ASSESSMENT OF GENETIC VARIATION IN WILD MYRTLE (*MYRTUS COMMUNIS* L.) GENOTYPES GROWING AROUND THE MEDITERRANEAN REGION OF TURKEY

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Abstract. Myrtle (*Myrtus communis*) grows naturally on the Mediterranean coast and the Aegean region of Turkey and is used as medicine, food and ornamental plant. Availability of genetic resources with genetic diversity constitutes an important material for researching genes that may be needed in future breeding studies. The goal of this study was to analyze the genetic diversity of the myrtle genotypes in order to develop further cultivation, regeneration or breeding strategies. Nine traits (length, width, weight and color of the fruit, number of seeds, weight of seeds, pulp weight, length, and width of the leaf) were evaluated. PCR amplification was performed using Simple Sequence Repeats (SSR) and Inter- Primer Binding (iPBS) primers. The variation was found between genotypes in terms of the morphological characters considered. Using molecular analyses, the genetic diversity among the genotypes was determined based on the 64 SSR and 80 polymorphic iPBS bands. The genetic variation obtained by morphological and molecular analyses shows that these genotypes may be useful for future breeding and cultivation practices and can be considered as valuable genetic resources. It has also been shown that some of the iPBS primers were highly polymorphic and can be used in phylogenetic analyzes and mapping studies of myrtle.
Keywords: *genetic relationship, genetic resources, iPBS, SSR, diversity*

Introduction

Myrtle (*Myrtus communis* L.) is a diploid (2n = 2x = 22), medicinal and aromatic plant and grows wildly on the rocky slopes, *Pinus brutia* forests and coastal regions of the Mediterranean Basin (Özkan and Güray, 2009). These plants can reach up to 5 m in height and grow at altitudes ranging from 50–500 m above sea level. It is an evergreen shrub with ovate lanceolate leaves, white flowers and is very aromatic because of the high oil content of its leaves (Agrimonti et al., 2006). The fruits have two different color (*Fig. 1*), a dark blue and a white (yellowish-white) form (Traveset et al., 2001; Messaoud et al., 2011; Serçe et al., 2010). Dark blue fruits contain higher polyphenolic content and antioxidant activity than white fruits and they are rich in α-pinene, linalool and α-terpineol whereas white fruits were rich in myrtenyl acetate and unsaturated fatty acids like linoleic and oleic acids (Messaoud et al., 2011) and myrtle pigmented berries are a good anthocyanin source. The myrtle has value as ornamental and aromatic plant and shows hypoglycemic, antimicrobial, antihemorrhagic properties (Özek et al., 2000; Sepici et al., 2004). The aromatic leaves contain essential oil which are used in the pharmaceutical, cosmetic, food industries (Mulas, et al., 1998; Messaoud et al., 2005) and for liquor production. Nineteen compounds, concerning mostly to polyphenol compounds and a new class of hydrolysable tannins were identified in these berries (mostly in seeds) for the first time by D'Urso et al. (2017). Composition of essential oils found quite variable with a number of compounds ranging from 31 to 78 depending on

cultivar (Usai et al., 2020). There is an increasing interest to myrtle for medicinal uses and food industries. The increasing demand is causing uncontrolled collection of leaves and berries from the wild plants. To protect the genetic resources and control of reduction of myrtle populations, a domestication program and a conservation program was carried out starting in 1995 in Sardinia island (Mulas and Cani, 1999) and evaluation of the selected genotypes and breeding programs within the scope of this program continues (Medda and Mulas, 2021). The same scenario is also true for Turkey. Uncontrolled harvesting of leaves, and fruits, cutting branches for ornamental usage, decoration at traditional wedding ceremonies (Özkan and Güray, 2009) and increased construction activity cause the reduction of diversity of genotypes. Genetic diversity is a major benchmark in the choice of genetic resources and wild genotypes are important particularly for tolerance to biotic and abiotic stresses. Well-characterized genetic resources play an important role in guiding breeding studies, since breeders are usually looking for plant material containing desirable agricultural traits (e.g. disease resistance, or fruit traits). Management of genetic resources by morphological and molecular characterization, evaluation and conservation is necessary to maximize the benefits of genetic resources. Analysis of genetic diversity in germplasm collections can provide reliable classification of genotypes and help to understand the capacity of the gene pool for specific breeding purposes. Therefore, morphological, and molecular characterization of germplasm is an essential tool and ideally molecular characterization should be carried out by a suitable a marker system that is polymorphic and can reveal genetic variations. A limited number of studies have been conducted on morphological characterization of myrtle (Ciccarelli et al., 2008; Serçe et al., 2010; Messaoud and Boussaid, 2011).
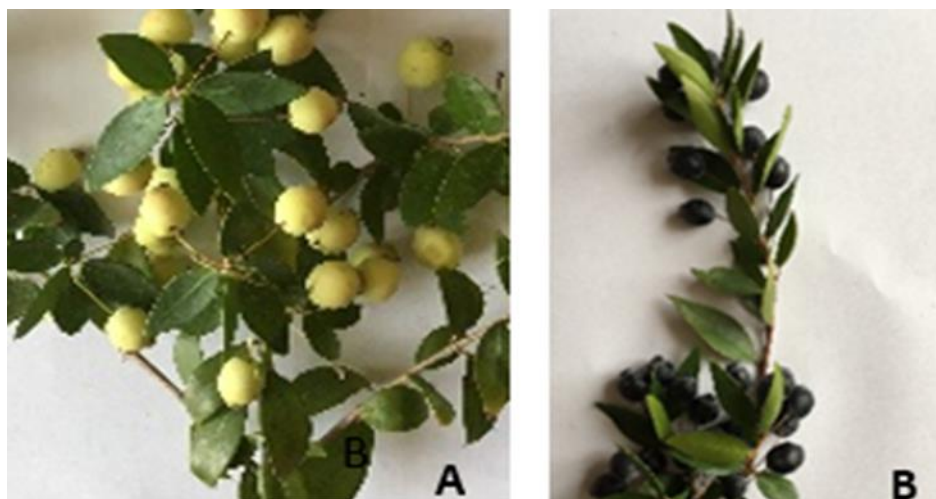

Figure 1. *Myrtus communis samples collected from: (A) Mersin, Erdemli-Kumkuyu, white fruited morphs, (B) Muğla, Marmaris-Armutalan, dark blue fruited morphs*

Studies at the molecular level for the identification, characterization and relatedness analysis of *Myrtus communis* (myrtle; Myrtaceae) have been performed using molecular markers, such as AFLP (Bruna et al., 2005; Agrimonti et al., 2006; Bruna et al., 2007; Albaladejo et al., 2009; Melito et al., 2014, 2016, 2017; Messaoud et al., 2011), RAPD (Messaoud et al., 2007), ISSR (Melito et al., 2013; Sımsek et al., 2019), SRAP

(Ghafouri and Rahimmalek, 2018) and SSR (Albaladejo et al., 2010; Nora et al., 2014; Corona et al., 2017; Mele et al., 2019).

The goal of this study was to analyse the genetic diversity of the naturally grown myrtle genotypes around the Mediterranean Region of Turkey based on morphological and molecular characterization in order to develop further cultivation, regeneration or breeding strategies. Molecular characterization was performed by using iPBS Kalendar et al. (2010) and previously described SSR primers by Albaladejo et al. (2010).

SSR markers have some advantages over other marker systems that include high potential for automation, co-dominant inheritance, distribution throughout the genome, high levels of polymorphism and repeatability. Small amount of DNA is sufficient for amplification of SSR markers (Park et al., 2009) and have been successfully used to examine genetic diversity, fingerprinting and genotyping in different plant species (Abdurakhmonov, 2016).

