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Original Article

Assessment of Genetic Relationship among Male and Female Fig Genotypes Using Simple Sequence Repeat (SSR) Markers

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Abstract

Fig (*Ficus carica* L.) is a traditional crop in Turkey and widely cultivated around the Mediterranean areas. The gynodioecious fig species is present in two sexual forms, i.e. the domesticated fig (female tree) and the caprifig (male tree). Caprifigs are crucial for high quality fig production and breeding while, the studies on assessment of genetic relationship among caprifigs is limited. The aim of this study was to determine genetic diversity among 45 caprifigs and 2 female figs collected from four provinces in Marmara and Aegean Sea Regions of Turkey using simple sequence repeat (SSR) markers. In this work, 24 SSR markers were tested, one was monomorphic and the remaining markers amplified 82 alleles. The number of polymorphic alleles per SSR marker ranged from 2 to 7. The observed heterozygosity (Ho) differed from 0.18 to 0.76 and expected heterozygosity (He) ranged between 0.24 and 0.81. The polymorphism information content (PIC) varied from 0.42 to 0.98. A UPGMA analysis based on Dice similarity matrix clustered fig genotypes into two main groups and similarly, STRUCTURE analysis placed fig genotypes into two different gene pools (K=2). Fig genotypes collected from the same region were not clustered together in a group indicating that the fig genotypes did not cluster on the basis of their collection sites. Our results demonstrated that caprifigs and female figs are not genetically distinct and they clustered together in a group. All fig genotypes had distinct SSR marker profiles suggesting that there were no synonyms or homonyms. These results revealed a high genetic variation among fig genotypes and 23 SSR markers were enough to discriminate all fig genotypes analysed in this study demonstrating that SSR marker system is suitable for genetic analysis in figs.

Keywords: caprifig, *Ficus carica* L., genetic variation, SSR marker, STRUCTURE analysis

Introduction

Fig (*Ficus carica* L.) has been cultivated since ancient times in the world (Janick, 2005) and Mediterranean region where Turkey is located is the origin of common figs (Caliskan and Polat, 2008). Traditionally, figs are an important ingredient in human diets in the region (Aksoy, 1998). Currently, they can be consumed as dried or fresh and excellent sources of vitamins, minerals, fiber and antioxidant compounds with health benefits (Caliskan *et al*., 2012; Mawa *et al*., 2013).

Due to its geographical and ecological conditions, Turkey is the major fig-producing country in the world. The world fig production in 2014 was 1.137.730 tons and Turkey was the leading country with the production of 300.282 t (26.3%) (FAOSTAT, 2014). According to Turkish Statistical Institute in 2015, the fig production in Turkey was mainly spread in the Aegean (76.3%), Marmara (9.6%) and Mediterranean (7.8%) regions. In addition, Turkey has a remarkable dried fig export potential with 69.3% of worldwide dried fig export (FAOSTAT, 2013).

Figs are morphologically gynodioecious but functionally dioecious species having a specific reproduction system (Stover *et al*., 2007; Flaishman *et al*., 2008). Based on pollination mechanism, there are two sexual forms of fig, i.e. the caprifig (male tree), contain both male flowers and short-styled female flowers (abortive) together so that they can be described as morphologically hermaphrodite. Therefore, caprifigs can be used only as a pollen source for pollination and they don't

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produce any edible fruits. Male fig plants give three types of fruit annually; profichi (in summer), mammoni (in autumn) and mamme (in winter). Profichi (the main crop) is used for pollination of main summer crop of female trees (Ferguson *et al*., 1990). However, female fig plants, called edible figs, have long-styled female flowers and they can produce commercial fruits. Pollination process, also called caprification in *ficus* species, occurs by transferring pollen from caprifigs to female flowers of edible figs by a pollinator wasp, *Blastophaga psenes* L. The wasp enters, oviposits and develops in caprifigs, so there has been a symbiotic relationship between *Blastophaga psenes* L. and caprifigs (Kjelberg *et al*., 1987).

