African Journal of Biotechnology Vol. 9(53), pp. 8970-8976, 27 December, 2010
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1557
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Genetic dissimilarities between wild olives by random amplified polymorphic DNA (RAPD) assay

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Accepted 19 November, 2010

Olive, being a fruit of subtropical climate and recognized to have significant importance in terms of human health, is being investigated with different aspects. Wild olives are valuable in the sense that they contain the sources of stability and they can be separated by using random amplified polymorphic DNA (RAPD) technique. In this study, a total of 59 primers were used for the purpose of determining the DNA fingerprints of 12 wild olives obtained from different provinces of Aegean region, and 92 polymorphic bands were yielded. Mean number of polymorphic bands per individual was calculated as 7.67 and number of polymorphic bands per scorable primer was calculated as 4.6. Specific DNA markers are important for determining the genetic relation among wild subspecies in olives. The purpose of this study is to identify the wild olives by using RAPD analysis. In conclusion, the genetic relation between wild olives were determined through Jaccard, Sorensen Dice, Simple Matching coefficients, (unweighted pair group method with arithmetic averages) UPGMA and neighbor joining method by using the data obtained with RAPD.

Key words: Random amplified polymorphic DNA (RAPD), *Olea europaea*, arbitrary primers, Jaccard, Sorensen Dice, Simple Matching, unweighted pair group method with arithmetic averages, neighbor joining.

INTRODUCTION

Olives are produced in two ways which includes vegetative and generative production. In generative production, seeds are taken from wild and cultivated. Wild olives are preferred in production as they are more resistant in hard natural conditions (MOARA, 2006).

In recent years, increase in the economic aspect of olive is required to define it in genetic terms. In this respect, after determining the random amplified polymorphic DNA (RAPD) (Williams et al., 1990), the assay is considerably useful in plant separation studies. As such, this method was used frequently (Hu and Quiros, 1991; Yang and Quiros, 1993; Khasa and Dancik, 1996; Sedra et al., 1998; Belaj et al., 2001; Rajora and Rahman, 2003). RAPD assay is also used for determining the genetic relations (Kaundun et al., 2000). The greatest advantage of RAPD assay is that it does not require any information regarding the genes of plant being examined; the primers are attached to DNA regions randomly during amplification (Welsh and McClelland, 1990). RAPD results to be yielded would be different since the genomic DNAs of different plants are different. Such differences allows for the comparison of organisms (Glick and Pasternak, 1998). As the number of primers increase, the number of bands obtained increases; thus, it becomes easy to separate related species (Mathieu-Daudé et al., 1997).

Besnard et al. (2001) used 43 decamer primers from Bioprobe on five olive species and 8 of the same (A1, A2, A9, C15, C9, E15 and O8) provided characterization of all species. Mekuria et al. (2002), obtained 119 polymorphic bands from six OP-Z primers (OP-Z 11, OP-Z 13, OP-Z 15, OP-Z 18 and OP-Z 20) as a result of RAPD they performed to compare 45 olive trees genetically. Belaj et al.

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Abbreviations: RAPD, Random amplified polymorphic DNA; **CTAB,** cetyl trimethyl ammonium bromide; **EDTA,** ethylene diamine tetraacetic acid; **UPGMA,** unweighted pair group method with arithmetic averages; **NJ,** neighbor joining.

Table 1. Locations of the samples.

Type of olive	Origin	Place of Supply	Province
Wild 1	Pinarcik	Natural setting	Pinarcik, Mugla, Turkey
Wild 2	Pinarcik	Natural setting	Pinarcik, Mugla, Turkey
Wild 3	Caglak	Natural setting	Akhisar, Manisa, Turkey
Wild 4	Caglak	Natural setting	Akhisar, Manisa, Turkey
Wild 5	Harlak	Natural setting	Akhisar, Manisa, Turkey
Wild 6	Sabancılar	Natural setting	Akhisar, Manisa, Turkey
Wild 7	Sabancılar	Natural setting	Akhisar, Manisa, Turkey
Wild 8	Bornova	ORI	Izmir, Bornova, Turkey
Wild 9	Bornova	ORI	Izmir, Bornova, Turkey
Wild 10	Yayakirildik	Natural setting	Akhisar, Manisa, Turkey
Wild 11	Bademli	Natural setting	Izmir, Dikili, Turkey
Wild 12	Karacakas	Natural setting	Soma, Manisa, Turkey

*ORI: Olive Research Institute, Izmir, Turkey.