The inter-primer binding site (iPBS) amplification technique utilizes the conserved parts of - primer binding site (PBS) sequences that are universally found almost all long terminal repeats (LTR) retrotransposon and used for determining the variations caused by retrotransposon movements or recombinations (Kalendar and Schulman, 2014). The LTR transposon elements contain a conserved region for binding of tRNA that act as a primer in the conversion of the retrotransposon RNA to DNA before enters its new position in the genome. iPBS markers (Kalendar et al., 2010; Kalendar and Schulman, 2014) have been used to investigate the genetic relationship among the genotypes of apricot (*Prunus armeniaca* L.) (Baránek et al., 2012) and apple (*Malus pumila* Mill.) (Kuras et al., 2013), guava (*Psidium guajava* L.) (Mehmood et al., 2015), grapevine (*Vitis vinifera* L. ssp. sativa D.C.) (Milovanov et al., 2019), Laurel (*Laurus nobilis* L.) (Karık et al., 2019), wild Cicer species (Andeden et al., 2013), pea (Pisum sativum) (Baloch, 2015), Motherwort (*Leonurus cardiaca* L.) (Borna et al., 2016). To our knowledge, no prior studies have examined iPBS markers in *Myrtus communis.*

Genetically diverse germplasm resources constitute an important material for researching genotypes that may carry the desired characters. Identification and protection of *Myrtus communis* genotypes which are widely grown naturally in the Mediterranean and Aegean coasts of Turkey, are important in terms of providing material for future breeding studies and preserving genetic diversity.

Material and methods

Plant material

The 48 myrtle genotypes were used as study material (*Table 1*). The leaves and fruit samples were collected from 20 different locations (included 8 provinces) covering an area of ~1200 km along the Mediterranean coastline and Aegean region of Turkey in the period of fruit ripening (October to January) in 2017 and 2018 from either very ancient myrtle trees or young shrubs (*Fig. 2*). The map in *Figure 3* and *Table 1* shows the sampling locations.

Morphological analysis

On each genotype nine morphological traits (length, width, weight and color of the fruit, number of seeds, weight of seeds, pulp weight, length and width of the leaf) were evaluated. The samples were examined morphologically using the method described by

Melito et al. (2016) and Uzun et al. (2016). The fruits were harvested and measured in full ripening stage and a sample of 50 fruits and leaves was taken from each genotype from all sides of the shrubs. The leaves were stored at − 70 °C for further molecular analysis. All analyses of morphological characteristic were performed using analysis of variance (ANOVA) in SPSS 20.0 software package (IBM, 2020). Normality test performed, and the data showed normal distribution. Variance analysis (one-way ANOVA-Duncan) was used to determine variations among the genotypes based on measured properties. Pearson's correlation analysis was performed to find correlation between the morphological characteristics and altitude.

Figure 2. (a) The 'Old myrtle' shrub (Antalya, Bahtılı), (b) myrtle shrub (Antalya-Göynük)

Figure 3. The sampling areas in the Mediterranean Region of Turkey

Table 1. *List of the studied genotypes, their identification codes, fruit color, location, geographical coordinates of the collection sites*

Genotype code	Fruit color	Introduced genotype location	Longitude	Latitude	Altitude (m)
Hty1B	Dark blue	Hatay, Samandağı, Fidanlı	E36°01'28.54"	N36°09'04.75"	141
Ant3B	Dark blue	Antalya, Kumluca, Belen	E30°23'34.80"	N36°22'28.66"	505
Ant4W	White	Antalya, Kumluca, Belen	E30°23'34.80"	N36°22'28.66"	505
Ant5W	White	Antalya, Kumluca, Belen	E30°23'34.80"	N36°22'28.66"	505
Ant6W	White	Antalya, Kumluca, Belen	E30°23'34.80"	N36°22'28.66"	505
Ant7B	Dark blue	Antalya, Alanya, Okurcalar	E31°41'51.00"	N36°39'00.00"	23
Ant8W	White	Antalya, Alanya, Okurcalar	E31°41'51.00"	N36°39'00.00"	23
Ant9B	Dark blue	Antalya, Göynük Kanyon	E30°32'02.75"	N36°40'57.40"	64
Ant10B	Dark blue	Antalya Göynük Kanyon	E30°32'02.75"	N36°40'57.40"	64
Ant11W	White	Antalya, Kemer, Arslanbucak	E30°33'35.14"	N36°36'10.05"	122
Ant12W	White	Antalya, Kemer, Kuzdere	E30°31'53.71"	N36°34'59.51"	45
Ant13W	White	Antalya, Kemer, Kuzdere	E30°31'53.71"	N36°34'59.51"	45
Ant14B	Dark blue	Antalya, Kemer, Çamyuva	E30°34'00.00"	N36°34'00.00"	9
Ant15B	Dark blue	Antalya, Kemer, Çamyuva	E30°34'00.00"	N36°34'00.00"	9
Ant16B	Dark blue	Antalya, Kemer, Tekirova	E30°31'35.01"	N36°30'06.93"	14
Ant17W	White	Antalya, Kemer, Tekirova	E30°31'35.01"	N36°30'06.93"	14
Isp18W	White	Isparta, Çobanpınar	E30°48'24.23"	N37°23'03.06"	339
Ant19B	Dark blue	Antalya, Alanya, Dereköy	E32°02'01.54"	N36°39'31.24"	659
Ant20W	White	Antalya, Manavgat, Hatipler	E31°24'38.94"	N36°48'40.10"	42
Ant21W	White	Antalya, Finike, Sahilkent	E30°12'17.06"	N36°20'15.72"	21
Ant22B	Dark blue	Antalya, Akdeniz Univ.	E30°39'07.97"	N36°53'31.48"	27
Ant23B	Dark blue	Antalya, Akdeniz Univ.	E30°39'07.97"	N36°53'31.48"	27
Izm24W	White	İzmir, Değirmen Dere	E27°08'58.03"	N27°37'37.83"	20
Izm25B	Dark blue	İzmir, Değirmen Dere	E27°08'58.03"	N27°37'37.83"	20
Izm26W	White	İzmir, Değirmen Dere	E27°08'58.03"	N27°37'37.83"	20
Mgl27W	White	Muğla, Datça	E27°41'09.04"	N36°43'34.38"	10
Mgl28B	Dark blue	Muğla, Datça	E27°41'09.04"	N36°43'34.38"	10
Mgl29W	White	Muğla, Marmaris, Armutalan	E28°14'15.13"	N36°51'23.64"	37
Mgl30B	Dark blue	Muğla, Marmaris, Armutalan	E28°14'15.13"	N36°51'23.64"	37
Izm31B	Dark blue	İzmir, Menemen, Yamanlar	E27°13'38.47"	N38°32'44.90"	946
Ayd32B	Dark blue	Aydın	E28°29'11.03"	N37°48'42.13"	1358
Ant33B	Dark blue	Antalya, Bahtılı	E30°34'27.71"	N36°53'16.82"	38
Ant34W	White	Antalya, Serik, Şatırlı	E30°34'27.71"	N36°53'16.82"	15
Ant35W	White	Antalya, Konyaaltı	E30°42'47.96"	N36°53'48.81"	56
Ant36W	White	Antalya, Geyikbayırı	E30°27'53.86"	N36°52'31.58"	614
Ant37B	Dark blue	Antalya, Gökçam	E30°32'46.99"	N36°53'41.11"	66
Ant38B	Dark blue	Antalya, Gökçam	E30°32'46.99"	N36°53'41.11"	66
Ant44B	Dark blue	Antalya, Gökçam	E30°32'46.99"	N36°53'41.11"	66
Mrs48W	White	Mersin, Erdemli, Kumkuyu	E34°12'00.00"	N36°32'00.00"	183
Mrs49W	White	Mersin, Erdemli, Kumkuyu	E34°12'00.00"	N36°32'00.00"	183
Mrs50B	Dark blue	Mersin, Erdemli, Ayaş	E34°11'00.00"	N36°29'00.00"	0
Ant51B	Dark blue	Antalya, Döşemealtı	E30°36'04.35"	N37°01'22.89"	302
Ant52B	Dark blue	Antalya, Döşemealtı	E30°36'04.35"	N37°01'22.89"	302
Ant53W	White	Antalya, Döşemealtı	E30°36'04.35"	N36°55'16.06"	302
Hty55B	Dark blue	Hatay, Erzin, Kuzuyuk	E36°16'12.10"	N37°01'22.89"	469
Hty56W	White	Hatay, Erzin, Kuzuyuk	E36°16'12.10"	N36°55'16.06"	469
Ant57B	Dark blue	Antalya, Alanya, Okurcalar	E31°41'51.00"	N36°39'00.00"	23
Mgl59W	White	Muğla, Fethiye	E29°07'34.85"	N36°39'33.29"	25

