



# Investigation of the genetic variation of anise (*Pimpinella anisum* L.) using RAPD and ISSR markers

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**Abstract** Anise (*Pimpinella anisum* L.) is an annual spice and medicinal plant belonging to the family Apiaceae. Only a limited number of anise breeding studies are available. Therefore, genetic variation among anise landraces has not been extensively examined with molecular markers. In this study, the molecular diversity of 15 Turkish anise landraces collected from different regions of Turkey and four foreign landraces obtained from Cyprus, Syria and France were analyzed using randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers. The discriminatory power of these markers was assessed by calculating various marker parameters, namely percent polymorphism, polymorphism information content (PIC), resolving power (RP), and marker index (MI). The genomic DNA of 19 anise landraces was amplified with nine RAPD primers that generated 71 polymorphic bands and five ISSRs primers that produced 45 polymorphic bands. The percentage of polymorphic loci was 59.8% in RAPD profiling and 51.5% in ISSR profiling. The average PIC values obtained using RAPD and ISSR markers were 0.40 and 0.38, respectively. The mean RP (2.90 and 2.67) and MI values (1.23 and 1.05) of the RAPD assay were found to be somewhat higher than those of the ISSR assay, but ISSR was more

favorable than the RAPD techniques in terms of presenting the phylogenetic relationships in anise landraces. A UPGMA cluster grouped the 19 anise landraces into two major clusters based on both markers. The RAPD and ISSR marker systems were found to be useful for determining genetic diversity in *P. anisum* L. and identifying variation among landraces.

**Keywords** Anise · *Pimpinella anisum* L. · RAPD · ISSR markers · Molecular characterization · Fingerprinting

## Introduction

Anise (*Pimpinella anisum* L.), belonging to the family Apiaceae (Umbelliferae), is an important spice and medicinal plant used for pharmaceuticals, perfumery, food and beverages industries. Anise is native to the Middle East and is grown in Turkey, Iran, Iraq, India, Egypt, South Africa, Latin America, and many other warm regions of the world (Arslan et al. 2004). In Turkish folk medicine, the seeds of anise have been used as an appetizer, tranquillizer, carminative, emmenagog and diuretic drug (Bayram 1992). Essential oils of anise have positive effects on the central nervous system and are also known to help sleep. Besides, these oils are characterized by antispasmodic, antioxidant, antimicrobial, insecticidal, and antifungal

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properties (Tunc and Sahinkaya 1998; Gülcin et al. 2003; İpek et al. 2004; Özcan and Chalchat 2006; Tepe et al. 2006; Tirapelli et al. 2007). Anise is an important agricultural crop in Turkey, extensively used in the production of “raki”, a famous national alcoholic beverage. In Turkey, anise has a cultivation area of approximately 12,450 ha and seed production of approximately 8700 tons (TUİK 2018), of which 70% are used in the manufacturing of raki and 30% are exported throughout the world (Baser 1997; Tabanca et al. 2006). As a spice plant, anise ranks second after cumin in terms of the planting area in Turkey. Anise grown in Turkey is usually known by the name of the cultivation area, such as Cesme Anise, Burdur Anise, and Denizli Anise. Although Turkey is an important producer of anise, “Göhlhisar” is the only variety registered by the Aegean Agricultural Research Institute in 1988 (Sarı and Oguz 2004). Today, this variety is not known or used by anise producers. For this reason, producers either procure the seeds from previous crops or other farmers (Gürbüz 2010). The production of anise is still carried out using landraces. The seeds used in production are composed of very mixed populations. Therefore, the anise industry experiences certain difficulties in obtaining products of standard quality. It is thus necessary to conduct further breeding studies in different ecologies to increase the seed yield and improve the quality characteristics of aniseed. The success of a breeding program depends, first, on sound genetic information about the breeding material. In addition, the methods for the fast and accurate identification of genotypes, determination of the uniformity of the cultivars, and evaluation of the genetic variability provide substantial benefits for the certification of cultivars with specific medicinal properties, and management of germplasm banks and breeding programs (Manica-Cattani et al. 2009). Molecular markers have been successfully used for this purpose.

Random amplified polymorphic DNA (RAPD) (Williams et al. 1990) and inter-simple sequence repeat (ISSR) markers (Zietkiewicz et al. 1994) are the two commonly used polymerase chain reaction (PCR)-based techniques for estimating genetic diversity in many plant species, including several medicinal and aromatic plants (MAPs), such as lemongrass (Bishoyi et al. 2016), sweet basil (Giachino et al. 2014), *Allium* (Mukherjee et al. 2013), yarrow (Ebrahimi et al. 2012), oregano (Tonk et al. 2010),

cannabis (Kayis et al. 2010), and Lippia (Manica-Cattani et al. 2009). These markers are highly sensitive in detecting genomic polymorphism and offer great potential to determine intraspecific and interspecific levels of variation. They involve a large number of loci and cover a large part of the genome. RAPD and ISSR markers, compared to the other marker systems, are time- and cost-efficient, do not require sequence information or use of radioactive substance, and are easy and fast to apply. However, to date, only a limited number of studies have examined the genetic relationships between *Pimpinella* species (e.g., Nurcahyanti et al. 2016; Wang et al. 2014; Maraklı 2018).

The objective of the present work was to identify genetic variation between *Pimpinella anisum* landraces of Turkish and different origin using RAPD and ISSR markers. In addition, estimates of genetic relationships between the anise landraces were determined and comparatively analyzed for both marker assays.

## Material and methods

The material of the study consisted of the seeds of 19 anise landraces (*P. anisum* L.) collected from different localities in Turkey (Balıkesir, Denizli, Konya, Isparta, Burdur, Eskisehir, and Cesme) and obtained from Cyprus, Syria, and France (Table 1). This study was carried out at Ege University Agriculture Faculty Central Laboratory (AGROLAB) in Izmir/Turkey.

## DNA isolation

Total genomic DNA of 19 anise landraces was extracted from approximately 100 mg fresh leaves using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich) following the instruction manual. Each score represents the fingerprint of 20 bulked plants. Purity and concentration of the DNA template was checked by the 260:280 nm absorbance ratios of a spectrophotometer. DNA integrity was confirmed by electrophoresis on 0.8% agarose gel. The DNA concentrations diluted to 10 ng/μl were determined based on the absorbance measured at 260 nm and stored at – 20 °C.

**Table 1** Origin of anise landraces used in the study

Code	Label	Origin/province	Country
1	Bal-1	Balikesir	Turkey
2	Bal-2	Balikesir	Turkey
3	Den-1	Denizli	Turkey
4	Den-2	Denizli	Turkey
5	Ko-1	Konya	Turkey
6	Ko-2	Konya	Turkey
7	Is-1	Isparta	Turkey
8	Is-2	Isparta	Turkey
9	Cyp-1	Famagusta	Cyprus
10	Cyp-2	Famagusta	Cyprus
11	Esk	Eskisehir	Turkey
12	Ces	Cesme	Turkey
13	Bur-1	Burdur	Turkey
14	Bur-2	Burdur	Turkey
15	Bur-3	Burdur	Turkey
16	Bur-4	Burdur	Turkey
17	Bur-5	Burdur	Turkey
18	Syr	Aleppo	Syria
19	Fra	Tarn	France

### RAPD analysis

A PCR analysis was performed in a final volume of 15  $\mu$ l containing 50 ng DNA template, 1  $\times$  Taq DNA polymerase buffer (100 mM Tris–HCl, pH: 8.3, 500 mM KCl and 0.01% gelatin), 200  $\mu$ M each dNTP, 3 mM MgCl<sub>2</sub>, 0.5  $\mu$ M primer (Operon Technologies, USA), and 0.5 unit of Taq DNA polymerase (Sigma). In total, 17 oligonucleotides arbitrary primers from (Operon Technologies, USA) were used for RAPD amplification. Nine primers were selected based on the repeatable and scorable banding patterns to detect the polymorphisms in *P. anisum* L. landraces (Table 2). PCR amplification was carried out in a Thermo Scientific Arktik thermal cycler with an initial denaturation at 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 42 °C for 1.30 min and extension at 72 °C for 1 min with a final extension at 72 °C for 10 min. The amplified PCR products were separated by TAE-buffered gel electrophoresis in 2% agarose, stained with ethidium bromide, and visualized under a UV transilluminator (Vilbert Lourmat Bio-Vision 1000/26MX). A 1 kb Plus DNA ladder (Thermo Scientific GeneRuler) was

used for the estimation of the molecular weights of the amplified products.