(2002) used 30 primers on 103 olive species, and obtained evaluable bands from 21 of the same. Some of the primers they obtained at the end are (OP-A 1, OP-A 19, OP-X 9, OP-Z 7 and OP-K 16).

Belaj et al. (2003) used 16 primers in their study they performed for comparing 19 Albanian olive species and 2 wild olive types (OP-A 1, OP-A 2, OP-A 3, OP-A 8, OP-A 19, OP-I 6, OP-I 12, OP-I 14, OP-K 7, OP-K 716, OP-K 717, OP-P 19, OP-Q 12, OP-X 1, OP-X 13 and OP-Z 10) and they obtained 76 polymorphic bands. The results of DNA based markers were evaluated with some multivariate methods such as clustering analysis (Duarte et al., 1999). For revealing genetic relationships between the genotypes, a genetic similarity or dissimilarity matrix should be constructed. These matrices can be calculated by several suggested similarities or distance coefficients in literature (Duarte et al., 1999). The genetic similarity coefficients such as Jaccard (1901), Sorensen-Dice (1948, 1945) and Simple Matching (Sokal and Michener, 1958) were the used binary matrix based on the presence (1) or absence (0) of electrophoretic bands for calculating the genetic similarity or dissimilarity matrices. These coefficients are built similarity matrices by pairwise comparison between co-occuring bands (1-1), different bands (1-0 or 0-1) or negative co-occurrences (0-0) (Jackson et al., 1989; Skroch et al., 1992; Da Silva et al., 2004). The correct selection of a similarity coefficient is an important step for constructing dendrograms which reveals phylogenetic relationships between genotypes because it affects the clusters obtained (Jackson et al., 1989).

Jaccard and Sorensen-Dice coefficients exclude negative co-occurrences in their equations while Simple Matching coefficient involves shared absence of DNA bands. In connection with the results of clustering and selection of the coefficients, the similarity coefficients using binary variables are suggested for dominant markers such as randomly amplified polymorphic DNA (RAPD) by Duarte et al. (1999). NJ (neighbor joining) is one of the commonly used clustering method for constructing dendrograms. However, UPGMA (unweighted pair group method with arithmetic averages) is an older clustering algorithm (Sneath and Sokal, 1973; Saitou and Nei, 1987) and different from the NJ because, producing trees from large data sets is easy and provides additional information on relationships between genotypes (Allendorf and Luikart, 2007).

The first objective of this study was to seek how to acquire information on the genetic structure of olive plant using DNA fingerprinting, and it was examined as to whether different primers used in RAPD analysis yielded bands. Therefore, second, the objective was to investigate how the three commonly used genetic similarity coefficients (Jaccard, Sorensen-Dice and Simple Matching) influenced clustering of the wild olive samples and affect of 2 clustering algorithms (UPGMA, neighbor joining) on dendrograms.

MATERIALS AND METHODS

Plant material

Samples of wild olives were obtained from villages of Manisa, Mugla and Izmir provinces, except the Bornova samples which were obtained from ORI (Olive Research, Institute, Bornova, Izmir, Turkey). Young leaves without necrotic areas or lesions were collected and stored in liquid nitrogen until DNA extraction. Table 1 shows the wild olives used in this study, their origins and places where they were supplied.

DNA extraction

Genomic DNA was extracted from young leaves by using Doyle and Doyle method (1987). Young plant tissues were ground with liquid nitrogen in mortar with pestle until no large parts of tissue remain. Ground tissues were immediately poured into 1.5 ml Eppendorf tubes and 700 µl preheated CTAB extraction buffer (2% CTAB, 20

Table 2. Base sequences of primers providing evaluable bands, number of bands they provided and base pair sizes.