Although some parthenocarpic female fig genotypes require no pollination, caprification has become a common practice in commercial fig production in order to obtain sufficient fruit yield and high quality fruit set. Various studies reported that caprified fig fruits were larger and had more phytochemicals than uncaprified figs; and also caprification increased fruit weight and yield (Rahemi and Jafari, 2008; Trad *et al*., 2013). Pollination is required for many important Turkish female fig cultivars such as 'Bursa Siyahı' and 'Sarılop' which are well-known in Marmara and Aegean Regions, respectively. 'Bursa Siyahı' is one of the best cultivars for fruit quality and fresh consumption and 'Sarılop' is the well-known cultivar for dried fig production.

Due to the easy propagation of fig via cuttings, fig genotypes or cultivars can be transferred from one region to another region without any data. Therefore, it is crucial to characterize the local fig germplasm and discover genetic diversity among the fig accessions for intensive fig cultivation (Flaishman *et al*., 2008).

There have been various studies on the characterization of fig genotypes using of morphological traits (Caliskan and Polat, 2008; Giraldo *et al*., 2010; Pérez-Sánchez *et al*., 2016). However, morphological characters can be influenced by environmental factors. To overcome this limitation, molecular marker based analysis have been carried out using isoenzyme markers (Cabrita *et al*., 2001); RAPDs (Khadari *et al*., 1995; Dalkilic *et al*., 2011); AFLPs (Baraket *et al*., 2009); ISSR (Guasmi *et al*., 2006; Ikegami *et al*., 2009). Microsattelites or SSRs have become the choice of marker due to their high polymorphism, co-dominance and reproducibility in recent genetic diversity studies for fig cultivars (Khadari *et al*., 2001; 2004; Giraldo *et al*., 2005; 2008; Achtak *et al*., 2009; Çalışkan *et al*., 2012; Perez-Jiménez *et al*., 2012; Ganopoulos *et al*., 2015; Ferrara *et al*., 2016)

However, genetic characterization of caprifig germplasms using molecular markers such as SSR and RAPD is limited to few studies (Dalkilic *et al*., 2011; Essid *et al*., 2015). Thus, the purpose of this study was to investigate genetic relationship between 45 caprifig genotypes and 2 common Turkish female fig cultivars with SSR markers. The best caprifig genotypes as pollinators of important Turkish fig cultivars such as 'Sarılop' and 'Bursa Siyahı' are needed and in this respect, caprifig collection in Uludag University can serve as germplasm resources. Therefore, this study is important to assess and characterize the genetic relationship among caprifigs in this collection.

Materials and Methods

Plant material

In this study, the genetic relationship between 47 fig genotypes was analyzed (Table 1). 'Bursa Siyahı' and 'Sarılop' are the common female fig cultivars while the remaining 45 genotypes were the caprifigs collected from Bursa, Yalova, Balıkesir and Aydın provinces of Turkey (Fig. 1). Caprifig genotypes were collected to determine best pollinator genotypes for fig cultivars such as'Bursa Siyahı' and 'Sarılop'.

