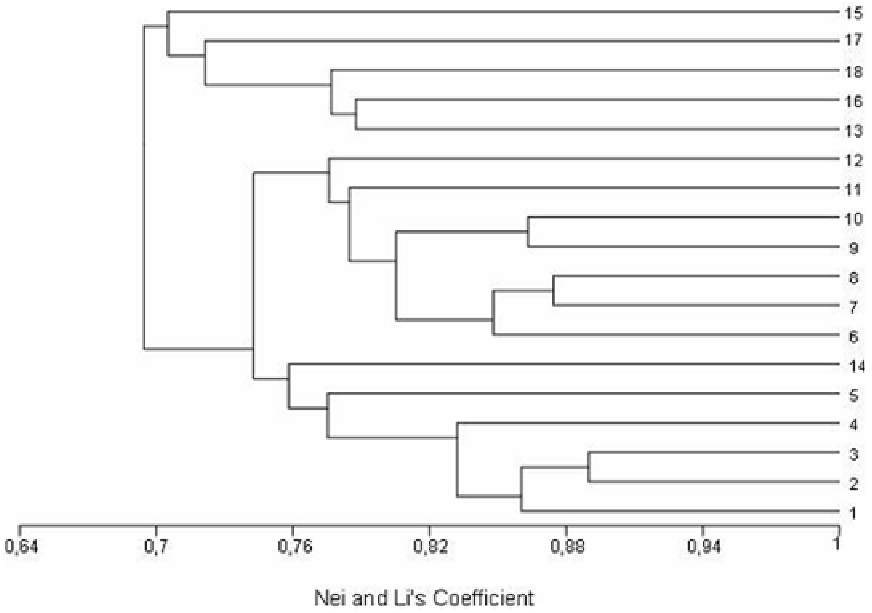

Figure 4. Dendogram (UPGMA) showing the genetic relationships among the samples.

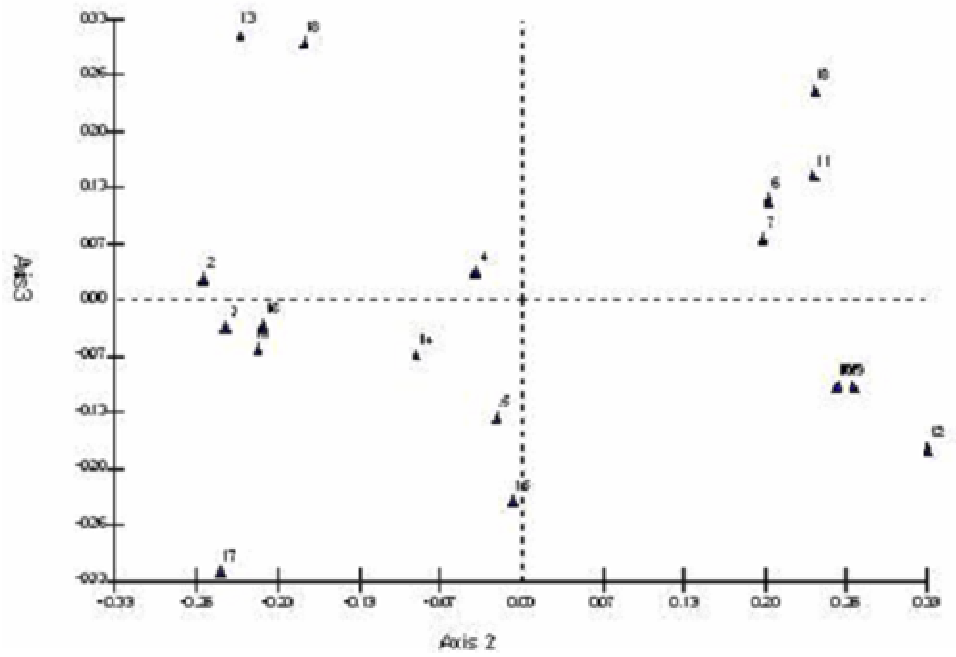

Figure 5. Association of the 18 *D. kaki* samples with principal coordinate analysis. Numbers given in the plot represents the sample order listed in Table 2.

