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Full Length Research Paper

Genetic diversity of male fig (*Ficus carica caprificus* L.) genotypes with random amplified polymorphic DNA (RAPD) markers

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The objective of this research is to characterize and relate 43 different male fig (*Ficus carica caprificus* L.) genotypes grown in Aydın Province, Turkey using random amplified polymorphic DNA (RAPD) markers. A total of 85 10-mer primers were tested with the RAPD technique. A total of 76 polymorphic bands out of 272 bands (27.9%) were observed from 36 primers. The molecular size varied from 200 - 3000 bp. Genotypes were placed in nine different groups in the dendrogram drawn from the similarity matrix. The closest genotypes are 'Yanako1' and 'Yanako2'. The relationship between the genotypes and their respective regions supported by the dendrogram is incongruent. Polymorphic information content (PIC) values ranged from 0.16 (OPD08) to 0.50 (OPC01 and OPC04) for the polymorphic data set. The larger source of variation was observed within populations (88.58%) followed by among populations (11.42%) in the analysis of molecular variance. The relationships among genotypes were defined by the first three principle components (PC), accounting for 40.91% of the total variation at the molecular level.

Key words: *Ficus carica caprificus*, genetic diversity, Aydın province, random amplified polymorphic DNA markers, fingerprinting, analysis of molecular variance, polymorphic information content, principle component analysis.

INTRODUCTION

Fig plants (*Ficus carica* L., 2n = 26) belong to the Moraceae family. Within the genus *Ficus*, approximately 400 monoecious and 350 gynodioecious (Parrish et al., 2004) or 800 (Al Malki and Elmeer, 2010) species exist. The Mediterranean Basin, including Anatolia, is the origin of cultivated figs. Fossils of dried fig fruit (syconia) and seeds (drupelets) dating back to 11,400 years from the present were excavated during archaeological surveys in the Gilgal Village of the Lower Jordan Valley (Kislev et al., 2006). Turkey is the world's leading producer country (205,067 t, 18.5%) in both the fresh and dried fig markets (1,108,398 t) (FAOSTAT, 2008). Aydın Province produces approximately 90% of the dried figs in Turkey (DIE, 2001) with the most widely grown 'Sarılop' (synonym 'Calimyrna').

Fig plant populations, consisting of female (morphologically and physiologically female) and male (morphologically hermaphrodite and physiologically male) trees, are considered as gynodioecious according to their functional flower parts (Condit, 1955; Storey, 1975). Within a male syconium, female flowers reside close to the peduncle, while male flowers are placed around the ostiolum (opening at the flower end of the receptacle). A male fig plant, called caprifig, gives fruit in three crop

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Abbreviations: PCR-RFLP, Polymerase chain reaction-restriction fragment length polymorphism; **RAPD,** random amplified polymorphic DNA; **AFLP,** amplified fragment length polymorphism; **CTAB,** cethyl trimethyl ammonium bromide.

cycles of each growing season, such as in summer (profichi, “ilek”), fall (mammoni, “ebe”), and winter (mamme, “boğa”). The main caprifig crop, referred to as “ilek” in the region, coincides with the main summer crop cycle of female trees (“iyilop”). Although most male fig trees do not produce edible fruit, they contribute a pollen source for caprification, meaning the transfer of pollen grains from male trees to female trees by a vector, *Blastophaga psenes* L.

Caprifigs play an important role in local markets. Since each genotype has unique characteristics such as different flowering times coinciding with the female flowers, the number and pollination quality (germinability) of pollen grains and tolerance/resistance to habitation by the insects causes sour rot of the edible crop (Ölçer, 1968; Öncel, 1969; Eroğlu, 1982; Kabasakal, 1990; Zeybekoğlu et al., 1998; Akaroğlu et al., 2004). While some female fig genotypes do not require caprification (persistent, parthenocarp), most do require outcrossing (caducous) for better fruit set. Since the caprifig fruit are sold in bulks of low and high quality fruit mixed within a basket or bag, the first caprification is insufficient to produce enough good figs. The producers need a second or even third caprification, spending two to three times as much labor and time. ‘Sarılop’ needs better quality caprification to obtain export quality.