Table 1. List of genotypes and their sites of collection

Genotypes	Locations	Genotypes	Locations
16-05-01	Işıklı, Mudanya, Bursa	16-ZF-02	Görükle, Bursa
16-05-03	Işıklı, Mudanya, Bursa	16-ZF-03	Görükle, Bursa
16-05-04	Işıklı, Mudanya, Bursa	16-ZF-04	Görükle, Bursa
16-05-08	Işıklı, Mudanya, Bursa	16-ZF-05	Görükle, Bursa
16-05-10	Işıklı, Mudanya, Bursa	16-ZF-06	Görükle, Bursa
16-05-12	Işıklı, Mudanya, Bursa	16-ZF-07	Görükle, Bursa
16-09-09	Nilüfer, Bursa	16-ZF-08	Görükle, Bursa
16-09-10	Nilüfer, Bursa	16-ZF-09	Görükle, Bursa
16-09-11	Nilüfer, Bursa	77-00-01	Yalova
16-08-05	Osmangazi, Bursa	77-00-01-B	Yalova
16-08-06	Osmangazi, Bursa	77-00-02	Yalova
16-08-07	Osmangazi, Bursa	Bursa Siyahı	Yalova
16-08-09	Osmangazi, Bursa	Sarılop	Yalova
16-08-10	Osmangazi, Bursa	Havran	Balıkesir
16-08-12	Osmangazi, Bursa	Kaba İlek	Ömerbeyli, Aydın
16-00-01	Hürriyet, Bursa	Elma İlek	İmamköy, Aydın
Bursa 1	Osmangazi, Bursa	Mor Demirtaş	Kuyucak, Aydın
16-03-06	Karabalçık, Osmangazi, Bursa	Taşlık	Bozdoğan, Aydın
16-03-07	Karabalçık, Osmangazi, Bursa	Çakın 1	Kuyucak, Aydın
16-03-08	Karabalçık, Osmangazi, Bursa	Kıbrıslı	İmamköy, Aydın
16-07-01	Gürsu, Bursa	Yanako	Gümüşköy, Aydın
16-07-05	Gürsu, Bursa	Ak İlek	İmamköy, Aydın
16-07-06	Gürsu, Bursa	Karabulut	İmamköy, Aydın
16-ZF-01	Görükle, Bursa		

Fig. 1. The signs on the map indicates the provinces where leaf samples were collected (Source: http://www.openstreetmap.org/)

DNA Extraction

DNA extraction from young leaf samples were collected and powdered with a paint shaker after lyophilization. DNA samples were extracted from 25 mg powdered leaf samples by using a modified CTAB method (Futterer *et al*., 1995). The concentration of each DNA sample was measured using a Quibit fluorometer (Invitrogen, USA) and adjusted to 50 ng/μL and stored at -80 °C until use.

SSR analysis

Twenty four SSR primer pairs previously developed by Giraldo *et al*. (2005) were used for SSR analysis (Table 2). Forward primers were tailed with M13 sequence (GACGTTGTAAAACGACGGCC) at the 5′ end (Schuelke, 2000). Each 20 μL PCR solution contained 1.0 U *Taq* DNA polymerase (Thermo Scientific, USA) with 1 × reaction buffer, 0.10 μM M13 sequence tailed forward primer, 0.20 μM reverse primer, 0.20 μM M13 primer labelled with infrared dye either 700 nm or 800 nm (Licor, USA), 0.2 mM dNTPs, and 50 ng of DNA. SSR makers were amplified with a Veriti 96 well thermal cycler (Applied Biosystems, USA) using the following thermal cycling condition: 2 min at 94 °C; 6 cycles of 40 s at 94 °C, 1 min at 60 °C (annealing temperature was reduced 1 °C after every cycle for touchdown PCR protocol), 1 min and 30 s at 72 °C; 28 cycles of 40 s at 94 °C, 1 min at 55 °C and 1 min and 30 s at 72°C; 7 cycles of 40 s at 94 °C, 1 min at 54 °C and 1 min and 30 s at 72 °C and final extension step of 10 min at 72 °C. The PCR products were separated on 7.5% polyacrylamide gels at 30 W for 2 to 3 h using a Li-COR 4300 automated sequencer system (LI-COR).

Data analysis

SSR markers were scored manually as present (1) or absent (0) and a genetic similarity matrix of fig genotypes was calculated using Dice coefficient (Dice, 1945) with the NTSYSpc v2.21 program (Exeter Software, New York, NY, USA). An unweight pair group method with arithmetic averages (UPGMA) dendrogram based on the Dice similarity matrix was constructed. Expected heterozygosity (He) and observed heterozygosity (Ho) were calculated according to the method of Levene (1949) using POPGEN32 software v.1.32 (Yeh *et al*., 1997). The polymorphism information content (PIC) was calculated using the formula:

$$PIC = 1 - \sum_{i=1}^{j} P_i^2 - 2\sum_{i=j+1}^{j}\sum_{j=1}^{i-1} P_i^2 P_j^2$$

where Pi and Pj are the frequencies of the ith and jth alleles at a locus with l allele in a population, respectively (Botstein *et al*., 1980).