Molecular analysis

DNA extraction

Genomic DNA was extracted from leaf tissues of 48 genotypes using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990). The leaves (200 mg) were cut into small pieces with a help of scalpel and grind to a fine

paste in a 600 µl CTAB buffer (2% CTAB, EDTA 20 mM, Tris-Cl 100 mM, NaCl 1.4 M, 0.2% Mercaptoethanol) using mortar and pestle. The CTAB/plant extract mixture transferred to the Eppendorf tube and incubated 1 h at 65 °C. After two times Chloroform: Isoamyl alcohol (24:1) treatment and 15 min centrifugation was performed at 13,000 × g, then upper aqueous phase was transferred to clean tube and 500 µl isopropanol alcohol added. Following the centrifugation for 15 min, the upper phase was discarded. The pellet was washed with 70% ethanol two times and leave to dry (approximately 15 min) at the room temperature. The quality of DNA was checked by 1% agarose gel electrophoresis with ethidium bromide staining.

DNA amplification with SSR

PCR amplification was performed using 12 SSR primer pairs developed by Albaladejo et al. (2010) (*Table 4*). PCR reactions were performed in a 15-µl volume containing 20-30 ng genomic DNA, 0.2 mM dNTPs, 1.5 mM MgCl2, 0.5 µM each primer, and 1 U Taq DNA polymerase (Thermo). An adaptor sequences (GGAAACAGCTATGACCAT) were ligated to at its 5' end of the reverse primers and fluorescently labeled with either IRDye 700 or IRDye 800 fluorescent dyes according to the protocol by Schuelke (2000).

Amplifications were conducted using a program with an initial denaturation step at 95 °C for 3 min. followed by 35 cycles at 94 °C for 45 s, 50–65 °C for 30 s and 72 °C for1 min with a final cycle of 72 °C for 5 min. A 1.5- to 2-µl aliquot of PCR product (depending on the performance of amplification of each primer pair) was mixed with10 µl of loading dye including 98% formamide, 10 mM EDTA, 0.25% bromophenol blue, 0.25% xylenecyanol and denatured at 95 °C for 4 min. and placed in ice until loaded. PCR products were separated on a 25 cm 6% polyacrylamide gel (0.25 mm thick) containing acrylamide: bis-acrylamide (19:1), 8 M urea, and TBE 1X using a 32-well square comb. The separated bands were visualized in a Li-Cor -IR2 4200 Genetic Analyzer (Li-Cor Biosciences).

DNA amplification with iPBS

Twelve iPBS primers were selected from the study of Kalender et al. (2010) (*Table 5*). DNA amplification was carried out by using a modified protocol of Kalendar et al. (2010). PCR was performed in a volume of 15 µL containing10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM of each dNTP, 10 pmol of each primer, 50 ng genomic DNA, and 1U Taq DNA polymerase (Thermo) using the following temperature profile: 95 °C for 3 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 50–60 °C (the annealing temperature varied depending on the primer used) for 45 s and 72 °C for 1 min, and an extension step at 72 °C for 5 min. PCR was performed two times for some primers to confirm band pattern consistency. Products were separated by gel electrophoresis in 2% (w/v) agarose gels and visualized by staining ethidium bromide under UV light.

Data analysis

The allelic data matrix of "1" or "0" was used to calculate the genetic analysis by using NTSYS (Numeracal Taxonomy Multivariate Analysis System, NTSYS-pc version 2.1 Exeter Software, Setauket, N.Y. USA (Rohlf, 1993). A genetic similarity matrix was constructed with in the SIMGEND module and similarity matrices were utilized to

construct the UPGMA (unweighted pair group method with arithmetic average) dendrograms. Principal component analysis (PCA) was performed to show the differences among the genotypes. Eigen values were calculated using the EIGEN module and based on the variance–covariance matrix. Two dimensional plots calculated between each two pairs of the 48 myrtle genotypes. The polymorphism information content (PIC) of SSR and iPBS markers was calculated as the mean of the PIC of each allele using the formula of Roldan-Ruiz et al. (2000) (*Eq. 1*).

$$PIC_i = 2f_i(1 - f_i) \qquad (Eq.1)$$

where "PIC_i" is the polymorphism information content of allele "I" and "f_i" is the frequency of occurrence of allele "I" (fragment present) in the 48 individuals.

Results and discussion

Morphological analysis

Morphological characterization of genetic resources provides the most important guiding information for identification, classification, and conservation of the genetic resources. Knowledge of morphological characters is the first step in the parental selection in the breeding programs. In this study the important morphological traits of the myrtle genotypes were determined in their natural ecosystems. Fifty fruits and leaves from each genotype were evaluated in terms of morphological characters and results are shown in *Table 2*.

The fruit weight, fruit length and fruit width ranged 0.05 to 1.18, 6.44 mm to 15.69 mm and 4.16 to 12.72 mm respectively. Results showed that the highest values for six out of the eight morphological characters (fruit weight, length, width, number of seed per fruit, seed weight, pulp weight) were recorded in genotype Ant8W. The lowest value for fruit weight, width, seed weight and pulp weight were measured in genotype Ant17W (*Table 2*). In the previous studies Melito et al. (2016) reported the fruit weight from 0.25 g to 0.34 g, fruit length 7.18 mm to 9.03 mm and fruit width 5.74 to 8.22 in Sicily. Uzun et al. (2016) measured highest values for fruit weight 1.33 g, fruit length 15.33 mm and fruit width 13.27 mm in the myrtle genotypes with white fruit color in coastal conditions of Antalya, Turkey. The highest and lowest values for fruit weight, length and width were recorded as 0.26 to 2.01, 7.52 to 16.73 mm and 5.52 to 14.74 mm respectively by Yıldırım et al. (2013) in the study carried out in Adana and Mersin province of Turkey. In the current study the number of seed for per fruit, seed weight and pulp weight are ranged 2.2-22.4, 0.02-0.21 g and 0.03-0.93 g respectively. Melito et al. (2016) reported a narrower range than we find between the highest and lowest values of the seed number for per fruit (10.34-18.43), seed weight (0.04 g-0.05 g) and pulp weight (0.20 g - 0.29 g). In the present work the seed weight ranged 0.02- 0.21 in genotypes with white berries and 0.02-0.13 in genotypes with black berries. The number of seeds ranged 2.2- 22.4 in genotypes with white berries and 2.2 − 10.8 in genotypes with black berries. Measurements varied between 13.95 (Ant22) and 32.85 mm (Ant13W) for leaf length, 5.5 (Ant23B) and 15.3 mm (Ant35W) for leaf width. The studied genotypes showed variation for all morphological traits evaluated. There were statistically significant differences ($p < 0.01$) among the genotypes for all fruit and leaf traits measured (*Table 3*). Samples were collected from different altitudes from 0 to 1358 m. According to the

results of the correlation analysis, there were negative and significant (p < 0.01) correlations between altitude and fruit length (r = -,088**), fruit weight (r = -,078**) and pulp weight (-,082**). Medda et al. (2021) found no effect of altitude on leaf total phenols, whereas a negative correlation was reported on berries total phenols and tannins concentrations.