Nursery practices of calling the same germplasm from different locations by different names (synonyms), causes suspicion for both nurserymen and consumers. One of the ways to solve such a problem is to analyze them using molecular markers polymorphic among different germplasm for identification of genetically distinct plants. Different molecular marker systems have been used to characterize germplasm although results are limited in *F. carica* compared to other plant species. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Saiki et al., 1985), random amplified polymorphic DNA (RAPD) (Welsh and McClelland, 1990; Williams et al., 1990) marker technology has been widely used in perennial woody plant population studies. In figs, besides other marker technologies used such as isozymes (Uzun et al., 2003), mtDNA RFLP (Khadari et al., 2005b), amplified fragment length polymorphism (AFLP) (Cabrita et al., 2001; Parrish et al., 2004), microsatellites (Khadari et al., 2001, 2003a, 2003b, 2005a; Saddoud et al., 2005; Salhi-Hannachi et al., 2004; Giraldo et al., 2005; Crozier et al., 2007; Giraldo et al., 2008), random amplified microsatellite polymorphisms (Chatti et al., 2007), RAPDs give sufficient polymorphism to determine within species similarity for local cultivars (Khadari et al., 1995; Elisiario et al., 1998; Galderisi et al., 1999; Cabrita et al., 2001; Papadopoulou et al., 2002; Aka-Kacar et al., 2003; Khadari et al., 2003a; De Masi et al., 2003; Hepaksoy et al., 2004; Sadder and Ateyyeh, 2006). Since there have not been any genetic diversity study results in the literature dealing with only male fig genotypes using RAPD marker system, which is easy, fast and convenient, saves money and time for routine DNA fingerprinting analyses, it makes the current research original in its area. The objectives of this research are (I) to characterize different male fig genotypes using RAPD markers and (ii) to relate the male fig genotypes and their respective locations in Aydın Province, Turkey.

MATERIALS AND METHODS

Young leaves of 43 caprifig genotypes gathered from nine different districts (Argavlı, Bozdoğan, Gümüşköy, İmamköy, Kuyucak, Naipli, Ortaklar, Ödemiş and Ömerbeyli) were collected from the Erbeyli Fig Research Institute, Aydın Province, Turkey. The summer crop of male figs matured between 08 (‘Şeytan1’) and 24 June (‘KaraErkek’) (Eroğlu, 1982).

The DNA extraction was performed according to the cethyl trimethyl ammonium bromide (CTAB) method modified from Doyle and Doyle (1990), Rogers and Bendich (1994), and Okuno and Fukuoka (1998) in the Science and Technology Research and Applied Center of Adnan Menderes University. The leaves were ground to powder with liquid nitrogen using a glass rod in 1.5 ml Eppendorf tubes. To each sample, 600 µl 1% CTAB was added. After vortexing, the samples were incubated at 65°C at 400 *g* for 10 min (Eppendorf Thermomixer Comfort, Hamburg, Germany). Then an equal volume (600 µl) of 24:1 chloroform : iso-amyl alcohol was added. After vortexing, centrifugation was performed at 11,200 *g* at 4°C for 5 min (Eppendorf Centifuge 5415R, Hamburg, Germany). After the supernatant was transferred and washed with choloroform : iso-amyl alcohol twice, 750 µl cold 2-propanol was added and inverted gently. The samples were incubated at 4°C for 10 min in the refrigerator. The final centrifugation was performed at 11,200 *g* at 4°C for 15 min, producing a pellet. The aqueous phase was decanted. The samples were rinsed with 500 µl 70% ethanol. The samples were dried at 26°C in the sterile hood with continuous air flow. The pellet was dissolved in 100 µl 1× TE and RNase (10 ng/µl) was added. Approximate DNA content was recorded at 260 nm wavelength (Shimadzu UV-1601 UV-visible Spectrophotometer, Tokyo, Japan). The DNA amount was equalized to 50 ng/µl for each sample with TE.