The population structure of the fig genotypes used in this study was determined using a model based Bayesian clustering implemented in STRUCTURE v.2.3.4 (Pritchard *et al*., 2000). Possible Ks (where K is an assumed fixed number of subpopulations in the entire population) from 1 to 12 were examined with 5 replicates. Each replication run was conducted with a burn-in period of 100,000 steps followed by 20,000 Monte Carlo Markov Chain (MCMC) replications using an admixture model and correlated allele frequencies options. The most likely number of subpopulations (K) was determined using the method described by Evanno *et al*. (2005).

Results and Discussion

A total of 24 SSR markers were tested to assess genetic variation between 45 caprifigs and two female Turkish fig cultivars (Table 2). While 23 of these SSR markers were polymorphic, LMFC12 was monomorphic with a single allele. The number of polymorphic alleles per SSR marker ranged from 2 (LMFC22-1, LMFC22-2, LMFC25, LMFC27, LMFC31 and LMFC37) to 7 (LMFC30) (Fig. 2). Besides, LMFC22 was considered to be multiple loci (Table 2), therefore, each locus was scored separately because the sizes of each loci were different. Aradhya *et al*. (2010) reported that the number of alleles ranged from 4 (LMFC22, LMFC31 and LMF35) to 9 (LMFC30) with a mean of 4.9. Similar results were also obtained by Essid *et al*. (2015) with 20 caprifig

Table 2. Number of polymorphic alleles, observed heterozygosity (Ho), expected heterozygosity (He) and polymorphism information content (PIC) of SSR markers

SSR Markers	Number of Alleles	Ho	He	PIC
LMFC11	3	0.40	0.59	0.70
LMFC12	monomorphic	-	-	-
LMFC13	5	0.35	0.64	0.90
LMFC14	3	0.42	0.57	0.80
LMFC15	4	0.33	0.67	0.68
LMFC17	3	0.36	0.64	0.73
LMFC18	4	0.30	0.70	0.42
LMFC19	5	0.25	0.74	0.54
LMFC20	5	0.58	0.41	0.72
LMFC21	3	0.63	0.37	0.72
LMFC22-1	2	0.54	0.45	0.78
LMFC22-2	2	0.49	0.50	0.98
LMFC23	5	0.34	0.65	0.82
LMFC25	2	0.59	0.41	0.63
LMFC26	3	0.70	0.30	0.72
LMFC27	2	0.51	0.49	0.71
LMFC28	5	0.27	0.72	0.74
LMFC30	7	0.18	0.81	0.81
LMFC31	2	0.76	0.24	0.74
LMFC32	5	0.48	0.51	0.78
LMFC35	3	0.43	0.57	0.62
LMFC36	3	0.37	0.62	0.82
LMFC37	2	0.72	0.28	0.78
LMFC38	4	0.34	0.65	0.70
Mean	3.56	0.45	0.54	0.73