### ISSR analysis

Of the nine ISSR primers (University of British Columbia, Canada) used for PCR, five provided evaluable bands (Table 3). The PCR analysis was performed following the protocol described by Zietkiewicz et al. (1994). All primers were amplified in a total volume of 10  $\mu$ l PCR mixture containing 10X Taq DNA polymerase buffer (100 mM Tris–HCl, pH: 8.3, 500 mM KCl and 0.01% gelatin), 2.5 mM MgCl<sub>2</sub>, 300  $\mu$ M dNTPs, 1 mM primer, 1 unit taq DNA polymerase (Sigma), and 50 ng DNA. Amplification reactions were performed in a Thermo Scientific Arktik thermal cycler using the following program: an initial cycle at 94 °C for 1.30 min, followed by 45 cycles at 94 °C for 45 s, 52–60 °C for 45 s, 72 °C for 1.30 min, and a final cycle at 72 °C for 7 min. The PCR products were electrophoresed in 2% agarose gel with ethidium bromide in 0.5XTBE buffer at 4 V/cm. The gels were then photographed using Vilbert Lourmat Bio-Vision 1000/26MX (France) imaging system. A 100 bp plus DNA ladder (Fermentas) was used as a standard marker for estimating the size of the PCR products.

### Data analysis

The RAPD and ISSR bands were scored for their presence (1) or absence (0) of amplified bands for each of the 19 anise landraces. The total number of the bands scored, the number of polymorphic bands, and the percentage of polymorphic bands were determined for each primer used. The discriminatory power of these markers was assessed by calculating various marker parameters, namely polymorphism information content (PIC), resolving power (RP), effective multiplex ratio (EMR), and marker index (MI). The PIC values were calculated according to Anderson et al. (1993), using the following formula for all primer combinations:  $PIC = 1 - \sum p_i^2$ , where  $p_i^2$  is the frequency of the *i*th allele. PIC provides an estimate of the discriminatory power of a locus by taking into account not only the number of alleles that are expressed but also the relative frequencies of those

**Table 2** List of the RAPD primers used in this study, their base sequences, total number of fragments (TF), number of polymorphic fragments (PF), polymorphic fragments ratios % (PFR%), polymorphism information content (PIC), resolving power (RP), effective multiplex ratio (EMR) and marker index (MI)

Primer	Sequence 5'–3'	TF	PF	PFR%	PIC	RP	EMR	MI
OPA-01	5'-CAGGCCCTTC-3'	10	5	50	0.38	2.74	2.50	0.96
OPA-03	5'-AGTCAGCCAC-3'	8	4	50	0.44	2.74	2.00	0.89
OPA-17	5'-GACCGCTTGT-3'	9	4	44.4	0.43	2.74	1.78	0.77
OPAA-10	5'-TGGTCGGGTG-3'	6	1	16.7	0.47	0.74	0.17	0.08
OPAB-02	5'-GGAAACCCCT-3'	10	8	80	0.42	5.16	6.40	2.69
OPAB-05	5'-CCCGAAGCGA-3'	9	7	77.8	0.33	3.58	5.44	1.78
OPAJ-07	5'-CCCTCCCTAA-3'	4	3	75	0.34	1.58	2.25	0.77
OPAJ-18	5'-GGCTAGGTGG-3'	9	7	77.8	0.37	4.00	5.44	2.03
OPK-10	5'-GTGCAACGTG-3'	6	4	66.7	0.43	2.84	2.67	1.15
Average		7.9	4.8	59.82 <sup>a</sup>	0.40 <sup>a</sup>	2.90 <sup>a</sup>	3.18 <sup>a</sup>	1.23 <sup>a</sup>
Minimum		4	1	16.7	0.33	0.74	0.17	0.08
Maximum		10	8	80	0.47	5.16	6.40	2.69
Total		71	43	–	–			

<sup>a</sup>Average of the column

**Table 3** List of the ISSR primers used in this study, their base sequences, annealing temperature (Temp °C), number of total fragments (TF), number of polymorphic fragments (PF),

polymorphic fragments ratios % (PFR%), polymorphism information content (PIC), resolving power (RP), effective multiplex ratio (EMR) and marker index (MI)

Primer	Sequence 5'–3'	Temp (°C)	TF	PF	PFR%	PIC	RP	EMR	MI
ISSR 4	5'-HVH(TCC) <sub>5</sub> -3'	60	15	11	73	0.32	5.05	8.07	2.55
ISSR11	5'-(GA) <sub>8</sub> YG-3'	56	8	5	62.5	0.44	3.37	3.13	1.38
ISSR 24	5'-(AC) <sub>8</sub> C-3'	52	9	4	44.4	0.32	1.89	1.78	0.58
ISSR 25	5'-(AC) <sub>8</sub> G-3'	52	8	3	37.5	0.34	1.58	1.13	0.39
ISSR 28	5'-(GA) <sub>8</sub> YC-3'	56	5	2	40	0.46	1.47	0.80	0.37
Average			9	5	51.48 <sup>a</sup>	0.38 <sup>a</sup>	2.67 <sup>a</sup>	2.98 <sup>a</sup>	1.05 <sup>a</sup>
Minimum			5	2	37.5	0.32	1.47	0.80	0.37
Maximum			15	11	73	0.44	5.05	8.07	2.55
Total			45	25	–	–	–	–	–

Y = G/C, H = A/T/C, V = 3A/G/C

<sup>a</sup>Average of the column

alleles. The RP of each primer was determined according to Prevost and Wilkinson (1999) as follows:  $RP = \sum Ib$ , where  $Ib$  is the band informativeness with  $Ib = 1 - [2 \times (0.5 - p)]$  and  $p$  is the proportion of 19 landraces containing the band. MI was obtained by multiplying the PIC with effective multiplex ratio (EMR), defined as the product of the fraction of polymorphic loci and the number of polymorphic loci (Powell et al. 1996; Milbourne et al. 1997) ( $MI = EMR \times PIC$ ). Genetic distance based on the Dice coefficient (Dice 1945) was calculated by making a pairwise comparison between all anise landraces using the Simqual module of NTSYS-pc software version

2.20j (Rohlf 2000). A principle coordinate analysis (PCoA) was also carried out using this software. The distance coefficients obtained were utilized to construct dendrograms utilizing the unweighted pair group method with arithmetic averages (UPGMA) and the sequential, agglomerative, hierarchical, and nested clustering (SAHN) algorithm in the same software package. To determine the goodness-of-fit of the clustering compared with the basic data matrix, the cophenetic correlation coefficient was computed using the normalized Mantel statistics Z test (Mantel 1967) via the COPH and MXCOMP procedures of NTSYS-pc, version 2.01e (Rohlf 2000).

## Results

### RAPD analysis

In the RAPD analysis of the 19 anise landraces, 17 random primers were screened, and nine were detected. From the nine primers evaluated, a total of 71 fragments were obtained with 43 (60.6%) bands being polymorphic. The base sequences, total number of fragments (TF), number of polymorphic fragments (PF), polymorphic fragments ratios % (PFR %), PIC, RP, EMR and MI values are presented in Table 2.