Each PCR was performed with 15 µl total solution in thin-walled (0.2 ml) tubes (Greiner Bio-One, Hamburg, Germany). The final concentration of each component in solution consisted of 500 mM KCl, 10 mM Tris-HCl pH 8.8, 25 mM $MgCl_2$, 1 unit *Taq* DNA polymerase (Fermentas, Vilnius, Lithuvania), 10 mM each of dNTP (dATP, dCTP, dGTP and dTTP), 10 µM 10-mer primer (OPA, OPB, OPC and OPD from QIAGEN Operon GmbH, Cologne, Germany and Dat2 and Dat5 from custom-made from Galderisi et al. (1999)), 50 ng/µl male fig genomic DNA and sterile ddH_2O (Millipore Simplicity 185, France). The PCR program was as follows: 30 s at 94°C initial denaturation, 25 s at 94°C denaturation, 45 s at 35°C annealing (the best band profile obtaining temperature), 60 s at 72°C extension for 35 cycles and 5 min at 72°C final extension (Eppondorf Mastercycler Gradient, AG 22331, Hamburg, Germany). PCR samples were died by adding 2 µl of 6 × loading dye solution and separated on 1.7% (w/v) agarose gels containing 2 µl (0.625 mg/ml) ethidium bromide in 0.5 × TBE buffer for 40 min at 96 V. Then, a photograph was taken under UV light using a color camera image system (EDAS 290, Eastman Kodak Company, Rochester, NY, USA).

Band presence (1), absence (0) and missing (-) were recorded from the gels corresponding to the respective marker, that is, OPA07-1700 where 1700 bp fragment shown in OPeron set-A primer

number 07. A similarity matrix was calculated using the Dice coefficient (Galderisi et al., 1999) with the unweighted pair group method using arithmetic average (UPGMA) as a clustering algorithm using NTSYSpc (Numerical Taxonomy and Multivariate Analysis System, Applied Biostatistics Inc., ABD). The cophenetic correlation with ultrametric distance method was calculated by using VARCOV, COPH, and MXPLOT modules. The stability of the dendrogram was determined by the bootstrap procedure based on 1,000 permutations using MEGA3 (Molecular Evolutionary Genetics Analysis (Kumar et al., 2004). The principle component analysis (PCA) was performed using EIGEN module of NTSYSpc. The polymorphic information content (PIC) was computed as:

$PIC_i = 2f_i(1-f_i)$

Where, PIC_i is the polymorphic information content 'i', f_i is the frequency of the amplified allele (band present) and $(1-f_i)$ is the frequency of the null allele (band absent) (Roldán-Ruiz et al., 2000; Soengas et al., 2006). The analysis of molecular variance (AMOVA, Excoffier et al., 1992, 2006) was performed by ARLEQUIN 3.1. The total variance was partitioned into three sources of variation where locations were considered as populations.