Li's (1979). Dendogram was constructed using Unweighted Pair-Group Method of the Arithmetic Average (UPGMA) according to the coefficients (Figure 4). In the dendogram persimmon samples formed two main clusters, and the second main cluster formed another two clusters. In the first main cluster, out of 5 samples, 4 of them were from around Samandağı County. According to the similarity index data (Table 5) samples number 7 and 8 which were collected exactly from the same region (Hisarcık village 40 km to Antakya) and took part in the second branch of the dendogram, manifested one of the highest similarity ratio of 0.874. Samples 2 and 3 showed the highest similarity of 0.89 was collected from trees just 20 km apart from each other in Yayladağı County. Sam-

Table 5. Similarity index (Nei and Li, 1979) of the tested samples.

Sample #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	1																	
2	0.85	1																
3	0.871	0.89	1															
4	0.808	0.828	0.86	1														
5	0.756	0.746	0.792	0.806	1													
6	0.766	0.786	0.819	0.804	0.773	1												
7	0.77	0.76	0.813	0.818	0.788	0.856	1											
8	0.734	0.724	0.769	0.792	0.75	0.84	0.874	1										
9	0.701	0.732	0.729	0.76	0.738	0.779	0.835	0.788	1									
10	0.735	0.714	0.771	0.763	0.762	0.782	0.837	0.81	0.863	1								
11	0.716	0.684	0.693	0.713	0.656	0.806	0.768	0.836	0.728	0.785	1							
12	0.646	0.677	0.696	0.714	0.66	0.764	0.758	0.761	0.792	0.814	0.766	1						
13	0.67	0.73	0.737	0.687	0.611	0.687	0.73	0.744	0.691	0.694	0.674	0.667	1					
14	0.743	0.762	0.787	0.76	0.738	0.739	0.752	0.766	0.776	0.768	0.75	0.72	0.772	1				
15	0.642	0.642	0.673	0.639	0.645	0.691	0.684	0.688	0.684	0.667	0.699	0.754	0.684	0.738	1			
16	0.674	0.684	0.723	0.67	0.667	0.68	0.705	0.708	0.684	0.72	0.732	0.691	0.788	0.779	0.763	1		
17	0.715	0.705	0.762	0.681	0.699	0.649	0.715	0.667	0.684	0.73	0.656	0.681	0.684	0.728	0.699	0.785	1	
18	0.66	0.701	0.709	0.698	0.684	0.687	0.701	0.746	0.67	0.695	0.717	0.656	0.784	0.694	0.674	0.77	0.695	1
Sample #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18

See Table 2 for the identity of the sample

ple 1 which is exactly from the same region with sample 3 (Yayladağı central region) also displayed a relatively high similarity ratio of 0.871 and formed a group with samples 2 and 3. Samples 6, 7, 8, 11 which collected from the same area of Hisarcık village, 40 km to Antakya from Harbiye road were all grouped in the 2 main clusters. Samples 9 and 10, which form a group in the tree, are also from same (very close) localities (Karaköse village, 34 km to Antakya from Samandağı road). The samples 12 and 18 although collected from the same region (Aslanyazı village, Yayladağı) located in distinct groups on different branches with a relatively low similarity ratio of 0.488.

Principle co-ordinate analysis was carried out in order to determine the genetic relationships among samples with a minimum distortion (Figure 5). The analysis also supported the data generated by the dendogram. An obvious exception was, the sample number 14 from Harbiye Şeyh Davut Road, 10 km to Antakya did not form a group with the samples 5, 4, 3, 2 and 1.

The study was conducted to identify the genetic variability *D. kaki* population around Hatay region, that posses the highest production potential of *D. kaki* among Turkey. Identification of genetic distances between cultivars is essential for breeders, in order to be able to make further selection studies among the genotypes for cross and control purposes and also for preservation of germplasm resources. The results of the current study indicate that RAPD data could be useful for the DNA fingerprinting of persimmon cultivars in agreement with previous studies performed by Badanes et al. (2003) and Yamagashi et al. (2005). All samples are collected from selected trees with their superior morphological characters like yield, fruit size. Although the morphological analysis have been continuing, preliminary data indicates that the trees analyzed in this study display characteristics that are above the average or very close to the maximum values given in Table 1. The data obtained from the current study might suggest the differences and similarities among the sample trees arise from their genetic constitution, rather than the ecological or growing conditions. According to the dendogram and PCA analysis, a geographical distribution has been noticed. However, this might not be the explanation of the genetic differences among the population since persimmons are cultivated in this area although the exact date of introduction is not known and the spacial scale is too limited to accumulate genetic differentiation depending on geographical differences. Similarities according to the geographical distribution of the cultivars might have originated from the cultivation of related varieties within the same regions rather than contribution of the different ecologies to genotype differences.

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