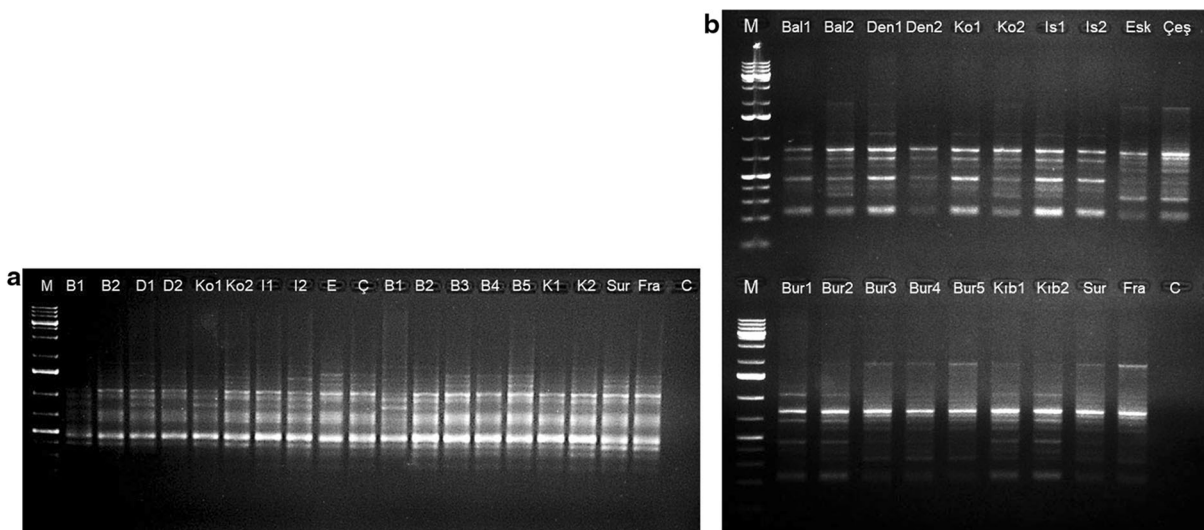
The numbers of bands of the primers that yielded results varied between 4 (OPAJ-07) and 10 (OPA-01 and OPAB-02). The number of PFs generated by each RAPD primer ranged from 1 (OPAA-10) to 8 (OPAB-02) (Fig. 1). The mean numbers of fragments and PFs per primer were 7.9 and 4.8, respectively. The level of polymorphism ranged from 16.7% (OPAA-10) to 80% (OPAB-02). The average PFR was found to be 59.8% (Table 2).

The PIC value of the RAPD primers varied from 0.33 (OPAB-05) to 0.47 (OPAA-10) with an average of 0.40. Five of the nine primers (OPA-03, OPA-17, OPAA-10, OPAB-02, and OPK-10) had a PIC value above the average (Table 2).

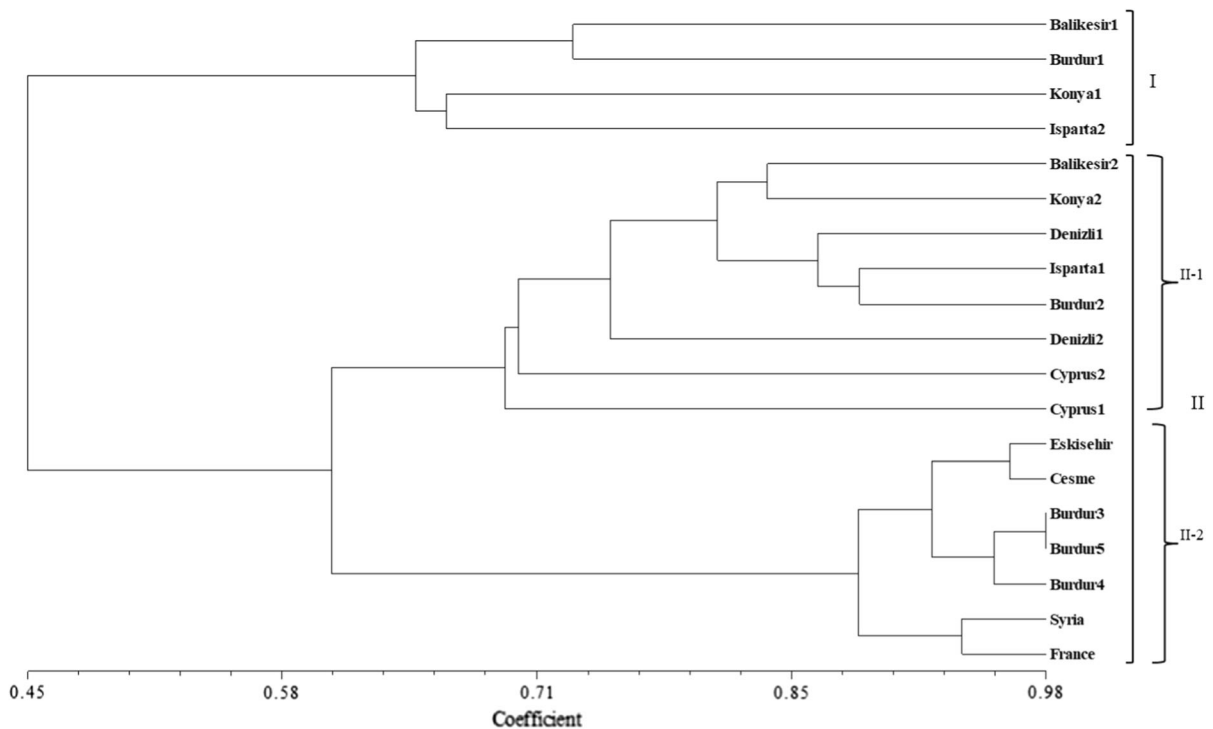
RP is another parameter used to indicate the discriminatory potential of selected primers or techniques (Prevost and Wilkinson 1999). In the current

study, the highest RP value was observed with the primer OPAB-02 (5.16) and the lowest with the primer OPAA-10 (0.74), with an average RP of 2.90 per primer. EMR depends on the fraction of polymorphic fragments. In this study, the highest EMR (6.40) was obtained from OPAB-02 and the lowest (0.17) from OPAA-10, with an average of 3.18 per primer. MI was calculated for each RAPD primer to determine the overall usefulness of the marker system used. The MI of the primers ranged from 0.08 (OPAA-10) to 2.69 (OPAB-02), with an average of 1.23. The most informative primers were found to be OPAB-02 and OPAJ-18 (Table 2).

The dendrogram obtained using the Dice's similarity index is shown in Fig. 2. The cophenetic correlation coefficient was fairly high (0.79), indicating a good fit of the cluster analysis performed. This shows that the clustering dendrogram was compatible with the similarity matrix. The anise landraces were divided into two main clusters based on RAPD markers (Fig. 2). Cluster I consisted of four Turkish landraces: Balıkesir1, Burdur1, Konya1, and Isparta2. The second cluster was further divided into two subclusters: Subcluster 1 comprising two foreign landraces, Cyprus2 and Cyprus1, and six domestic landraces, Balıkesir2, Konya2, Denizli1, Isparta1, Burdur2 and Denizli2, and Subcluster 2 containing the remaining foreign and domestic landraces: Syria, France, Eskisehir, Cesme, Burdur3, Burdur5, and



**Fig. 1** Amplification patterns of anise landraces generated using arbitrary primers OPAA-10 (a) and OPAB-02 (b). M indicates the 1 kb plus size marker (Fermentas) and C is the PCR-control



**Fig. 2** The UPGMA dendrogram based on the similarity index among the 19 anise landraces based on RAPD markers

Burdur4. In the dendrogram, Burdur3 and Burdur5 landraces were observed as the closest landraces with the Dice values of 0.98.