RESULTS AND DISCUSSION

This is the first report distinguishing 43 male fig genotypes contained in Erbeyli Fig Research Institute, Aydın Province, Turkey with RAPD markers. Out of a total of 85 primers (OPA01-A20, B01-B20, C01-C20, D01-20 and Dat1-5*)*, 49 primers gave scorable bands in the first round of PCR performed using one male fig's genomic DNA. In the second run, 43 male fig genotypes were screened with 49 primers producing 357 total bands. Only 36 primers gave a total of 272 bands, ranging from 200 - 3000 bp in length, out of these, 76 clearly amplified polymorphic bands (ranging from one to six bands) were obtained (9.1% in OPD08-75.0% in OPB18 with an average of 27.9%) (Table 1 and Figure 1). OPA07-1700 does not exist in 'Kızılay-1', OPA20-650 does not exist in 'Çaçaron' and 'Siyah', OPB05-700 does not exist in 'Kızılay-1' and 'Hamza', OPB16-1200 does not exist in 'Hamza' and 'Kavun', OPB20-950 does not exist in 'Gabalı' and 'Damarlı', OPC01-1900 does not exist in 'Siyah' and 'MorDemirtaş', OPB15-400 exists in 'KKonkur' ('KüçükKonkur'), 'Kızılay-2', and 'Karaerkek',OPD20-1200 exists in 'MorDemirtaş' and 'Damarlı'. While an average band number per individual was 8.3, the average for polymorphic bands was 1.8. The highest number (6) of polymorphic bands was obtained from primers OPB20 and OPC04. When De Masi et al. (2005) worked with 39 samples of *F. carica* in Italy, seven of 20 arbitrary oligonucleotide primers clearly differentiated the fig trees (35.0%). In the current study, 36 out of 49 primers gave polymorphism (73.5%). This might be due to the use of different genotypes and more arbitrary primers in the current study. Lower values (less than 0.90) indicate that more chance seedlings have been used for caprifig reproduction. Therefore, the caprifig genotypes collected from different districts in the current study most probably were chance seedlings.

Each individual genotype displayed a characteristic banding pattern of identity confirming that any respected individual is not synonymous at the same germplasm collection site reported by the previous study of Eroğlu (1982). From the custom ordered primers used in this study, primers Dat2, Dat4 and Dat5 gave 1 (7), 0 (4), and 5 (8) polymorphic (total) bands, respectively. Galderisi et al. (1999) used the same primers and obtained 4 (6), 8 (11) and 1 (5) polymorphic (total) bands, respectively. These band number and polymorphism differences might be due to the differences either in PCR conditions (35°C compared to 40°C in primer annealing temperature) or in gel reading sensitivity between laboratories. The different genotypes used in these two studies can be considered as another source of variation.

A dendrogram was constructed according to calculated genetic similarities with the highest cophenetic correlation coefficient (r = 0.5526) (Figure 2). The dendrogram is superficially divided into nine groups in an attempt to represent the sampling locations of the genotypes. While 'Kızılay2' was placed in Group I, other male fig genotypes were in eight groups (II-IX). 'Yanako1' and 'Yanako2' selected from Gümüşköy District were found to be the most similar (0.92) male fig genotypes. The lowest genetic similarity (0.56) was observed between 'Gabalı' (from Naipli District) and 'Kızılay2' (from Argavlı District). In a study by Khadari et al. (2005a), all eight male fig genotypes were placed in the same group in the dendrogram of four groups. Exact differentiation could not be reached since three mis-labeled and four homonym genotypes were determined. The genotypes collected from one region were placed in different groups in the dendrogram. Also, Giraldo et al. (2008) reported that since accessions from different geographic areas were placed in the three main groups, geographic origin was not the main criterion. In the current study, it was similarly found that the genotypes collected from the same regions were placed in different groups in the dendrogram.

A relationship between the 43 male fig genotypes and their respected locations was weakly supported in this study. While 6 genotypes, namely 'AyarDolduran', 'AkErkek1', 'AkErkek2' and 'Armut' (0.91), and 'Conkurt' and 'KaraErkek' (0.84), form Ödemiş District were together in group II; 'HacıAbdullah' and 'Çiçekli2' were placed in groups V and VIII, respectively. While two of the genotypes ('Ak' and 'Afyoncu', 0.83) collected from İmamköy District were placed in group V; 'Kıbrıslı', 'Karabulut' and 'KKonkur' (0.82), 'Elma' and 'Mor' (0.85), and 'BKonkur' ('BüyükKonkur') were placed in groups IV, VI, VIII, and IX, respectively. 'KaraErkek2' and 'Sarı' (0.80) from Bozdoğan District were in group VII. The other three genotypes, 'Taşlık', 'Kızılburun' and 'Frenk', from Bozdoğan District were placed in group IX. In the work done by Saddoud et al. (2005), the differences

Table 1. Results of 36 arbitrary primers in RAPD analysis of male fig genotypes.