Primer	Evaluable bands	Number of bands	Base pair sizes (bp)
OP- Q 1	GGGACGATGG	1	5333
OP-Q 2	TCTGTCGGTC	2	2649-2627
OP-Q 3	GGTCACCTCA	1	6333
OP-Q 4	AGTGCGCTGA	1	5000
OP-Q 11	TCTCCGCAAC	3	4615-4385
OP-Q 12	AGTAGGGCAC	4	4643-4286
OP-Q 13	GGAGTGGACA	1	4571
OP-Q 14	GGACGCTTCA	2	4500-4357
OP-Q 15	GGGTAACGTG	3	4600-4467
OP-Q 16	AGTGCAGCCA	3	4300-4200
OP-Q 17	GAAGCCCTTG	6	4882-4388
OP-Q 18	AGGCTGGGTG	5	4500-4063
OP-Q 19	CCCCCTATCA	4	4290-4097
OP-Q 20	TCGCCCAGTC	3	4433-4333
OP-I 4	CCGCCTAGTC	3	875-833
OP-I 14	TGACGGCGGT	14	1171-304
OP- I 15	TCATCCGAGG	2	1255-953
OP- I 16	TCTCCGCCCT	14	1568-585
OP- I 17	GGTGGTGATG	17	1263-312
OP-F 15	CCAGTACTCC	3	4500-4200

mM EDTA, 1.4 M NaCl, 100 mM Tris-HCl pH 8.0, 2% β-mercapto-ethanol) was added into leaf powder, and mixed several times by gentle inversions. Samples with CTAB buffer were incubated for 30 min in 65°C. Tubes were mixed by inversions in every 5 min. Tubes were removed from water bath, and allowed to cool down, and then added to 700 µl of cold mixture of chloroform : isoamyl alcohol (24:1). Tubes were spun for 10 min at 10000 rpm/min in a refrigerated centrifuge. Supernatants were transferred into new tubes; 600 µl of cold mixture of chloroform : isoamyl alcohol (24:1) was added, and mixed by gentle inversions for 5 min. Samples were spun for 10 min at 10000 rpm in a refrigerated centrifuge again and supernatants were transferred to fresh tubes including 10 M ammonium acetate and 3 M sodium acetate; 500 µl of cold isopropanol was added and mixed by shaking very gently for DNA precipitation. Precipitated DNA was removed with pipette, and washed with 70% ethanol. DNAs were dried and re-suspended in 50 µl EDTA. RNAase was added against the possibility of contamination, in the content of 100 µl DNA solution and 1 µl RNAase.

Spectrophotometric analysis

For the determination of DNA quality and concentration of DNA samples, samples were both subjected to spectrophotometric analysis and run in 0.8% agarose gels. In spectrophotometric analysis, each sample of DNA was calculated by their optical density values at 230, 260 and 280 nm. Optical density ratios were evaluated and only good quality DNA samples were used in polymerase chain reaction (PCR) (Wu et al., 2004).

Polymerase chain reaction (PCR) analysis

Fifty five arbitrary decamer primers obtained from Operon Technologies (Alameda, CA, USA) were used for RAPD assay. OP-Q (1-20), OP-I (1-20), OP- F (11, 12, 13, 14, 15), OP-X (1, 2, 3, 4, 5, 6, 7, 8, 9, 10) primer sets and 4 primers from Mekuria et al. (2002) (GC-1 5'-CAGGCCCTTC; GC-2 5'-AGGGGTCTTG; GC-185'-AGGTGACC GT; GC-20 5'-GTTGCGATCC, Qiagen, Operon) were used for PCR.