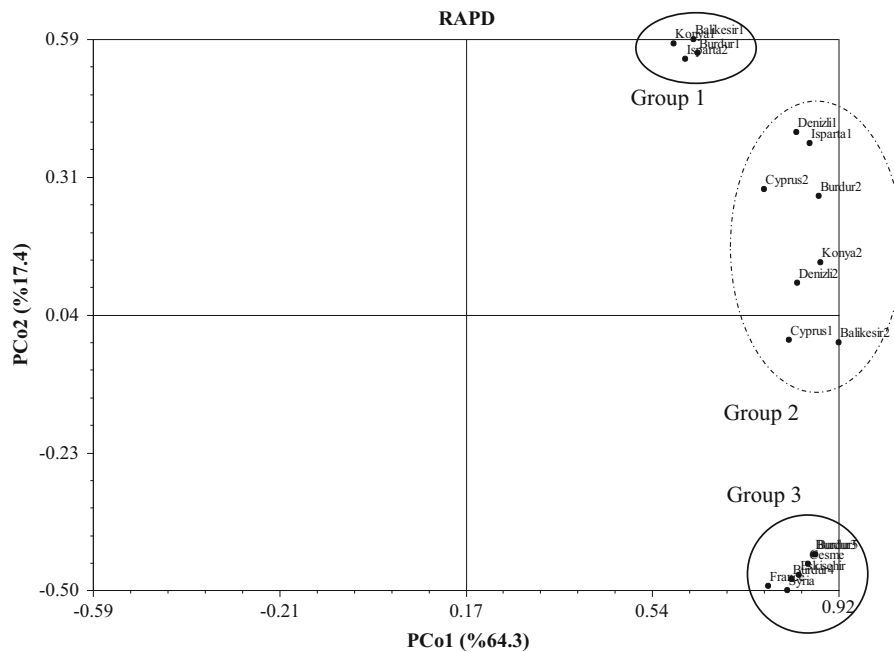
The genetic similarity values varied between 0.19 and 0.98. The average genetic similarity among the landraces was 0.62. Genetically, the most similar landraces were Burdur3 and Burdur5 with a value of 0.98 whereas the most distant landraces were Konya1 and Burdur4, with a value of 0.19, followed by Syria and Balıkesir1, and France and Isparta2, both pairs with a value of 0.20. The principal coordinate analysis was performed to confirm the clustering pattern of the RAPD analysis. The results of the PCoA analysis were in good agreement with the cluster analysis. The PCoA plot of RAPD clearly showed the two main clusters and one subcluster of anise landraces. PCoA derived from the RAPD data showed that the first two principal coordinate components had 64.3 and 17.4% variation, respectively, accounting for 81.7% of the genetic similarity variance (Fig. 3). Interestingly, PCo2 appeared to be much more informative, clearly separating the three different groups while on PCo1, two groups clearly overlapped. The first principal axis, which generated the most variation (64.3%),

contributed the least to the separation of Anise landraces. The second axis representing only 17.4% of the total variation was considerably loaded to discriminate the cluster and subcluster. This may be due to the fact that genetic variation is higher in group 2 than in group 1 and group 3. Most of the alleles within these groups were either observed or absent in most genotypes.

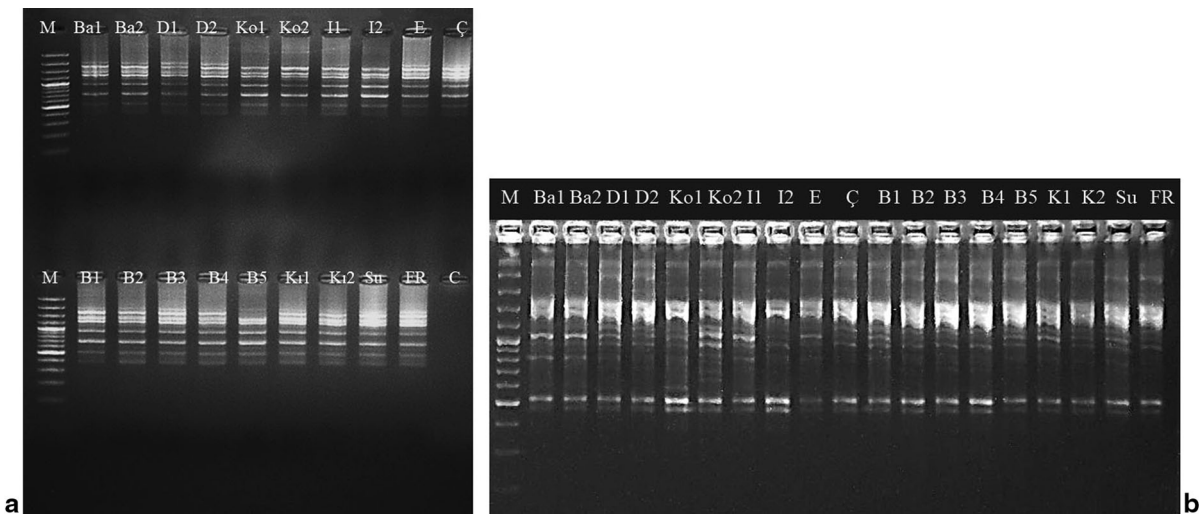
#### ISSR analysis

Table 3 presents the results of the ISSR analysis of the 19 anise landraces in terms of the number of ISSR primers used, base sequences, annealing temperature (°C), TF, PF, PFR (%), PIC, RP, EMR and MI values. Of the nine ISSR primers screened, five were detected and found to produce a total of 45 fragments across anise landraces. Twenty-five fragments were polymorphic, accounting for 55.6% of TF. The number of fragments ranged from 5 (ISSR 28) to 15 (ISSR 4). The average numbers of loci and polymorphic loci per primer were 9 and 5, respectively. PFR across the *P. anisum* landraces ranged from 37.5% (ISSR 25) to 73% (ISSR 4), with an average of 51.48% per primer





**Fig. 3** The two-dimensional plot of the principal coordinate analysis of 19 anise landraces using Dice's similarity coefficient of the RAPD data



**Fig. 4** Amplification patterns of anise landraces generated using ISSR primers ISSR-25 (a) and ISSR-04 (b). M indicates the 100 bp plus DNA ladder size marker (Fermentas), and C is the PCR-control

(Fig. 4). In this study, the highest PIC value was 0.46 obtained from the primer ISSR28 and the lowest was 0.32 observed in primers ISSR 4 and ISSR 24, with the average value being 0.38. The RP of the ISSR primers ranged from 1.47 for ISSR28 to 5.05 for ISSR4. The average RP value was determined as 2.67. The highest EMR (8.07) was obtained from ISSR4 and the lowest

(0.80) from ISSR28, with an average of 2.98 per primer. The overall mean MI was estimated as 1.05, with the highest value observed in ISSR4 (2.55) and lowest in ISSR28 (0.37).

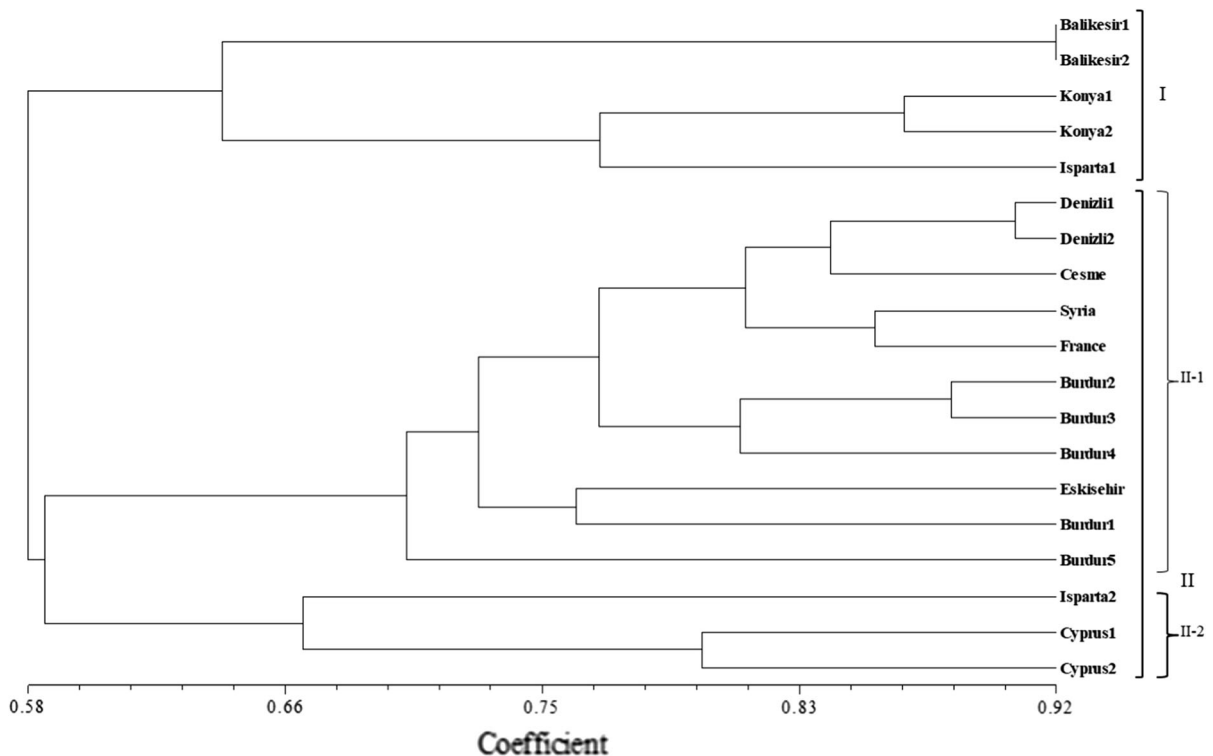
The ISSR bands were scored for presence (1) or absence (0) among the landraces and used for UPGMA analysis. Dice's similarity coefficient ranged from