Table 2. *Means of fruit and leaf characteristics for wild myrtle genotypes (mean ± SE)*

Genotype code	Fruit length (mm)	Fruit width (mm)	Fruit weight (g)	Number of seed per fruit	Seed weight (g)	Pulp weight (g)	Leaf length (mm)	Leaf width (mm)
Hty1B	8.55 ± 0.16	6.81 ± 0.17	0.19 ± 0.01	6.06 ± 0.43	0.06 ± 0.00	0.12 ± 0.009	23.40 ± 0.64	9.59 ± 0.38
Ant3B	9.05 ± 0.10	6.52 ± 0.09	0.19 ± 0.00	4.12 ± 0.22	0.05 ± 0.00	0.14 ± 0.004	25.44 ± 0.83	8.16 ± 0.34
Ant4W	14.8 ± 0.16	10.89 ± 0.13	0.83 ± 0.02	19.87 ± 1.18	0.17 ± 0.01	0.66 ± 0.02	32.06 ± 1.25	14.3 ± 0.57
Ant5W	9.17 ± 0.10	6.53 ± 0.09	0.20 ± 0.00	4.68 ± 0.33	0.05 ± 0.00	0.14 ± 0.005	26.01 ± 1.1	8.75 ± 0.35
Ant6W	9.3 ± 0.13	8.30 ± 0.11	0.32 ± 0.01	6.75 ± 0.50	0.09 ± 0.00	0.23 ± 0.008	19.79 ± 0.80	9.37 ± 0.29
Ant7B	13.56 ± 0.14	8.99 ± 0.12	0.55 ± 0.02	5 ± 0.30	0.09 ± 0.00	0.45 ± 0.01	24.35 ± 0.73	9.51 ± 0.26
Ant8W	15.69 ± 0.26	12.72 ± 0.20	1.18 ± 0.04	22.4 ± 1.19	0.21 ± 0.01	0.93 ± 0.04	28.35 ± 1.04	12.94 ± 0.40
Ant9B	9.36 ± 0.11	6.53 ± 0.09	0.20 ± 0.00	8.48 ± 0.32	0.08 ± 0.00	0.12 ± 0.006	27.20 ± 0.92	9.39 ± 0.33
Ant10B	9.28 ± 0.19	4.99 ± 0.12	0.12 ± 0.00	6.48 ± 0.37	0.04 ± 0.00	0.08 ± 0.006	23.39 ± 1.29	9.37 ± 0.51
Ant11W	14.28 ± 0.20	10.15 ± 0.12	0.66 ± 0.02	11.08 ± 0.73	0.10 ± 0.00	0.55 ± 0.01	21.17 ± 0.89	8.64 ± 0.38
Ant12W	8.16 ± 0.15	5.91 ± 0.11	0.13 ± 0.00	7.42 ± 0.50	0.05 ± 0.00	0.08 ± 0.005	25.44 ± 0.98	11.37 ± 0.39
Ant13W	8.54 ± 0.22	6.13 ± 0.23	0.16 ± 0.01	4.7 ± 0.35	0.04 ± 0.00	0.12 ± 0.007	32.85 ± 0.70	12.92 ± 0.29
Ant14B	10.65 ± 0.22	7.98 ± 0.18	0.32 ± 0.01	5.52 ± 0.40	0.08 ± 0.00	0.24 ± 0.01	25.75 ± 0.82	10.72 ± 0.42
Ant15B*							22.87 ± 1.03	8.54 ± 0.40
Ant16B*							23.45 ± 0.79	8.95 ± 0.47
Ant17W	6.67 ± 0.11	4.16 ± 0.11	0.05 ± 0.00	2.66 ± 0.26	0.02 ± 0.00	0.03 ± 0.003	22.91 ± 0.77	8.47 ± 0.43
Isp18W	13.62 ± 0.26	10.75 ± 0.18	0.77 ± 0.03	12.46 ± 1.15	0.15 ± 0.01	0.61 ± 0.02	23.60 ± 0.70	9.26 ± 0.28
Ant19B	11.07 ± 0.15	7.73 ± 0.15	0.33 ± 0.01	8.08 ± 0.86	0.09 ± 0.00	0.23 ± 0.009	25.33 ± 0.58	8.55 ± 0.21
Ant20W	13.67 ± 0.26	9.95 ± 0.17	0.62 ± 0.03	14.12 ± 1.50	0.13 ± 0.01	0.49 ± 0.02	27.65 ± 0.75	12.74 ± 0.31
Ant21W	12.8 ± 0.16	10.10 ± 0.14	0.65 ± 0.02	8.86 ± 0.50	0.10 ± 0.00	0.55 ± 0.02	24.56 ± 0.74	10.95 ± 0.27
Ant22B*							13.95 ± 0.33	6.19 ± 0.14
Ant23B	9.09 ± 0.12	6.99 ± 0.06	0.18 ± 0.00	5.44 ± 0.31	0.07 ± 0.00	0.11 ± 0.004	18.41 ± 0.33	5.50 ± 0.10
Izm24W	8.86 ± 0.07	6.26 ± 0.06	0.17 ± 0.00	5.14 ± 0.23	0.06 ± 0.00	0.11 ± 0.004	23.44 ± 0.94	8.87 ± 0.27
Izm25B	7.84 ± 0.15	5.29 ± 0.11	0.10 ± 0.00	4.76 ± 0.29	0.06 ± 0.00	0.04 ± 0.003	26.99 ± 1.07	9.67 ± 0.34
Izm26W	8.58 ± 0.2	6.38 ± 0.21	0.21 ± 0.01	6.06 ± 0.45	0.07 ± 0.00	0.13 ± 0.01	29.79 ± 0.94	11.01 ± 0.38
Mgl27W	10.47 ± 0.18	8.17 ± 0.13	0.36 ± 0.01	13.28 ± 0.72	0.12 ± 0.00	0.23 ± 0.01	16.89 ± 0.82	7.75 ± 0.35
Mgl28B	11.81 ± 0.07	8.11 ± 0.15	0.35 ± 0.01	10.8 ± 0.67	0.13 ± 0.00	0.22 ± 0.009	20.88 ± 0.87	6.73 ± 0.17
Mgl29W	10.69 ± 0.17	8.33 ± 0.15	0.41 ± 0.01	9.46 ± 0.63	0.11 ± 0.00	0.29 ± 0.01	32.10 ± 0.72	12.35 ± 0.37
Mgl30B	11.68 ± 0.15	9.13 ± 0.13	0.51 ± 0.01	7.64 ± 0.56	0.07 ± 0.00	0.44 ± 0.01	28.21 ± 1.04	11.89 ± 0.42
Izm31B	7.82 ± 0.12	6.42 ± 0.14	0.15 ± 0.00	3.4 ± 0.26	0.04 ± 0.00	0.11 ± 0.007	22.27 ± 0.60	8.08 ± 0.17
Ayd32B	10.71 ± 0.18	8.16 ± 0.15	0.30 ± 0.01	9.51 ± 0.64	0.10 ± 0.00	0.20 ± 0.01	27.88 ± 0.76	11.23 ± 0.22
Ant33B	9.5 ± 0.26	6.26 ± 0.14	0.20 ± 0.01	4.84 ± 0.29	0.05 ± 0.00	0.15 ± 0.01	26.24 ± 0.35	10.57 ± 0.18
Ant34W	13.26 ± 0.29	10.28 ± 0.22	0.68 ± 0.03	11.68 ± 0.76	0.15 ± 0.00	0.53 ± 0.03	28.79 ± 0.81	12.74 ± 0.28
Ant35W	11.03 ± 0.24	9.89 ± 0.17	0.53 ± 0.03	15.24 ± 1.11	0.17 ± 0.01	0.36 ± 0.01	32.03 ± 0.65	15.30 ± 0.33
Ant36W	12.08 ± 0.22	9.59 ± 0.16	0.54 ± 0.02	7.71 ± 0.90	0.08 ± 0.001	0.46 ± 0.02	25.93 ± 0.6	11.25 ± 0.30
Ant37B	7.59 ± 0.19	5.72 ± 0.14	0.13 ± 0.00	2.26 ± 0.21	0.03 ± 0.002	0.10 ± 0.007	27.45 ± 0.65	11.77 ± 0.30
Ant38B	6.44 ± 0.19	4.77 ± 0.15	0.09 ± 0.00	2.22 ± 0.13	0.02 ± 0.001	0.06 ± 0.007	24.04 ± 0.69	9.08 ± 0.33
Ant44B	7.58 ± 0.14	6.85 ± 0.17	0.21 ± 0.01	3.25 ± 0.21	0.06 ± 0.004	0.14 ± 0.01	17.54 ± 0.30	6.94 ± 0.16
Mrs48W	12.46 ± 0.16	10.54 ± 0.12	0.67 ± 0.02	15.3 ± 1.03	0.19 ± 0.01	0.47 ± 0.02	24.25 ± 1.09	10.57 ± 0.42
Mrs49W	9.35 ± 0.16	8.31 ± 0.18	0.35 ± 0.02	2.26 ± 0.14	0.03 ± 0.003	0.32 ± 0.02	20.79 ± 0.71	7.45 ± 0.27
Mrs50B	11.73 ± 0.16	6.90 ± 0.10	0.22 ± 0.00	6.58 ± 0.55	0.07 ± 0.005	0.14 ± 0.005	23.41 ± 0.84	9.29 ± 0.28
Ant51B	8.54 ± 0.17	7.52 ± 0.25	0.28 ± 0.01	5.12 ± 0.47	0.05 ± 0.002	0.23 ± 0.01	22.46 ± 0.75	9.77 ± 0.37
Ant52B	11.33 ± 0.11	7.18 ± 0.08	0.29 ± 0.00	5.3 ± 0.37	0.07 ± 0.004	0.21 ± 0.005	28.57 ± 0.83	9.87 ± 0.22
Ant53W	8.18 ± 0.22	6.72 ± 0.25	0.19 ± 0.01	12 ± 1.34	0.10 ± 0.009	0.09 ± 0.01	22.15 ± 0.62	9.71 ± 0.31
Hty55B	9.53 ± 0.12	7.34 ± 0.11	0.24 ± 0.00	8.14 ± 0.40	0.09 ± 0.003	0.14 ± 0.008	22.29 ± 0.53	9.90 ± 0.27
Hty56W	11.41 ± 0.29	8.52 ± 0.28	0.43 ± 0.03	9.13 ± 0.90	0.10 ± 0.01	0.33 ± 0.02	28.84 ± 0.77	13.71 ± 0.31
Ant57B	12.5 ± 0.27	6.11 ± 0.16	0.16 ± 0.01	7.04 ± 0.60	0.07 ± 0.005	0.08 ± 0.006	32.06 ± 1.19	7.87 ± 0.34
Mgl59W	13.41 ± 0.45	11.22 ± 0.39	0.81 ± 0.06	14.45 ± 2.04	0.12 ± 0.01	0.69 ± 0.05	27.39 ± 0.77	13.38 ± 0.41