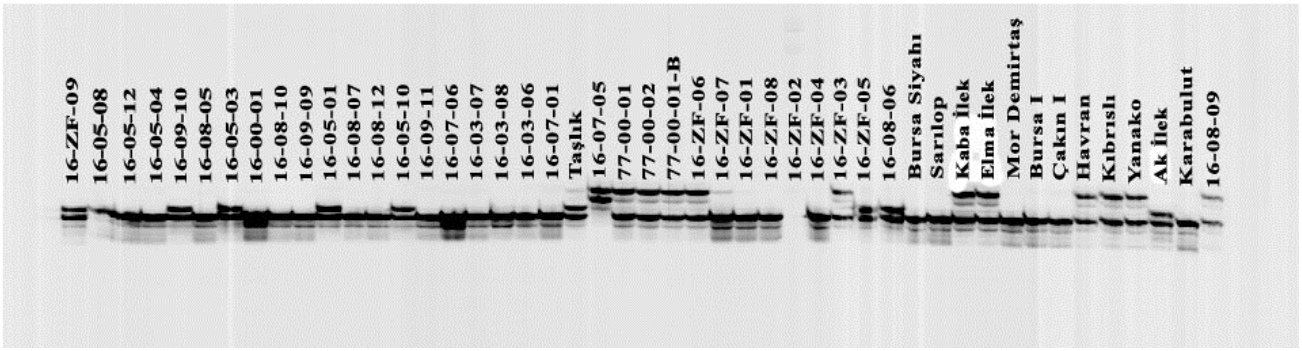

Fig. 2. Polyacrylamide gel picture demonstrating the alleles of LMFC20 SSR marker among45 caprifig and 2 female fig genotypes

accessions that the number of alleles per locus ranged from 2 (LMFC32, LMFC15, LMFC21, LMFC31, LMFC18, LMFC27, LMFC23) to 6 (LMFC30). Thus, results from present study and previous demonstrated that LMFC30 produced the highest number of alleles per locus.

Among 47 genotypes, 82 polymorphic SSR alleles were amplified, and the average number of alleles per SSR marker was 3.56 (Table 2). Ho values of each SSR marker varied from 0.18 (LMFC30) to 0.76 (LMFC31) with an average of 0.45 while He values ranged from 0.24 (LMFC31) to 0.81 (LMFC30) with an average of 0.54. Ikegami *et al.* (2009) found that the average He and Ho values were 0.44 and 0.44, respectively in 19 European and Asian fig varieties. In another study assessing genetic variation among caprifig cultivars, it was reported that the average He and Ho values were 0.29, 0.33, respectively (Essid *et al.*, 2015). On the other hand, Khadari *et al.* (2004) reported an average He of 0.60 and average of 6.3 alleles per locus with six SSRs and 72 fig cultivars. Our results are also comparable to the results of previous studies and also confirmed that genetic diversity among fig genotypes was large.

The Ho was higher than the He for LMFC20, LMFC21, LMFC22-1, LMFC25, LMFC26, LMFC27, LMFC31 and LMFC37 while Ho was lower than He for the remaining SSR markers in this study. A heterozygote excess (Ho>He) was observed for the LMFC15, LMFC31, LMFC18, LMFC27 loci whereas a heterozygote deficiency (Ho<He) was observed in LMFC24 and MFC2 loci in a study dealing with genetic variation of caprifigs (Essid *et al.*, 2015). According to Giraldo *et al.* (2008), for all SSRs except LMFC15 and LMFC21, Ho values were higher than the expected.

The polymorphism information content (PIC) value ranged from 0.42 (LMFC18) to 0.98 (LMFC22-2) with an average of 0.73 in this study. Ikegami *et al.* (2009) found that the highest, the lowest and the average PIC values were 0.82 (LMFC30), 0.26 (LMFC15) and 0.39, respectively. Ferrara *et al.* (2016) reported that, the lowest PIC observed was 0.07 for LMFC23 and LMFC27 primers while the highest was 0.91 for Frub422 SSR marker. These results suggested that the genetic variation within fig genotypes in this study is high.

The number of gene pools indicated by the STRUCTURE analysis was estimated to be K=2 with the highest ΔK value (29.86) (Fig. 3), followed by K=6 and K=3 with ΔKs of 3.81 and 1.63, respectively. According to the STRUCTURE analysis, 47 fig genotypes were divided into

two different gene pools. As shown in Fig. 3, gene pool 1 contained 25 genotypes collected from Aydın (7), Balıkesir (1), Bursa (13) and Yalova (4) provinces while genepool 2 consisted of 22 individuals collected from Bursa (19); Aydın (2); Yalova (1) provinces. Female cultivars ('Bursa Siyahı' and 'Sarılop') were in gene pool 1. On the other hand, the genotypes from Aydın and Yalova provinces were mostly placed in gene pool 1. Caprifig genotypes from Bursa province were present in both gene pools although about 86% of genotypes in gene pool 2 is from Bursa. Similarly, in another study, 90 fig cultivars were placed in two gene pools based on STRUCTURE analysis (Ganopoulos *et al*., 2015).