0.36 (Eskisehir vs. Isparta2) to 0.92 (Balıkesir1 vs. Balıkesir2). The average genetic similarity values of the anise landraces were found as 0.65. Figure 5 presents the dendrogram constructed based on the UPGMA analysis of the ISSR data. The cophenetic correlation coefficient was 0.78, indicating that the cluster result was compatible with the genetic similarity matrix. The 19 anise landraces were grouped into two clusters. Cluster I comprised Balıkesir1, Balıkesir2, Konya1, Konya2, and Isparta1. Cluster II was further divided into two subclusters: Subcluster 1 containing all landraces of Burdur (1 to 5), two foreign landraces (Syria and France), and Denizli1, Denizli2, Cesme and Eskisehir, and Subcluster 2 with Isparta2 and two foreign landraces Cyprus1 and Cyprus2. In the dendrogram, Balıkesir1 and Balıkesir2 were observed to be the closest landraces with values of 0.92. The landraces from the same location were grouped in the same cluster. This demonstrates that the ISSR dendrogram is generally coherent with the geographical distribution of anise landraces. Three-dimensional plots obtained from the PCoA of the ISSR data (Fig. 6) showed consistency with the UPGMA

dendrogram, with the anise landraces being similarly divided into three groups. The first three principal coordinate components accounted for 66.9, 8.3 and 5.7% of total variance, respectively, explaining 81.0% of the genetic similarity variance. In the dendrogram and on the PCoA plots, anise landraces were combined according to their geographic origin. In the PCoA plot, the second group, which mainly consisted of local Turkish, and two foreign landraces (Syrian and French), had more variation as compared with the first and third groups, including other local Turkish and Cyprus landraces.

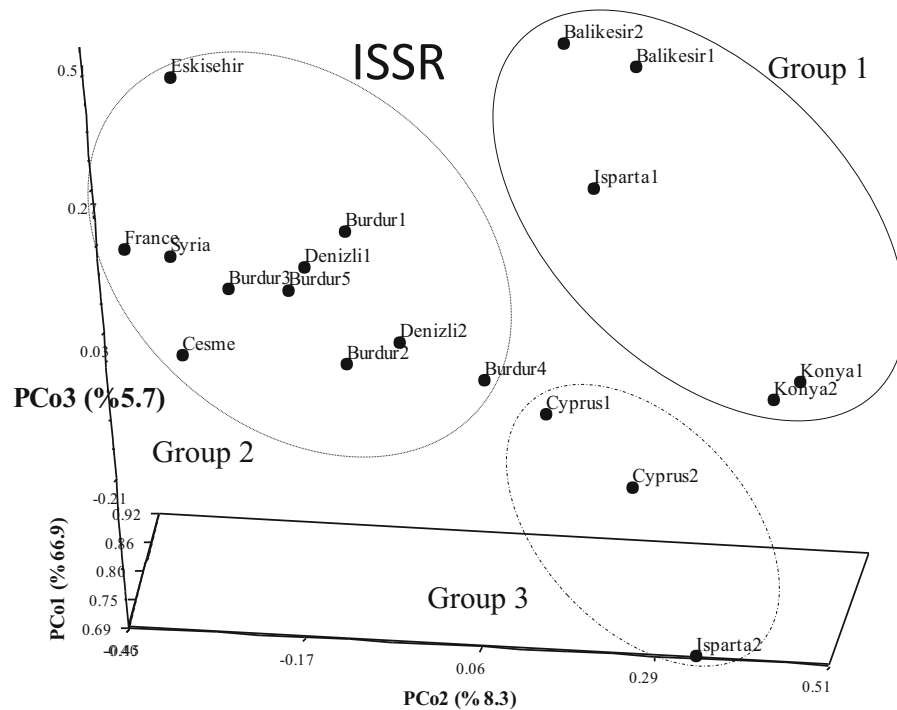
#### Combined RAPD and ISSR data analysis

Combining the analysis of RAPD and ISSR markers, a total of 14 primers were examined. A total of 116 DNA fragments were produced, of which 68 (58.6%) were polymorphic, with an average of 4.9 PF per primer. The mean PIC, RP, EMR and MI values observed for all primers were 0.39, 2.79, 3.08 and 1.14, respectively (Table 4). Dice's similarity coefficient, calculated using both RAPD and ISSR data,



**Fig. 5** The UPGMA dendrogram based on the similarity index among the anise landraces using ISSR markers





**Fig. 6** The three-dimensional plot of the principle coordinate analysis of 19 anise landraces using the ISSR data

**Table 4** Comparative analysis of genetic variability in *Pimpinella anisum* landraces using RAPD, ISSR and combined data

	RAPD	ISSR	RAPD + ISSR
Total number of fragments	71	45	116
Number of polymorphic fragments	43	25	6.8
Average of total fragments	7.9	9	8.3
Average of polymorphic fragments	4.8	5	4.9
Polymorphic fragments ratios	59.8	51.48	55.64
Polymorphism information content	0.40	0.38	0.39
Resolving power	2.90	2.67	2.79
Effective multiplex ratio	3.18	2.98	3.08
Marker index	1.23	1.05	1.14
Dice's similarity coefficient	0.19–0.98	0.36–0.92	0.29–0.91
Average Dice's similarity coefficient	0.62	0.65	0.63