Primer	Total DNA band	Polymorphic DNA band	Polymorphism ratio (%)	Band (bp)	PIC
OPA07	8	2	25.0	550-2900	0.38
OPA08	6	1	16.7	400-2800	0.28
OPA11	8	1	12.5	250-2100	0.22
OPA15	9	2	22.2	300-2000	0.35
OPA17	7	2	28.6	500-1600	0.20
OPA19	6	1	16.7	650-3000	0.28
OPA20	7	1	14.3	600-2000	0.25
OPB01	6	1	16.7	400-2500	0.28
OPB04	10	3	30.0	250-2000	0.42
OPB05	6	1	16.7	700-2000	0.28
OPB06	7	1	14.3	350-2000	0.25
OPB07	6	1	16.7	300-1800	0.28
OPB11	8	1	12.5	350-2100	0.22
OPB15	8	1	12.5	350-1450	0.22
OPB16	5	3	60.0	900-1600	0.48
OPB18	4	3	75.0	550-1500	0.38
OPB20	9	6	66.7	450-2000	0.44
OPC01	8	4	50.0	300-1900	0.50
OPC02	6	2	33.3	400-1500	0.44
OPC04	11	6	54.5	300-2500	0.50
OPC07	8	2	25.0	450-1600	0.38
OPC10	7	4	57.1	700-2500	0.49
OPC11	7	1	14.3	350-1500	0.25
OPC14	10	3	30.0	200-1300	0.42
OPC15	9	3	33.3	400-1800	0.44
OPC16	8	3	37.5	500-2400	0.47
OPC18	5	1	20.0	500-1500	0.32
OPC20	5	3	60.0	500-2400	0.48
OPD02	7	1	16.7	400-1500	0.28
OPD05	7	1	14.3	320-1300	0.25
OPD08	11	1	9.1	300-2000	0.16
OPD11	11	2	18.2	300-2500	0.30
OPD15	7	1	14.3	450-2800	0.25
OPD20	10	1	10.0	300-2500	0.18
Dat2	7	1	14.3	200-1500	0.25
Dat5	8	5	62.5	300-2800	0.47
Total	272	76			

among geographical regions were low. With the factorial relationship analysis, positive relationships between 16 cultivars and their geographical origin could not be determined.

PIC values ranged from 0.16 (OPD08) to 0.50 (OPC01 and OPC04) for the polymorphic data set (Table 1). The small differences among genotypes also were attributable to frequency differences in variable markers as observed in ryegrass (Roldán-Ruiz et al., 2000). The analysis of molecular variance (AMOVA) showed that two sources of variation were significant ($P < 0.001$) (Table 2). The larger source of variation was observed within populations (88.58%) followed by between populations (11.42%). The percentage of variation within groups (34.0%) was also higher than that between groups (15.5%) in a study of *Brassica napus* (Soengas et al., 2006). The relationships among genotypes were defined by the first three principle components (PC) (eigenvectors), accounting for 40.91% of the total variation at the molecular level (Figure 3). The genotypes were placed 0.12-0.36 and – 0.28-0.22