PCR was performed on an Eppendorf MasterCycler Thermal Cycler in a total volume of 25 µl. The PCR mix included 25 ng template DNA, 2.42 µl 10 X PCR reaction buffer with $MgCl_2$ (Sigma), 0.44 µl dNTP (Sigma), 1 µM primer and 0.13 µl Taq DNA polymerase (Sigma). The amplification reactions were carried out for 60 s at 94°C as an initial denaturation. The PCR program comprised 35 cycles of 20 s at 94°C for denaturation, 20 s at 35°C for annealing and 20 s at 72°C for extension, and a final extension was performed at 72°C for 5 min.

Electrophoresis and data analysis

PCR products were separated 1.5% agarose gels (Sigma) in 0.5 X TBE buffer with 0.5 µg/ml ethidium bromide at 100 V constant voltages. For evaluating the base pair length of bands, DNA ladder (Fermentas) was loaded on first lane of each gel. Data statistics such as fixed and private bands in groups were calculated with FAMD 1.123 program (Schluter and Harris, 2006).

RAPD bands were used for the estimation of the binary matrix, using absence (0) and presence (1) of bands (Wu et al., 2004). For the calculation of genetic similarity from binary matrix, each pair of samples were compared with Jaccard (1901), Sorensen-Dice (1948, 1945) and Simple Matching (Sokal and Michener, 1958) genetic similarity coefficients. Genetic similarities originated from pairwise comparing of binary matrix values which were transformed into genetic dissimilarities and applied for the construction of corresponding dendrograms (Duarte et al., 1999). The dendrograms of 12 wild olives were constructed by using UPGMA (unweighted pair group mean arithmetic method) and NJ (neighbour joining) methods (Sneath and Sokal, 1973; Saitou and Nei, 1987). The genetic dissimilarity matrices and dendrograms were calculated with FAMD 1.123 program (Schluter and Harris, 2006).

Table 3. Distribution of bands among samples based on the primers in OP-Q set.

Primer	Wild 1	Wild 2	Wild 3	Wild 4	Wild 5	Wild 6	Wild 7	Wild 8	Wild 9	Wild 10	Wild 11	Wild 12
OP-Q 1							■	■	■			■
OP-Q 2								■				
OP-Q 2		■										
OP-Q 3								■				
OP-Q 4								■				
OP-Q 11									■			
OP-Q 11								■				
OP-Q 11							■					
OP-Q 12									■			
OP-Q 12							■					
OP-Q 12								■				
OP-Q 12			■									
OP-Q 13						■						
OP-Q 14							■	■				■
OP-Q 14								■				
OP-Q 15												■
OP-Q 15										■		
OP-Q 15		■		■		■		■				
OP-Q 16		■							■			
OP-Q 16							■	■				
OP-Q 16			■									
OP-Q 17									■			■
OP-Q 17										■		
OP-Q 17				■								
OP-Q 17			■				■	■				
OP-Q 17					■							
OP-Q 17		■										
OP-Q 18												■
OP-Q 18							■	■	■			
OP-Q 18				■								
OP-Q 18			■		■							
OP-Q 18	■											
OP-Q 19												■
OP-Q 19					■		■		■			
OP-Q 19								■				
OP-Q 19				■								
OP-Q 20									■			■
OP-Q 20					■		■	■				
OP-Q 20		■										

RESULTS AND DISCUSSION

A total of 92 bands were obtained from twelve wild olive samples. Thirty five of the OPERON primers were not given scorable bands (OP-Q 5, OP-Q 6, OP-Q 7, OP-Q 8, OP-Q9, OP-Q10 for OP-Q set; OP-I 1, OP-I 2, OP-I 3, OP-I 5, OP-I 6, OP-I 7, OP-I 8, OP-I 9, OP-I 10, OP-I 11, OP-I 12, OP-I 13, OP-I 18, OP-I 19, OP-I 20 for OP-I set; OP-X 1, OP-X 2, OP-X 3, OP-X 4, OP-X 5, OP-X 6, OP-X 7, OP-X 8, OP-X 9, OP-X 10 for OP-X set; OP-F 11, OP-F 12, OP-F 13, OP-F 14 for OP-F set). Also, four designated primers from Mekuria et al. (2002) did not provide evaluable bands.