A UPGMA dendogram showing the genetic relationships among 47 fig genotypes was developed using Dice similarity matrix (Fig. 4). According to the dendogram, the fig genotypes divided in two distinct clusters (A and B), representing the two gene pools identified by STRUCTURE analysis. Among caprifig genotypes, the highest similarity was between the genotypes '16-ZF-02' and '16-ZF-03' (0.91). Both female cultivars were included in the cluster A with other caprifig genotypes indicating that female and male fig trees are not genetically distinct. Similarly, Saddoud *et al*. (2005) also found two main groups among 16 Tunisian fig cultivars. In the study of Dalkilic *et al*. (2011) with RAPD markers, authors determined nine different groups among 43 caprifig genotypes. Essid *et al*. (2015) identified three main groups among 20 caprifig genotypes.

It has been reported that there is a high number of homonymy, mislabeling and synonymy problems in fig genotypes worldwide. For example, Khadari *et al*. (2004) observed five cases of mislabeling and homonyms among 75 Moroccan fig accessions using SSR and ISSR markers. Similarly, Dalkilic *et al*. (2011) also determined three mislabeled and four homonym genotypes among 43 Turkish

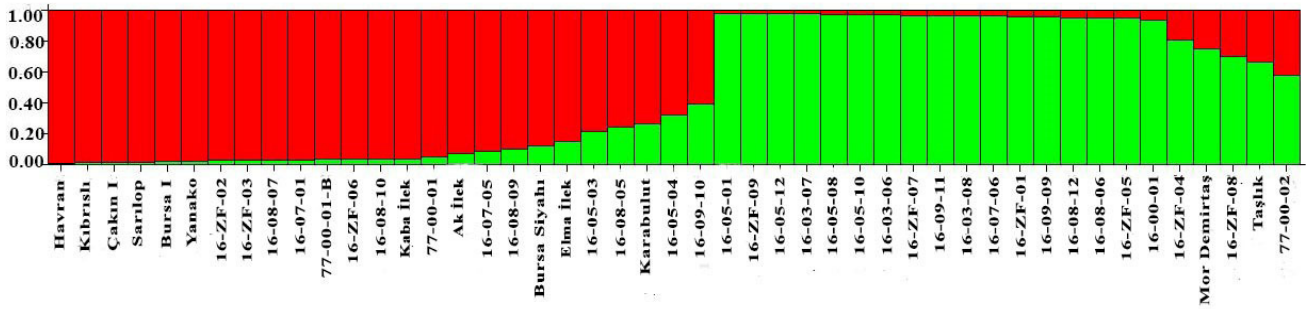

Fig. 3. STRUCTURE bar plot based on 23 SSR markers. Different colours represent different gene pools