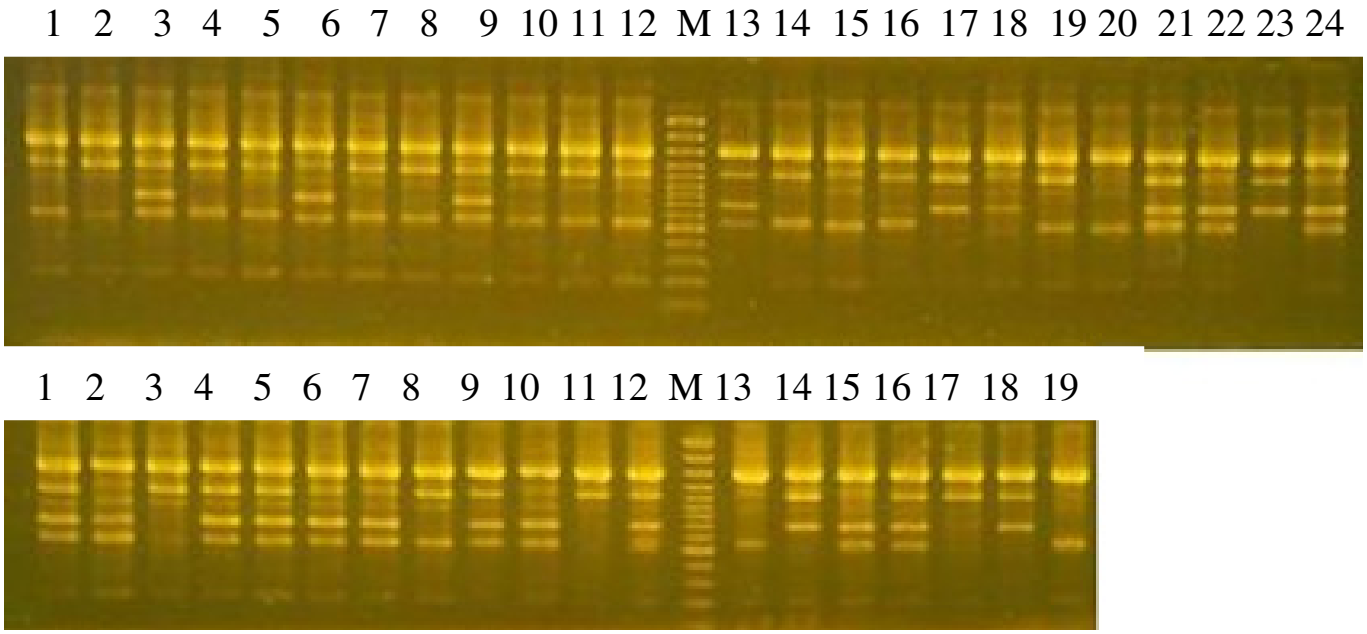

Figure 1. A RAPD pattern of OPB18 primer with 43 male fig genotypes. From left to right (upper row): 1: 'Çakın1', 2: 'Şeytan2', 3: 'Çaçaron', 4: 'DervişAli', 5: 'Kızılay1', 6: 'Siyah', 7: 'Kuyucak', 8: 'Bostancı', 9: 'Kaba', 10: 'Yanako1', 11: 'Taşlık', 12: 'KKonkur', M: 100 bp (Fermentas, GeneRuler™ DNA Ladder Plus), 13: 'Mor', 14: 'Afyoncu', 15: 'Kıbrıslı', 16: 'Şeytan1', 17: 'MorDemirtaş', 18: 'Hamza', 19: 'Çakın2', 20: 'Ak', 21: 'Elma', 22: 'Kavun', 23: 'Kızılay2' and 24: 'Karabulut'. (lower row): 1: 'Kara Erkek', 2: 'AkErkek1', 3: 'Çiçekli2', 4: 'AkErkek2', 5: 'Conkurt', 6: 'Armut', 7: 'AyarDolduran', 8: 'Yanako2', 9: 'Gabalı', 10: 'HacıYusuf', 11: 'Damarlı', 12: 'BKonkur', M: 100 bp, 13: 'HacıAbdullah', 14: 'Kızılburun', 15: 'Sarı', 16: 'KaraErkek2', 17: 'Frenk', 18: 'Adalı' and 19: 'Körpe'.

in dimension-1 and in dimension-2, respectively. However, the genotypes do not organize according to their respective locations. PCA can be used in yield and quality components, taxonomic similarities, and association between genetic and environmental attributes in horticultural crops (Iezzoni and Pritts, 1991). By comparison, the first three PC of 12 accessions of ryegrass (Roldán-Ruiz et al., 2000) and that of five PC of 10 accessions of European chestnuts (Ertan, 2007) explained together 51.00 and 86.44% of the total variation at the molecular level, respectively. Using Inter Simple Sequence Repeat (ISSR), fig varieties were similarly grouped to axis 1 of the PCA in agreement with UPGMA clustering (Salhi-Hannachi et al., 2004).