Scorable bands were obtained from 20 of a total of 59 primers. Thirty nine bands were determined in OP-Q series; 50 in OP-I series; and 3 in OP-F 15 primer. The number of bands per primer was 2.785 in OP-Q primers; 10 in OP-I primers; and 3 for OP-F 15. When evaluated based on the number of active total primers, the number of bands per primer was found to be 4.6. Table 2 shows

Table 4. Distribution of bands among samples in accordance with the primers in OP-I set and in OP-F 15 primer.

Primer	Wild 1	Wild 2	Wild 3	Wild 4	Wild 5	Wild 6	Wild 7	Wild 8	Wild 9	Wild 10	Wild 11	Wild 12
OP-I 4											■	
OP-I 4										■		
OP-I 4		■	■									
OP-I 14											■	■
OP-I 14												■
OP-I 14											■	
OP-I 14											■	
OP-I 14												■
OP-I 14							■				■	
OP-I 14												■
OP-I 14												■
OP-I 14											■	
OP-I 14											■	■
OP-I 14											■	
OP-I 14												■
OP-I 14												■
OP-I 14											■	■
OP-I 15										■		
OP-I 15										■		
OP-I 16											■	
OP-I 16												■
OP-I 16										■		
OP-I 16									■			
OP-I 16				■							■	
OP-I 16											■	■
OP-I 16												
OP-I 16									■			
OP-I 16				■							■	
OP-I 16									■			
OP-I 16				■								
OP-I 16				■								
OP-I 16											■	
OP-I 16								■				
OP-I 17											■	
OP-I 17	■	■		■			■					
OP-I 17			■									
OP-I 17					■	■						
OP-I 17		■		■			■					
OP-I 17	■		■									
OP-I 17									■			
OP-I 17										■	■	
OP-I 17							■					
OP-I 17							■				■	
OP-I 17	■	■				■				■		
OP-I 17			■	■								
OP-I 17									■	■	■	■
OP-I 17			■	■			■					
OP-I 17						■						
OP-I 17	■											
OP-F 15	■	■										
OP-F 15								■	■			
OP-F 15						■	■					