*Sufficient number of fruits could not be collected for Ant15B, Ant16B and Ant22B

Table 3. ANOVA of morphological characteristics

	Sum of squares	df	Mean square	F	Sig.
Fruit length	7269.623	44	165.219	137.175	.000
Fruit weight	84.074	44	1.911	147.834	.000
Number of Seed	28091.839	44	638.451	44.178	.000
Seed weight	2.932	44	,067	47.557	.000
Pulp weight	59.594	44	1.354	152.736	.000
Leaf length	39853.370	47	847.944	25.153	.000
Leaf width	46946.082	47	998.853	1.562	.009

The mean differences are significant 0.01 level

Molecular analysis

Molecular analysis was done separately with two marker system as well as in combination of SSR and iPBS data sets.

Simple sequence repeats (SSR)

Eleven out of the 12 markers designed for *Myrtus communis* (Albaladejo et al., 2010) were polymorphic in 48 wild myrtle genotypes and produced a total of 64 alleles. The number of fragments varied from one to nine with an average 5.3 per SSR primer pair (*Table 4*). In line with previous studies of Corona et al. (2017) and Mele et al. (2019) five SSR primer pairs, namely *Myrcom2, Myrcom4, Myrcom7, Myrcom8, Myrcom11*, were highly polymorphic, each producing 7-9 alleles. Three primers *Myrcom5, Myrcom6, Myrcom9* were moderately polymorphic, each producing 4 to 5 alleles. The remaining four SSR primer pairs, *Myrcom1, Myrcom3, Myrcom10, Myrcom12*, were less polymorphic by producing two to three alleles each (*Table 3*). The findings for *Myrcom1* and *Myrcom12* primers of current study are in accordance with findings of Corona et al. (2017) with 2 alleles for each primer and a similar band pattern was obtained by Albaladejo et al. (2010) for the Myrcom1, Myrcom5, Myrcom7 primer pairs. The PIC values of SSR primers ranged from 0.45 (Myrcom1, Myrcom6) to 0.99 (Myrcom8) with an average of 0.70 (*Table 4*). Similarity index ranged from 0.31 to 1.00 based on the similarity matrix (data not shown). A phylogenetic tree obtained based on the similarity matrix for SSR markers shown in *Figure 4*. The genetic distance coefficients ranged from 0.63 to 0.97 with an average of 0.82. Based on phylogenetic analysis, the 48 genotypes were clearly clustered into two major clades (A and B) at the 0.63 similarity coefficient level. Clades A divided two groups (A1 and A2) at the similarity index of 0.65. Seven different genotypes collected from Antalya province are clustered together in cluster A1. Similar pattern was observed in accession from İzmir and Aydın (both located on the Aegan region) that grouped together in cluster A.2. The cluster A2. 1 contained 18 accessions 13 of which have white fruit color. Seven genotypes clustered in A2.1. clade namely Ant4W, Ant8W, Ant11W, Ant20W, Ant34W, Mrs48W, Hty56W found genetically similar by SSR markers. B1.2.2 included 13 genotypes with both black and white berries and collected from different regions.

Inter-primer binding site (iPBS)

Initially 30 primers were screened with bulk DNA, which was prepared by taking equal amounts of DNA samples from each genotype and 12 polymorphic primers were

selected for further investigations (*Table 5*). The iPBS analysis was performed on 48 genotypes revealing 135 total and 80 polymorphic bands with a mean of 11.25 total bands for each tested primer. Maximum 14 fragments were generated with primers 2378 and 2394, minimum 8 fragments were generated with 2078 and 2221. A total of 80 polymorphic bands were obtained with a mean of 6.66 bands for each primer and 2378 was the most polymorphic one with 13 bands, while primer 2221 produced only two polymorphic bands. The sizes of reproducible and scorable bands ranged from175 bp to 2000 bp. Percentage of polymorphism ranged from 25.0% (2221) to 92.8% (2378), with an average polymorphism of 57.26% across all the genotypes. Similarity index ranged from 0.44 to 1.00 based on the similarity matrix (data not shown). The PIC observed for 2078 marker was the highest thus, this marker could be more efficient in genotypic differentiation of myrtle genotypes. A dendrogram based on UPGMA analysis with iPBS data is shown in *Figure 5*. The 47 genotypes were grouped into one cluster (B) at the 0.58 similarity coefficient level whereas one genotype (Ant19) separated from the population (A) showing less similarity coefficient (0.58) with other genotypes studied. Genotypes within clade B are further grouped into two subclusters, B1 and B2 at the 0.63 similarity coefficient level. Clade B2 comprised 4 genotypes and clade B1 comprised 43 genotypes and separated into two sub-group. Six genotypes with white fruit color, collected from different geographical locations namely: Ant4W, Ant21W, Ant35W, Mrs48W, Hty56W, Mgl59W found genetically similar and located in the subcluster B1.1. The genotypes Ant11W, Isp18W, Ant20W, Ant34W placed in the cluster B1.1 also found genetically similar with iPBS markers (*Fig. 5*). Ant4W, Mrs48W, Hty56W and Ant11W, Ant20W genotypes were found genetically similar by both SSR and iPBS markers.
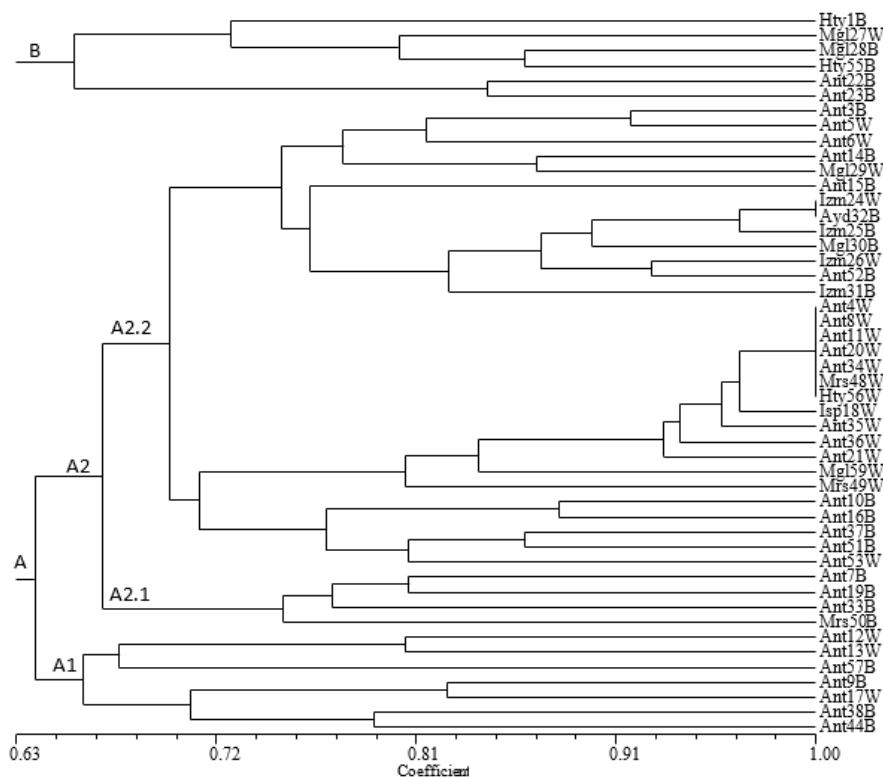