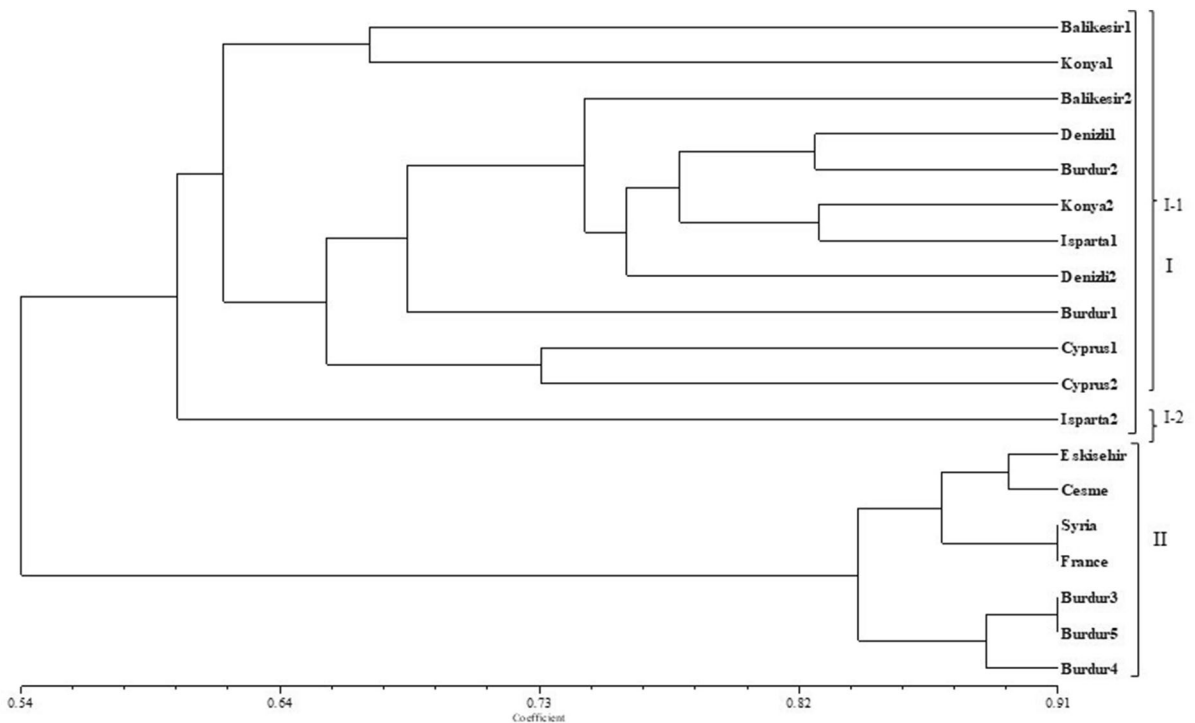
ranged from 0.29 (Isparta2 vs. France) to 0.91 (Syria vs. France and Burdur3 vs. Burdur5) (Table 5). The average genetic similarity values of the anise landraces were found as 0.63. The Mantel test between the two similarity matrices produced  $r = 0.74$ , indicating a good agreement between the RAPD and ISSR marker systems. The dendrogram based on the

UPGMA analysis with the combined RAPD and ISSR based data is represented in Fig. 7. According to this analysis, all the landraces were grouped into two major clusters. Balikesir1, Konya1, Balikesir2, Denizli1, Burdur2, Konya2, Isparta1, Denizli2, Burdur1, Cyprus1, Cyprus2 and Isparta2 landraces were included in Cluster I. In this group, Balikesir1-Konya1

**Table 5** The similarity matrix of the anise landraces using Dice's coefficient based on the RAPD and ISSR bands

	Bal1	Bal2	Den1	Den2	Ko1	Ko2	Is1	Is2	Esk	Ces	Bur1	Bur2	Bur3	Bur4	Bur5	Cyr1	Cyr2	Syr	Fra
Bal1	1.00																		
Bal2	0.64	1.00																	
Den1	0.67	0.71	1.00																
Den2	0.57	0.73	0.79	1.00															
Ko1	0.67	0.55	0.66	0.54	1.00														
Ko2	0.59	0.76	0.74	0.77	0.70	1.00													
Is1	0.71	0.76	0.82	0.72	0.73	0.83	1.00												
Is2	0.55	0.51	0.69	0.63	0.66	0.61	0.67	1.00											
Esk	0.42	0.73	0.61	0.66	<b>0.31</b>	0.59	0.59	<b>0.30</b>	1.00										
Ces	0.33	0.72	0.63	0.73	0.34	0.63	0.56	0.41	<b>0.89</b>	1.00									
Bur1	0.64	0.56	0.75	0.73	0.57	0.62	0.73	0.63	0.47	0.49	1.00								
Bur2	0.58	0.75	0.82	0.76	0.60	0.76	0.79	0.63	0.61	0.67	0.69	1.00							
Bur3	0.39	0.70	0.66	0.71	0.37	0.64	0.62	0.38	<b>0.89</b>	<b>0.90</b>	0.49	0.73	1.00						
Bur4	0.36	0.70	0.58	0.68	0.37	0.66	0.59	0.38	0.77	0.83	0.44	0.70	0.88	1.00					
Bur5	0.39	0.71	0.61	0.68	<b>0.31</b>	0.62	0.62	0.33	0.83	0.84	0.47	0.66	<b>0.91</b>	<b>0.89</b>	1.00				
Cyr1	0.57	0.73	0.68	0.63	0.54	0.67	0.62	0.47	0.61	0.63	0.46	0.71	0.68	0.65	0.71	1.00			
Cyr2	0.64	0.67	0.70	0.61	0.60	0.65	0.71	0.56	0.46	0.51	0.58	0.72	0.56	0.54	0.58	0.73	1.00		
Syr	0.33	0.68	0.61	0.66	0.34	0.57	0.54	<b>0.30</b>	0.85	<b>0.89</b>	0.44	0.64	<b>0.89</b>	0.82	0.88	0.63	0.55	1.00	
Fra	0.33	0.65	0.62	0.65	0.34	0.55	0.53	<b>0.29</b>	0.56	0.87	0.42	0.60	0.84	0.77	0.81	0.62	0.50	<b>0.91</b>	1.00

The bold values indicate the maximum and minimum genetic similarity values among the landraces



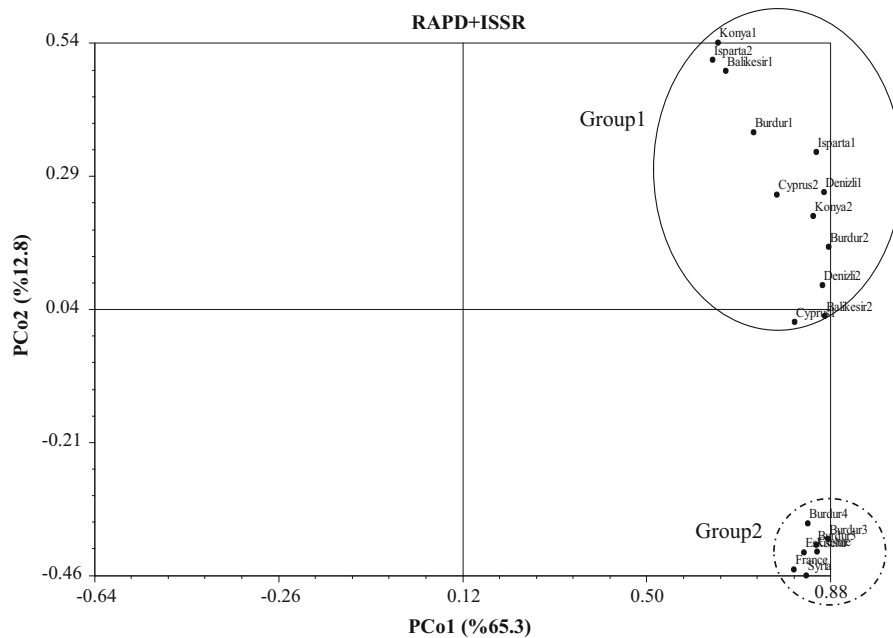
**Fig. 7** The UPGMA dendrogram based on Dice's similarity index among 19 anise landraces using the combined RAPD and ISSR data

(genetic similarity values, GS; 0.67), Denizli1-Burdur2 (GS; 0.82), Konya2-Isparta1 (GS; 0.83) and Cyp1-Cyp2 (GS; 0.73) formed a pairwise combination, while Balikesir2, Denizli2 and Burdur1 surrounded them at a relatively lower point. Isparta2 was located separately in this cluster. In cluster II were Eskisehir and Cesme with a similarity value of 0.89, Syria and France with a similarity value of 0.91, and three Burdur (3, 5 and 4) landraces. Burdur3 and Burdur5 had a close similarity value of 0.91. The results of PCoA for the combined data are shown in Fig. 8. The first two principal coordinates (having Eigenvalues > 1) accounted for 65.3 and 12.8%, respectively of the total molecular variation. This explained 78.1% of the total variation (Table 6). The results of the PCoA analysis were almost in agreement with the cluster analysis, similarly dividing the anise landraces into two groups. Differently, Isparta2 was found to be separately located in the subcluster 2 of cluster I in the UPGMA dendrogram; however, Isparta2 could not be differentiated in PCoA. Furthermore, PCo2, representing only 12.8% of the total variation, appears to be much more informative, as in

the PCoA of RAPD. This may be due to the high intra-group variation.