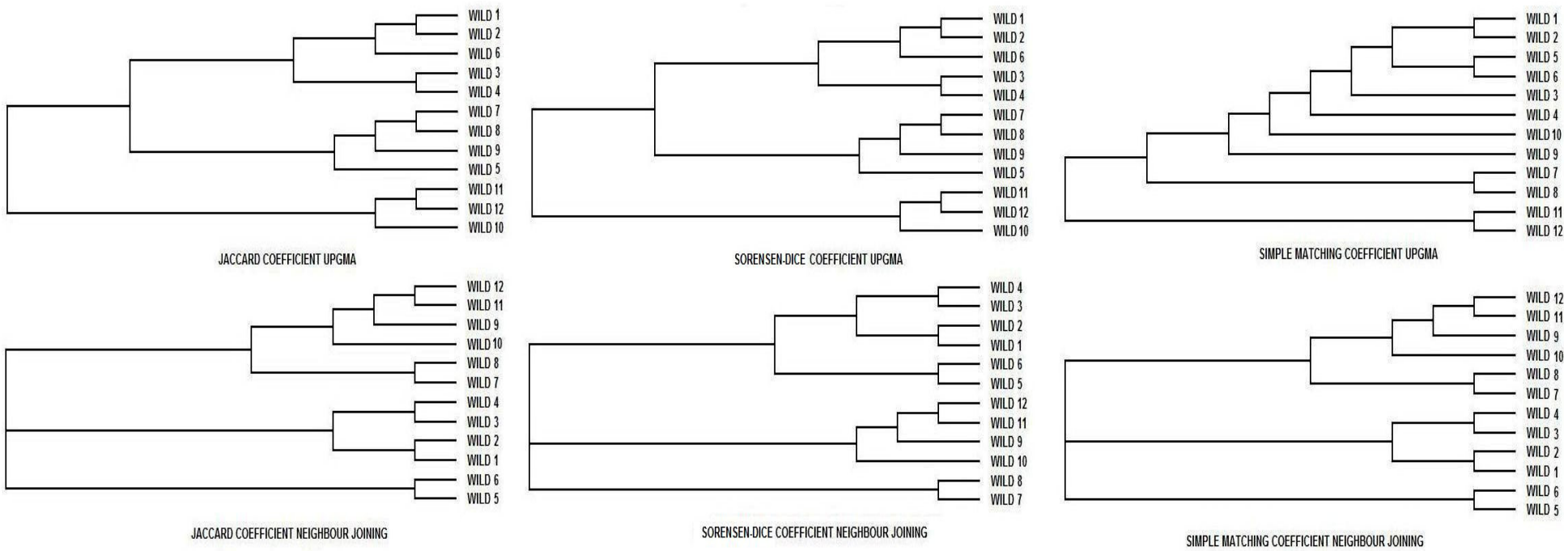

Figure 1. The dendrograms based on UPGMA and NJ algoritms.

primers that provide evaluable bands, the band sequences of primers, number of bands they provided and molecular sizes of such bands in base pairs.

When the bands were provided by primers, it was determined that the molecular sizes in OP-Q primers varied between 6333 and 2627 bp; between 1568 and 304 bp in OP-I primers, and between 4500 and 4200 bp in OP-F 15 primer. OP-I primers provided band with less molecular weight as compared to OP-Q primers and OP-F 15 primer; in addition, the primers that provided maximum bands in terms of total band providing primers were OP I-14, OP I-16 and OP I-17. Minimum numbers of bands were obtained from OP-Q 1, OP-Q 3, OP-Q 4 and OP-Q 13 with one band each; and maximum bands were obtained from OP-Q 17 with six bands. In OP-I set, minimum bands were obtained from OP-I 15 with two bands, and maximum bands were obtained from OP-I 17 primer with seventeen bands.

Total number of polymorphic bands is 92; and no monomorphic bands were determined. Mean number of polymorphic bands per sample was calculated as 7.67; and the number of polymorphic bands per active primer was obtained as 4.6. Bands were grouped as a Manisa (Caglak, Harlak, Sabancilar, Yayakirildik and Karacakas), Izmir (Bornova and Bademli) and Mugla (Pinarcik); 6 private bands were found in Mugla group, 23 in Izmir group and 36 in Manisa group. Also, 3 fixed bands were found in group Manisa. Table 3 shows the distribution of bands among the samples based on the primers in OP-Q set; and Table 4 shows the distribution of bands among primers in OP I set and OP-F 15 primer. The closest samples based on their genetic dissimilarity values have been found as wild 1 and wild 2, wild 7 and wild 8 and the most distant ones have been found as wild 10 and wild 12 in both of Sorensen - Dice and Jaccard coefficients. In the coefficient including negative co-occurrences such as Simple Matching, the closest samples based on their genetic dissimilarity values were determined as wild 5 and wild 6 and the most distant samples as wild 8 and wild 11 which are different from Jaccard and Sorensen – Dice coefficients. Figure 1 shows the dendrograms

based on UPGMA and NJ algoritms.

While previous morphological characters were used for identifying the plants, today DNA, which is not affected by external factors is used (Gonzalo-Claros et al., 2000). In conclusion, wild olive samples grown in Manisa, Mugla, and Izmir provinces were examined in terms of bands by using DNA fingerprinting; their band distributions were defined based on the primers; and optimum conditions showed that they would be useful for further studies. In addition, the ability to obtain effective results was discussed through the dendrograms developed in determining the relation between wild olives by means of similarity coefficients and developed based on such coefficients. Accordingly, it was determined in this study that Jaccard and Sorensen Dice, and Simple Matching were suitable similarity co-efficients as mentioned by Rabie (2010) and that the NJ method was more feasible in developing phylogenetic tree as compared to UPGMA which was determined by Saitou and Nei (1987).

ACKNOWLEDGEMENT

This research was supported by the Turkish Republic State Planning Organization.

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