Figure 4. Dendrogram for 48 Myrtus communis accessions derived from a UPGMA cluster analysis based on 12 SSR markers

Table 4. *List of SSR primers and sequence, number of bands and polymorphic bans, allele size range (bp) and polymorphic information content value generated by each primer*

Primer	Primer sequences 5'-3'	NTA	NPA	PPA	Size (bp)	PIC
Myrcom1	F: CGTGATGCACACTGAACTGA R: AACCCCTTTTGCCAACATTT	3	3	100	225-230	0.45
Myrcom2	F: ATAGCTCTTACCCGCCATTG R: GTGCATGGTCCTCGATAGGT	9	9	100	213-240	0.88
Myrcom3	F: GGCAGCTACCAGTCATACCC R: TTTGCAGCATTTCAAAGTGG	2	2	100	183-187	0.77
Myrcom4	F: CAACCACATCCACCCATAGA R: CCACAGTCAAGAGGGAGAGC	8	7	100	162-187	0.52
Myrcom5	F: TGAGAGATCAGCAACCAAAAAG R: CATGAATGGCAACGATGAAA	5	5	100	253-269	0.75
Myrcom6	F: AAATGAAAAAGCTAAAAGTTAAAC R: AACAGGAAGAGCAAGCCAAG	4	4	100	179-181	0.45
Myrcom7	F: AGACATGCTCAAACTTGTATGC R: AATGTATCCCAACATGTCAGA	9	9	100	177-213	0.87
Myrcom8	F: TGCTCGGTCATTAATTGGTGT R: TCAAAACCGTCTCCATGAAA	9	9	100	230-270	0.99
Myrcom9	F: GAAAGTTGCACTGTTTATTTCCAA R: TCTTCCTTCCAATCCTCATCA	4	4	100	181-187	0.67
Myrcom10	F: TTAAGTGCCTTTGGCATTTGT R: AGAGGACCTCGCGATAGACA	1	-	100	166	
Myrcom11	F: GCAAATAAAAAGCGAGTTAAATGA R: CCACACTTTTAAGAATTTGTGGTC	8	8	100	230-250	0.97
Myrcom12	F: CCCTCCATTTTTCCCTTCTC R: AGCCGAAGCTCCAAGAAAC	3	3	100	140-146	0.49

NTA: number of total allele, NPA: number of polymorphic allele, PPA: percentage of polymorphic allele, PIC: polymorphic information content

Table 5. *List of iPBS primers and sequence, number of bands and polymorphic bands, band size range (bp) and polymorphic information content value generated by each primer*

Primer	Primer sequences 5'-3'	NTB	NPB	PPB	Size (bp)	PIC
2078	GCGGAGTCGCCA	8	5	62.5	250-1300	0.63
2079	AGGTGGGCGCCA	11	3	27.2	250-1500	0.13
2080	CAGACGGCGCCA	12	6	50	300-1500	0.46
2081	GCAACGGCGCCA	12	8	66.6	350-1450	0.51
2095	GCTCGGATACCA	9	5	55.5	245-1750	0.31
2221	ACCTAGCTCACGATGCCA	8	2	25	245-1500	0.38
2270	ACCTGGCGTGCCA	12	8	66.6	245-1750	0.36
2277	GGCGATGATACCA	12	10	83.3	250-1500	0.31
2376	TAGATGGCACCA	12	6	50	250-1650	0.22
2378	GGTCCTCATCCA	14	13	92.8	175-2000	0.25
2383	GCATGGCCTCCA	11	4	36.3	295-1500	0.49
2394	GAGCCTAGGCCA	14	10	71.4	250-1500	0.40

NTB: number of total bands, NPB: number of polymorphic bands, PPB: percentage of polymorphic band, PIC: polymorphic information content
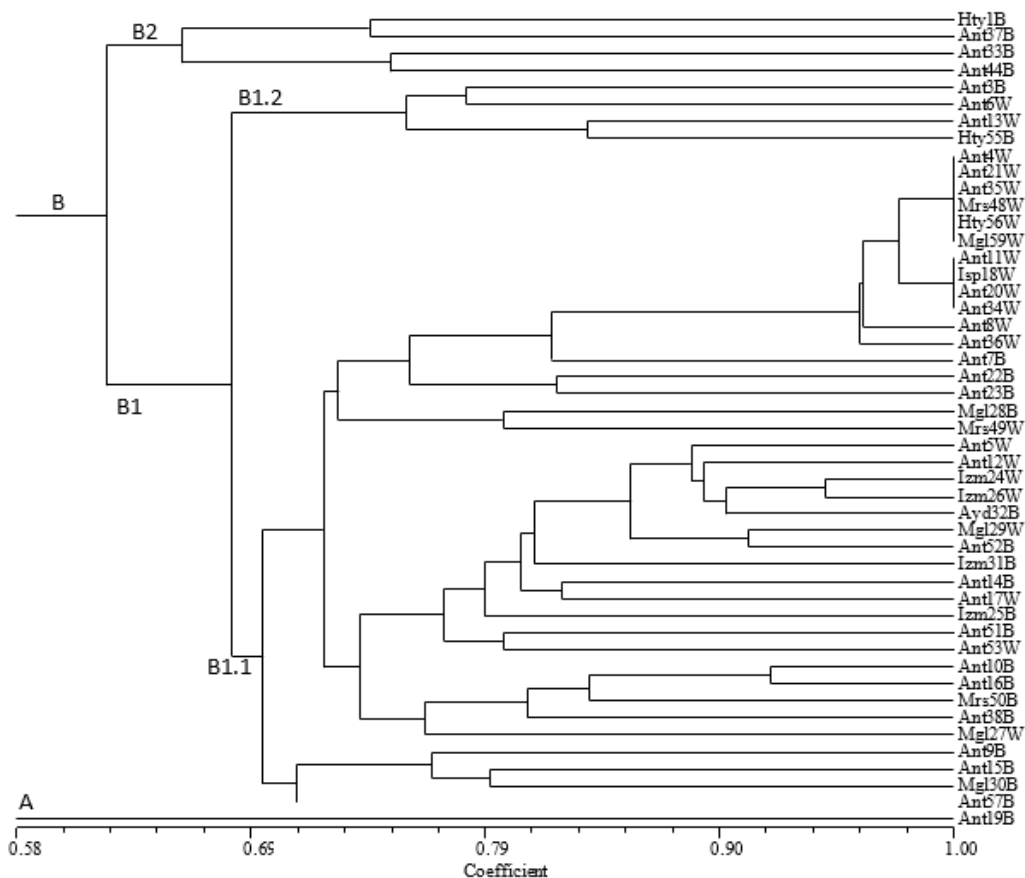
Figure 5. *Dendrogram for 48 Myrtus communis accessions derived from a UPGMA cluster analysis based on 12 iPBS markers*