## Discussion

Detailed characterization of the genetic structure of plants is one of the primary and important stages in the application of breeding programs, as well as efficient protection and use of plant genetic resources (Tripathi et al. 2012; Izzatullayeva et al. 2014). Molecular marker analysis is considered as the most reliable and powerful method for such characterization. To date, only a limited number of studies have examined the genetic relationships between *Pimpinella* species using a PCR-based approach (e.g., Tabanca et al. 2005; Nurcahyanti et al. 2016; Wang et al. 2014; Maraklı 2018). Furthermore, only one research (Sri-lakshmi et al. 2015) reported the genetic fidelity of micropropagated wild *Pimpinella candolleana* plants verified by RAPD and ISSR analyses to confirm the true-to-type nature of the plantlets. In this study, the genetic diversity of 15 cultivated anise landraces collected from different regions in Turkey (Balikesir,



**Fig. 8** The two-dimensional plot obtained using principal coordinate analysis based on the combined data of RAPD and ISSR of 19 anise landraces

**Table 6** Eigenvalue, percentage of variability explained by each coordinate and cumulative variation for the RAPD and ISSR data

Principal coordinate	Eigenvalue	Percent variation	Cumulative percent variation
<i>RAPD</i>			
1	12.22	64.32	64.32
2	3.31	17.41	81.72
<i>ISSR</i>			
1	12.73	66.99	66.99
2	1.58	8.33	75.32
3	1.09	5.74	81.06
<i>RAPD + ISSR</i>			
1	12.41	65.32	65.32
2	2.44	12.84	78.16

Denizli, Konya, Isparta, Burdur, Eskisehir, and Cesme) and four foreign landraces obtained from Cyprus, Syria and France were analyzed using two different marker systems, RAPD and ISSR. To the best of the author's knowledge, this is the first report on the use of RAPD, ISSR and combined RAPD and ISSR molecular markers for the characterization of genetic variation and determination of genetic relationships in anise (*P. anisum* L.) landraces. The data generated from both analyses were combined in order to obtain more balanced values for the genetic variation among the landraces. The combined use of several marker

types enables coverage of different parts of the genome and provides more informative data than the use of individual markers alone (Kumar et al. 2009; Elmeir et al. 2017).

In the present investigation, nine RAPD primers produced a total of 71 reliable fragments, and five ISSR primers produced 45 fragments. The PFR values obtained using RAPD (59.8%) were slightly higher than revealed by the ISSR data (51.5%). This may be due to the lower number of ISSR markers used in the study compared to RAPD and/or the two marker techniques targeting different parts of the genome.

Nevertheless, the mean TF and PF values detected by each ISSR primer combination was 9 and 5, respectively, which are slightly higher compared to the RAPD primers (7.9 and 4.8, respectively). Similar results were reported by Mohd et al. (2009) for *Dalbergia sissoo* and Kumar et al. (2014) for *Justicia adhatoda* L. It has been suggested that the ability to resolve genetic variation may be more directly related to the number of polymorphisms detected by the marker system (Sivaprakash et al. 2004). Moreover, the average polymorphism demonstrated by these markers was around %50–60. Such moderate-low polymorphism has been previously seen in flax germplasm (Smýkal et al. 2011; Fu 2002; Fu et al. 2003), spine gourd landraces (Rana and Das 2016), and shisham (Arif et al. 2009). These variations may be mainly due to the interaction of several factors, including the mating system, which is one of the strongest predictors of population genetic diversity and structure in plants (Hamrick and Godt 1996; Nybom 2004; Glemin et al. 2006) and the uncontrolled exchange of cultivated anise seeds among producers in different provinces in Turkey.

PIC reflects a discriminating ability of the marker and depends on the number of known alleles and their frequency distribution, thus representing genetic diversity. The maximum PIC value for dominant markers is 0.5. It should be noted that for the markers with an equal distribution in the population, the PIC values are higher. The PIC value is also much higher for markers with multiple alleles, but also depends on the frequency distribution of the alleles (Bolaric et al. 2005; De Riek et al. 2001). The measure of PIC is an important component in the planning of breeding programs and one of the key information and statistical indicators in their implementation (Chesnokov and Artemyeva 2015). In the current study, the analysis of the PIC values for the two marker systems indicated ranges of 0.33 (OPAB-05) to 0.47 (OPAA-10) for the RAPD primers and 0.32 (ISSR4 and ISSR24) to 0.46 (ISSR28) for the ISSR primers. The mean PIC values were calculated as 0.40 for RAPD and 0.38 for ISSR, and even if the RAPD markers had slightly higher PIC than the ISSR markers, both marker systems were equally effective in determining polymorphisms. Similar results are obtained by Muthusamy et al. (2008), Kayis et al. (2010), Arif et al. (2009), Gajera et al. (2011), and Kumar et al. (2014). However, in the current study, the average PIC

value (0.39) was much higher than previously reported. Tonk et al. (2011) determined the PIC values of *Hypericum perforatum* clones to range from 0.15 to 0.42.

RP is another parameter used to measure the ability of primers or techniques to distinguish genotypes. In this study, the average RP values of the RAPD primers (2.90) were observed to be slightly greater than those of the ISSR primers (2.67). Similar results were reported for *J. adhatoda* L. (Kumar et al. 2014), *Ocimum* species (Chen et al. 2013), and *Zingiber officinale* Roscoe (Baruah et al. 2019). In the literature, ISSR was found to provide higher RP values than RAPD in accessions of *Crocus* L. (Zarini et al. 2019), *Nilgirianthus ciliatus* (Rameshkumar et al. 2019), and alfalfa (*Medicago sativa* L.) (Touil et al. 2016). Comparing the average EMR and MI between the marker systems used in the present study, there were only minor differences (3.18 and 1.23, respectively for RAPD, 2.98 and 1.05, respectively for ISSR), which is in agreement with the results of Kumar et al. (2014), Tagizad et al. (2010) and Mukherjee et al. (2013).

The cluster analyses undertaken based on the two DNA marker profiles (RAPD and ISSR), as well as the combined data (RAPD + ISSR) generally grouped the 19 landraces of anise into two clusters with some exceptions. However, the composition of the subclusters within the main cluster varied between RAPD and ISSR. The clustering patterns were more similar for the RAPD data and the combined RAPD + ISSR data, but less similar for the ISSR data. Subcluster 2 in the RAPD dendrogram and cluster II in the combined dendrogram had almost the same grouping as the Eskisehir-Cesme, Burdur 3–4–5, and Syrian-French landraces. The remaining landraces were grouped differently in the other cluster and subcluster. The landraces of Turkish origin did not create a separate cluster from other countries, displaying a similar pattern throughout the clusters in all three dendrograms. There was no clear grouping according to geographical origin, indicating limited or no location specificity among the anise landraces. Similar results were previously revealed in castor (*Ricinus communis* L.) (Gajera et al. 2010) and shisham (*Dalbergia sissoo*) (Arif et al. 2009). Moulin et al. (2012), in a study using RAPD and ISSR markers, similarly did not find any correlation between geographic distance and genetic differences among sweet potato accessions. Possible explanations for the absence of the

relationship between geographic origin and the clustering of different landraces could be the reasons below;

- The exchange of cultivated anise seeds among producers in different provinces in Turkey,
- Plants could have been diffused from their origin due to human activities, during different periods (Iruela et al 2002),
- Associated with strong genetic flow (Izzatullayeva et al. 2014).