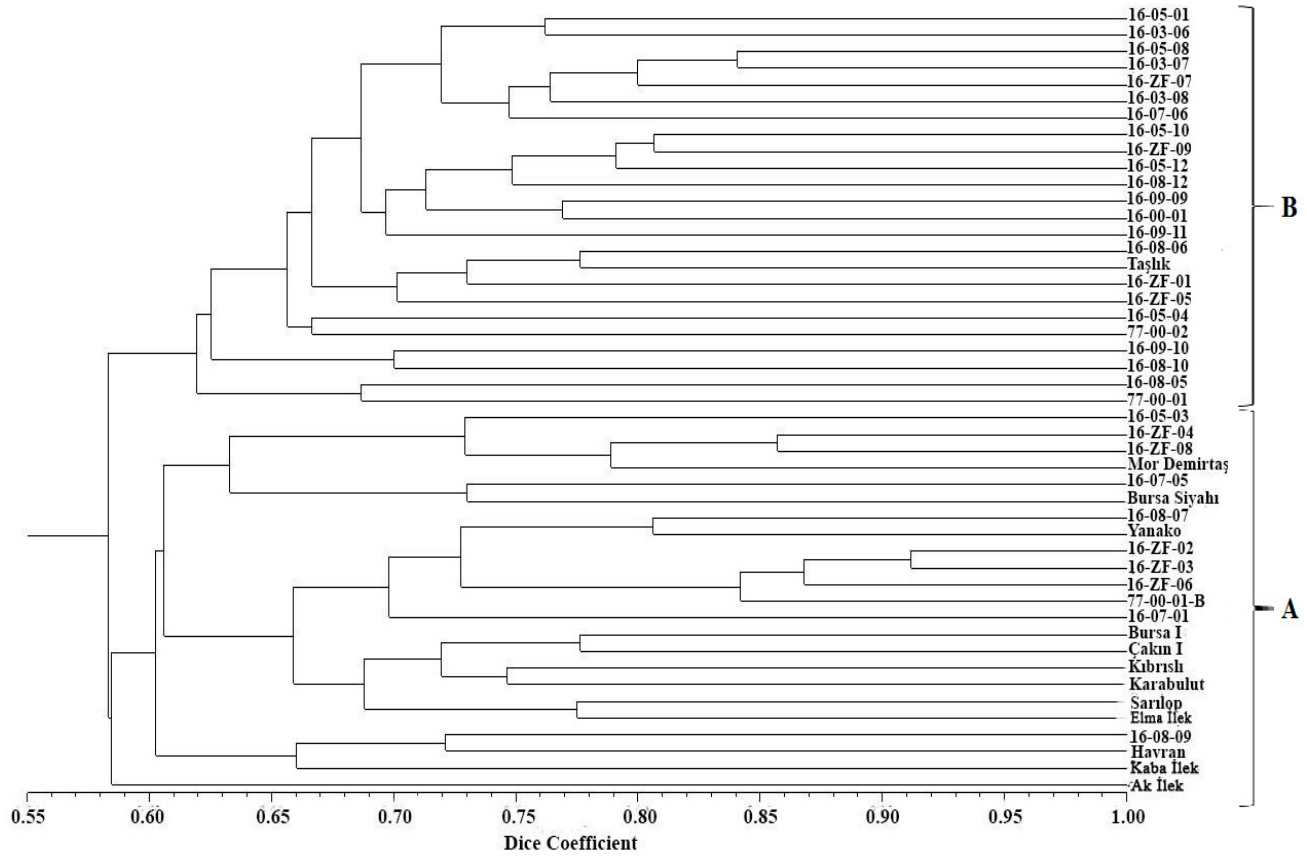

Fig. 4. UPGMA dendrogram based on the Dice similarity of 23 SSR markers showing the genetic relationships among 45 caprifig and 2 female fig genotypes

caprifig genotypes using RAPD markers. Besides, Essid *et al*. (2015) detected three undistinguished accessions due to synonyms and analyzed several homonyms within 20 Tunisian caprifig genotypes using SSR markers. However, our results showed that there were no homonyms, mislabeling or synonymies. Thus, all fig genotypes analyzed in this study were unique.

In addition, there was no clear distinction among fig genotypes based on their sites of collection in the present study. Similarly, Giraldo *et al*. (2008); Dalkilic *et al*. (2011) and Essid *et al*. (2015) also found that genotypes from the same regions were placed in different groups. On the other hand, Aradhya *et al*. (2010) reported that fig accessions from Turkmenistan are genetically different from the Mediterranean and the Caucasus figs.

Conclusions

The present study proved that SSR markers are reliable technique for identifying genetic diversity of both caprifig and female fig genotypes. Our results showed a high genetic diversity among 45 caprifig genotypes and 2 female cultivars commonly grown in Turkey and these results highlighted that all fig genotypes analyzed in this study were unique. In this respect, this genetic variation could be useful for constituting a caprifig gene bank for future breeding programs and fig production via caprification.

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