Evaluation of combined data of SSR and iPBS results with morphological findings

Similarity coefficient ranged from 0.64 to 1.00 in the dendrogram based on UPGMA analysis performed with combined data of SSR and iPBS primers and the population divided to two main group (A and B) at the similarity value of 0.64. Clade B comprised 8 genotypes, all with black fruit, and 7 of these genotypes were collected from Antalya province except Hty1B. Clade A divided into sub-clusters and sub-cluster A2 comprised 39 genotypes. Only one genotype (Ant 57) placed in sub-cluster A1 and appeared to be distinct from all others based on the combined SSR and iPBS based data sets (*Fig. 6*). The genotypes Ant4W, Mrs48W, Hty56W and Ant11W, Ant20W, Ant34W were found genetically very close (similarity index 1.00) with SSR and iPBS markers and placed together in the same clade with Ant 21W, Ant 36W and Mugl59W, Ant8W, Ant35W and Isp18W at the 0.92-0.98 similarity index value (*Fig. 6*). All these 12 genotypes have berries with white color and larger fruits (ranged from 0.43 g to 1.18 g with average fruit weight 0.65) than the other genotypes which have an average fruit weight of 0.23 g. The average number of seeds per fruit and seed weight of these 12 genotypes 13.41 and 0.13 g respectively, the remaining genotypes have an average 6.77 seed per fruit and 0.07 g seed weight. These genotypes with white and larger berries may be semi-cultivated and selected genotypes and probably genetically very similar or identical. Our results demonstrated that the genotypes are found to be genetically indistinguishable usually consisted of berries with white color. Uzun et al. (2016) also mentioned a selected

genotype (Hambeles) usually grown at the edges of the land with white fruit color and average 1.06 g fruit weight. In the current study fruit weight of the genotypes with white berries ranged from 0.06 to 1.28 g with average 0.48 g and genotypes with black berries ranged from 0.20 to 0.60 with average 0.25 g respectively. Messaoud et al. (2005) and Messaoud and Boussaid (2011) reported that the white fruits are smaller and showed high number of seeds per fruit than the dark blue fruit. The genetic relationship within the population of white berries and within the population with black berries were also analyzed with combined data of iPBS and SSR markers. The similarity co-efficient of genotypes with white berries and black berries ranged 0.69 to 1.00 and 0.61-0.90 respectively (*Figs. 7a* and *8a*). As seen in dendrograms the genotypes with black berries have higher genetic diversity than white ones. This result ties well with previous studies wherein similar results reported with dark blue morph population in Tunisia (Messaoud et al., 2011). In the current study the genotypes with white berries show less diversity than genotypes with black berries. At a similarity index value of 0.69 the genotypes with white berries were divided into two major clusters and first clade 6 genotypes. The second clade includes 17 genotypes and 11 of those were genetically most related and form a subgroup at the similarity index value of 0.96 (*Fig. 7a*). The 2D plot generated from the PCA of the combined iPBS and SSR data (*Figs. 7b* and *8b*) supported the clustering pattern of the UPGMA dendrogram. As with the scatter plot of the principal component analysis, overlapping in grouping was observed in accessions with white berries (*Fig. 7b*) and genotypes with black berries are dispersed (*Fig. 8b*).
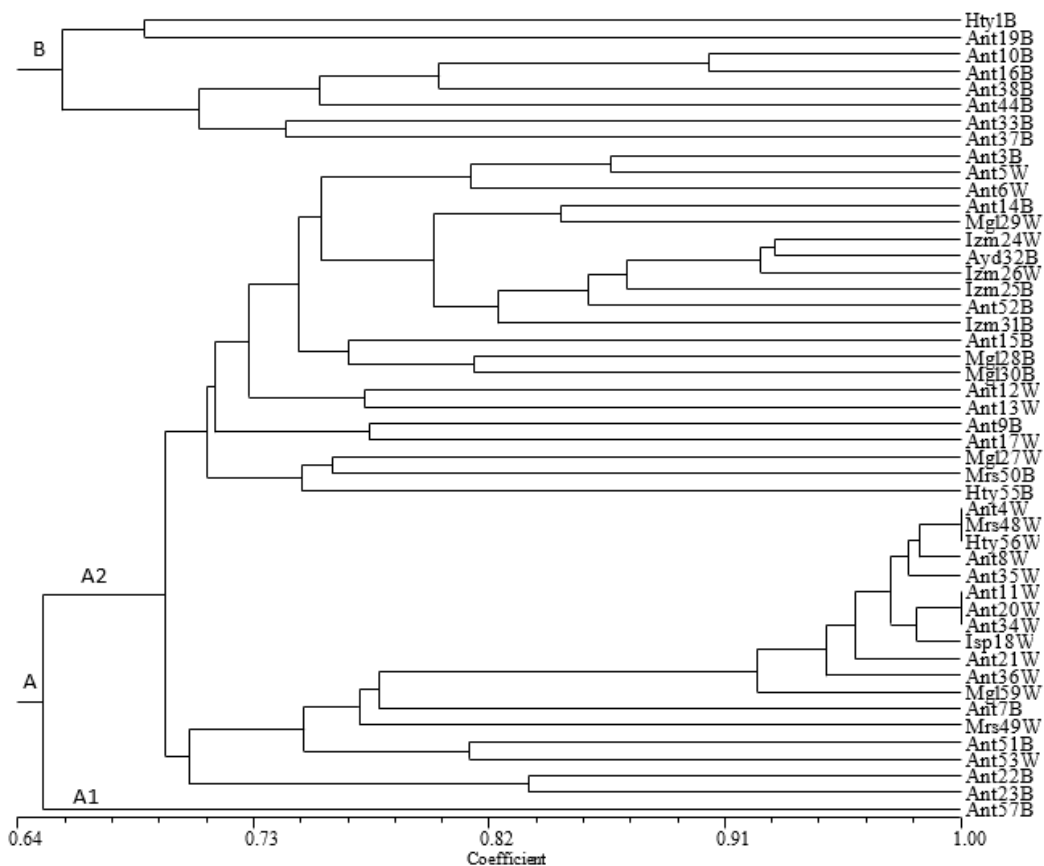