Since fig plants have been propagated vegetatively by means of cuttings for centuries (Kislev et al., 2006; Giraldo et al., 2008), genetic differentiation has been limited. On the other hand, the gynodioecious nature of fig flowers requires outcrossing. Since plants raised from seeds in nature are different from each other (Papadopoulou et al., 2002), a wide genetic diversity has been established. One of the ways for diversity to arise is the seed dispersal by frugivour birds and animals, which contributes to distant seed dispersal of fig plants from one region to another, after passing through their digestive systems. Another way of dispersal is by humans who transfer cuttings of plants having desired characteristics. During this transfer, however, care must be taken to carry the correct name of the individual. This study showed that RAPDs can easily be used for detecting different male fig genotypes, just before nursery plant propagation if there is any question of mis-labeling of individuals.

In conclusion, RAPD markers can be easily used to differentiate male fig genotypes. Unfortunately, the dendrogram weakly supported the relationships between the genotypes and their respective regions.

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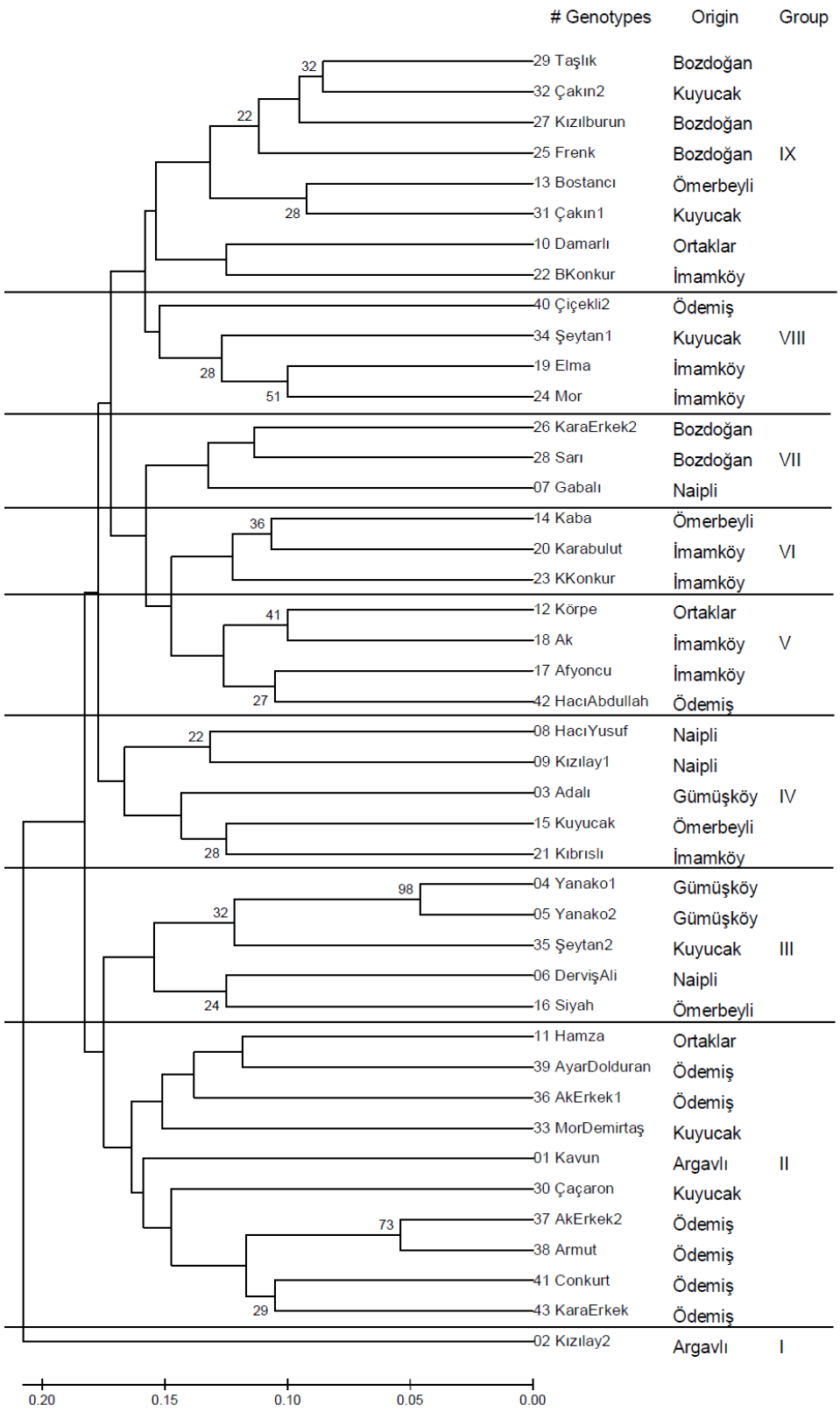