According to the locations from which the anise landraces were obtained, the ISSR data provided more favorable results in comparison to the other two dendrograms because the ISSR cluster results were more or less in agreement with the geographical distribution of anise landraces. The landraces obtained from the same location formed a pairwise combination (Balıkesir1-Balıkesir2, Konya1-Konya2, Denizli1-Denizli2, Cyprus1-Cyprus2) except Isparta landraces (Isparta1 and Isparta2), which were grouped in separate clusters. Isparta2 was also located separately in RAPD + ISSR clusters, indicating divergence from the remaining landraces. The local landraces, Burdur1 to Burdur5, were all within the same cluster on the ISSR-based dendrogram. In other words, the landraces from the same location were grouped in the same cluster. The RAPD-based and combined RAPD + ISSR dendrograms grouped these landraces in separate subclusters. Furthermore, Burdur3 and Burdur5 appeared to very close in the RAPD dendrogram, but in the ISSR dendrogram, the results were very different. Similarly, Balıkesir1 and Balıkesir2 were very close to each other with a similarity value of 0.92 in the dendrogram generated using ISSR markers, while they were located in separate clusters with a similarity value of 0.49 in the dendrogram generated with RAPD markers. This may be due to the RAPD and ISSR marker techniques targeting different parts of the genome. Although both marker techniques are based on DNA amplification by PCR, it was expected that due to the nature of the primers and reaction, the techniques would differ in the specific sequences targeted. The RAPD markers were considered to be uniformly distributed throughout the genome, while ISSR was found only between the microsatellite loci. In addition, the sequence of the ISSR primers was larger compared to the RAPD primers, and PCR in RAPD was performed using a low annealing

temperature than ISSR (Williams et al. 1990; Zietkiewicz et al. 1994; Malik et al. 2014).

To the best of the author's knowledge, none of the analyzed anise landraces had been previously evaluated for genetic relationships. No clear geographic separation was found for the foreign anise landraces, with only minor groupings including two foreign anise landraces. The landraces from Syria and France were included in the same clusters or subclusters and formed a dual combination according to all three dendrograms. The highest genetic similarity values were observed for these landraces [0.86 for RAPD, 0.94 for ISSR (data not shown), and 0.91 for the combined data]. The Cyprus1 and Cyprus2 landraces also had high similarity values [(0.80 for RAPD, 0.80 for ISSR (data not shown) and 0.73 for the combined data]; however, they were included in different clusters or subclusters from the other two foreign landraces (Syrian and French) according to all three dendrograms. The highest genetic similarity values of these landraces were probably associated with their resemblance in the amplified region. The results also showed that the foreign anise landraces were closely grouped with the indigenous landraces. This could be explained by the presence of a common genetic origin among the cultivars tested despite their great diversity (Haider et al. 2012). The Eskisehir-Cesme and Syria-France landraces were also located in the same cluster in RAPD-based and combined dendrograms and were observed as genetically close landraces. In addition, a high similarity (Eskisehir-Cesme 0.96 for RAPD, 0.89 for combined) was seen among these landraces. These results are in agreement with some of the previous studies, which concluded that the accessions from diverse geographical regions could be genetically similar (Naik et al. 2017).

The cophenetic correlation between the Dice similarity matrix and corresponding dendrogram obtained by RAPD ( $r = 0.79$ ), ISSR ( $r = 0.78$ ), and the combined analysis ( $r = 0.74$ ) revealed a good fit (Mantel 1967). The average Dice similarity coefficients were found to be relatively similar by different marker systems (0.65 by ISSR, 0.62 by RAPD and 0.63 by RAPD + ISSR). This level of moderate genetic diversity in anise landraces may be due to the uncontrolled genetic exchange in anise landraces, as well as closely related species. Similar results were reported by Saran et al. (2015), who determined Jaccard's genetic similarity values in the RAPD and



ISSR in the range of 0.30–0.99 (average: 0.65) and 0.26–0.95 (average: 0.61), respectively, in papaya germplasm lines. The mean similarities detected using ISSR were slightly greater than those observed with RAPDs in anise landraces. However, the widest range of similarity was found in the RAPD analysis (0.19 to 0.98), suggesting that higher genetic variations existed in the target genome regions than those targeted by ISSR (0.39 to 0.92) and combined (0.29 to 0.91) markers (Table 4). In a previous study, similar average Jaccard's similarity coefficients were observed among *ocimum* species (0.596 by ISSR, 0.581 by RAPD and 0.566 by SRAP markers), and a wide range of similarity was found in the ISSR analysis (0.065 to 1) compared to RAPD (0.21 to 0.99) and SRAP (0.28 to 0.95) (Chen et al. 2013).

The results of the PCoA analysis were in good agreement with the cluster analysis. The PCoA of the RAPD, ISSR and RAPD + ISSR data produced similar results, dividing the anise landraces into two main groups. The PCoA of the individual RAPD and ISSR data further generated subgroups, while no subgroup was observed in the PCoA of the RAPD + ISSR data. Differently, in the two-dimensional plot of PCoA based on the combined data, the Isparta2 landrace was grouped together with the remaining landraces of group 1, whereas in UPGMA clustering, it was distinctly separated (in the second subcluster of cluster I (I-2). Besides, in the PCoA of RAPD, the landraces were situated along the right side of the chart, significantly overlapping the landraces in group 1 and the group 3. In the PCoA of RAPD + ISSR, only the landraces in group 2 overlapped. Furthermore, in all PCoA plots, the first principal axis that produced the most variation contributed the least to the separation of aniseed landraces. It may be due to the high genotypic difference and variation within the group separated by the other axis. Similarly, Yılmaz et al. (2009), in their studies based on ISSR data, reported that the third PCoA axis with low variation was heavily loaded to distinguish subgenera and sections of the genus *prunus*. This multivariate approach was used to complement the information obtained from the cluster analysis because it was more informative regarding the distances between the major groups (Naik et al. 2017).

In this study, where the genetic variation was assessed and the genetic relationships between the anise landraces were determined, RAPD, ISSR and

combined markers were used for the first time, and the RAPD markers were found to provide slightly higher values than ISSR markers in terms of many parameters, namely the total number of fragments (RAPD = 71; ISSR = 45), the number of polymorphic fragments (RAPD = 43; ISSR = 25), polymorphic fragment ratios % (RAPD = 0), PIC (RAPD = 0.40; ISSR = 0.38), RP (RAPD = 2.90; ISSR = 2.67), and EMR (RAPD = 3.18; ISSR = 2.98), MI (RAPD = 1.23; ISSR = 1.05). However, ISSR was more favorable than the RAPD technique in demonstrating the phylogenetic relationships between the 19 anise landraces while the ISSR dendrogram was generally compatible with the geographical distribution of the anise landraces. Previous studies on the date palm cultivar revealed that ISSR markers were better tools than RAPD for phylogenetic studies, but RAPD provided more accurate results for the genetic diversity between the date palm species (Elmeer et al. 2017). Parthasarathy et al. (2013) concluded that ISSR markers could be used to identify unknown species in *Garcinia* whereas RAPD markers can be utilized to determine variations based on geographical or morphological features within the same species.

## Conclusion

Anise is an important aromatic and spice plant in Turkey, ranking second after cumin in terms of planting area. However, producers do not have a registered variety and anise production is still carried out with landraces. The seeds used in anise production are provided through exchange between farmers in different regions and are far from offering standard quality due to the mixed nature of populations. For these reasons, it is vital that further breeding studies are carried out in different ecologies for the development and cultivation of superior anise varieties. The phylogenetic information of this crop is scarce, and therefore such a study can make a strong contribution to the knowledge of the genetic materials currently used in anise breeding. In this study, RAPD and ISSR markers were used for determining the genetic relationships in anise. The results of this study demonstrate that genetic variation exists between the investigated anise landraces. RAPD and ISSR markers can be successfully used to determine the diversity of *P. anisum* L. Various marker parameters, namely

percent polymorphism, PIC, RP, EMR, and MI of the RAPD assay were found to be slightly higher than those of the ISSR assay. RAPD provided more prominent results in differentiating landraces based on their genetic diversity compared to ISSR markers; however, ISSR performed better than the RAPD technique in explaining the phylogenetic relationships and geographical distribution in anise landraces. Genetic variations among anise landraces could be useful in selecting parents to be crossed to generate suitable populations intended for breeding strategies. The information obtained as a result of this study will shed light on the future improvement works.

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#### Compliance with ethical standards

**Conflict of interest** The author declares that they have no conflict of interest.

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