***Figure 6.** Dendrogram for 48 Myrtus communis accessions derived from a UPGMA cluster analysis based on combined data of iPBS-SSR markers*
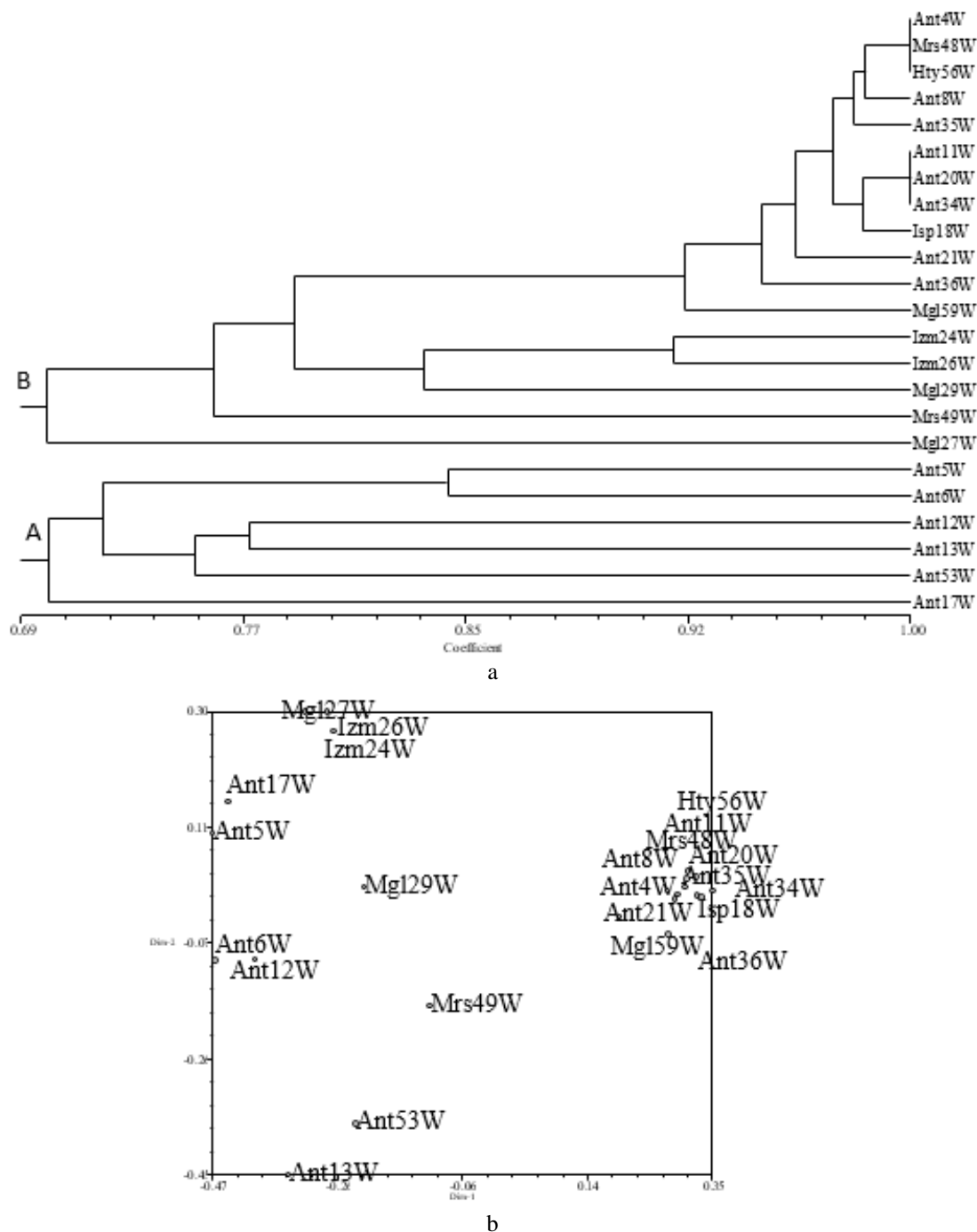
a

b

Figure 7. *(a) Dendrogram for 23 Myrtus communis accessions with white fruits derived from a UPGMA cluster analysis based on combined data of iPBS-SSR markers. (b) Two-dimensional plot of principal components (PC) 1 and 2 based on combined data of iPBS-SSR markers*

Both marker techniques (iPBS and SSR) proved to be effective in discriminating the 48 genotypes. To our knowledge, iPBS primers for *Myrtus communis* were used for the first time in this study and according to the results obtained ıPBS primers can be used in phylogenetic analyzes and mapping studies of myrtle.
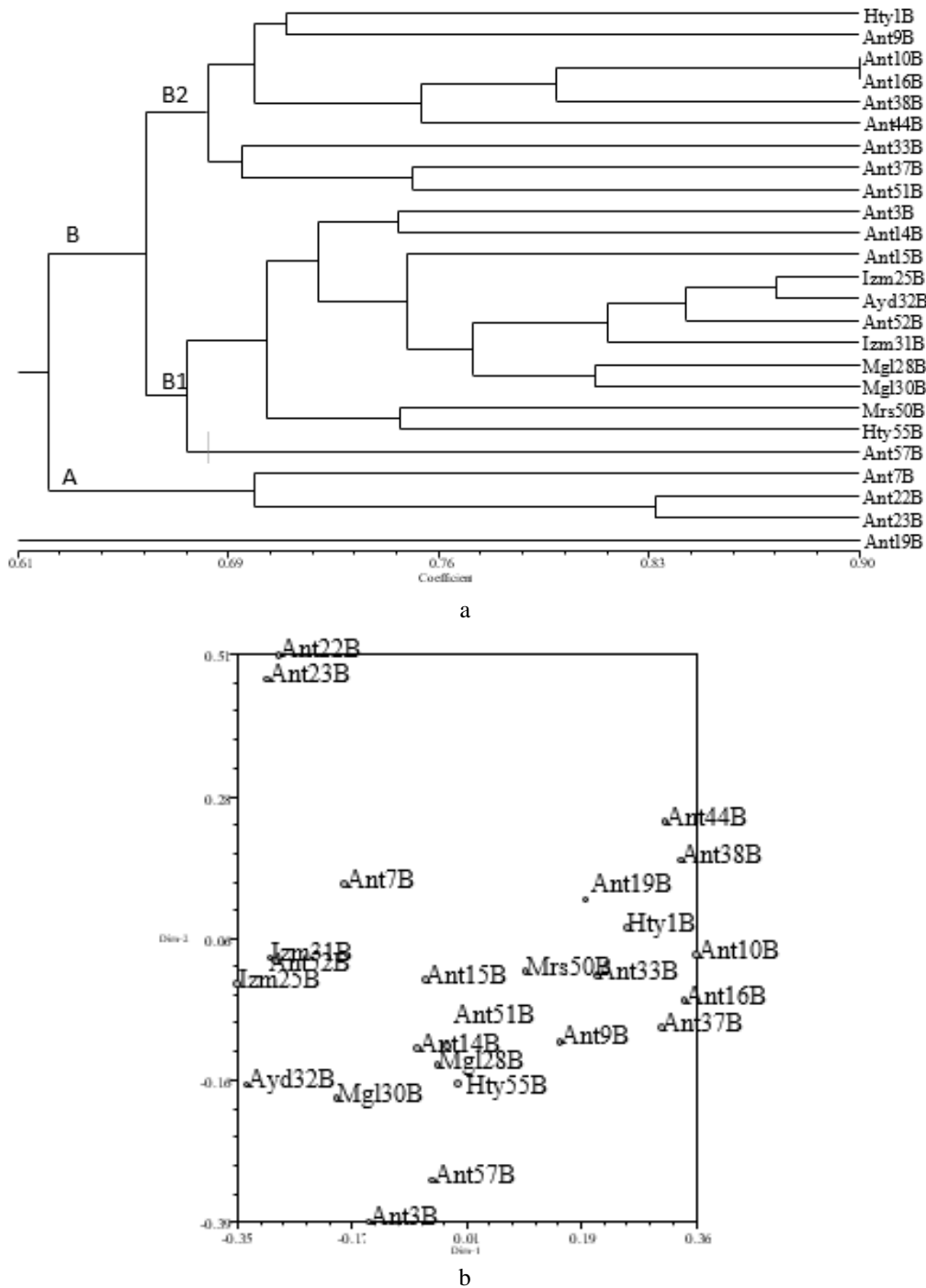
a

b

Figure 8. *(a) Dendrogram for 25 Myrtus communis accessions with dark blue berries derived from a UPGMA cluster analysis based on combined data of iPBS-SSR markers. (b) Two dimensional plot of principal components (PC) 1 and 2 based on combined data of iPBS-SSR markers*

Conclusion

Genetic diversity is fundamental for germplasm collections. Natural floras in the Mediterranean Sea Coastal of Turkey have a significant amount of myrtle (*Myrtus communis*) genotypes. Using genetic resources available, new cultivars can be improved

by gathering of plants from natural flora, selection of superior species that have characteristics desired for further cultivation and breeding practices.

Characterization and evaluation of myrtle is an imperative step for germplasm conservation and utilization in the breeding programs. Moreover, characterization of available germplasm is very crucial to identify desired traits or genes. The genetic variation obtained by morphological and molecular analyzes between genotypes shows that these genotypes can be useful for future breeding and cultivation practices and can be considered as valuable genetic resources that need to be protected. These results are valuable for further reproduction and conservation programs for *Myrtus communis*. Additional studies to understand the genetic characteristics of the genotypes regarding the genes associated with important traits are required.

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