Figure 2. UPGMA dendrogram based on the Dice similarity coefficient among 43 male fig genotypes as determined by RAPD markers using NTSYSpc. Bootstrap values out of 1,000 replicates are shown when it is 20% or higher.

Table 2. Analysis of molecular variance (AMOVA) results for 43 male fig genotypes based on RAPD markers using Arlequin.

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation	*P* value
Between population	8	136.40	1.37 V_a	11.42	<0.001
Within population	34	361.93	10.64 V_b	88.58	<0.001
Total	42	498.33	12.02		

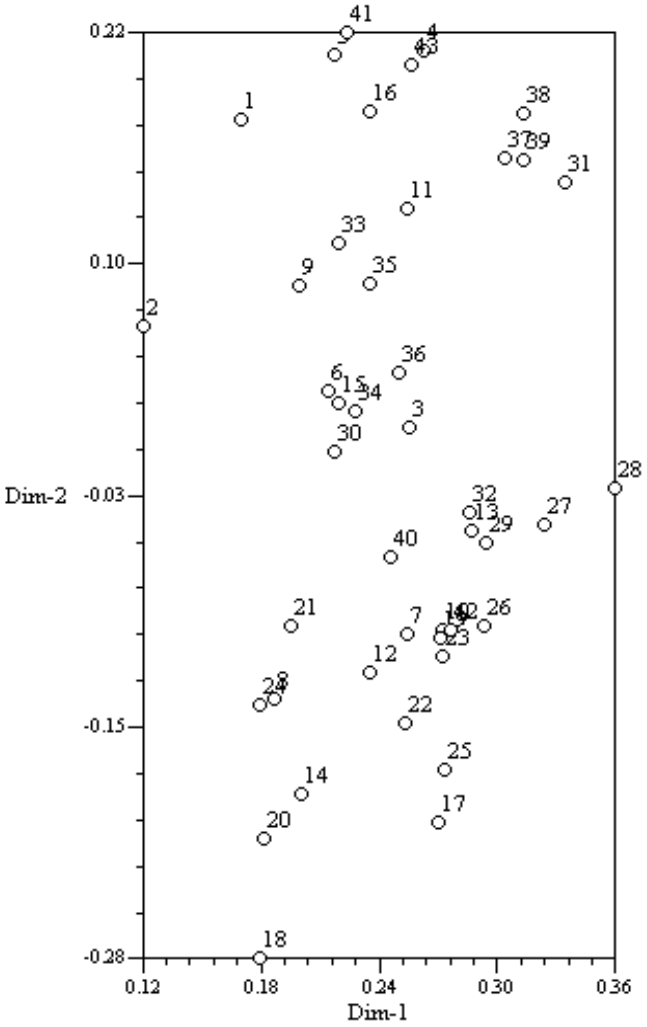

Figure 3. Principle component analysis (PCA) of 43 male fig genotypes.

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