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Mapping of QTLs associated with frost tolerance in olive (*Olea europaea* L.)

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Abstract: The olive (*Olea europaea* L.) is widely distributed due to its high adaptability to different environmental conditions. However, in recent years, sudden extreme temperature decreases and frost damage have caused significant yield and quality losses in some regions. This situation has led to the development of varieties with high frost tolerance, suitable for regions where cold damage may occur. It takes many years to develop varieties using classical breeding methods in olives. Therefore, early selection should be made using biotechnological methods. Markers about any trait provide a great advantage, especially in plant species with a very long juvenile sterility period, as they allow for a selection of nonpurpose plants in the early stage of the breeding process. The elucidation of the molecular mechanism associated with frost stress is important in laying the groundwork for breeding studies. To determine the locations of genes controlling frost tolerance in olives on chromosomes, linkage Quantitative Trait Loci (QTL) and association mapping studies were performed on leaf tissues of Memecik × Uslu hybrids (104 genotypes) subjected to frost tests under controlled conditions in the laboratory. Twenty-three linkage groups (LG) exactly matching the haploid chromosome number (n = 23) of the olive were obtained for the maternal parent Memecik and 26 LG for the paternal parent Uslu. A total of 4377 markers were mapped for the Memecik cultivar and 4664 markers for Uslu. The total number of markers mapped for both parents was 9041. This study investigated candidate QTLs associated with frost tolerance in the olive.

Key words: Olive (*Olea europaea* L.), frost stress, cultivar, breeding, biotechnology

1. Introduction

The olive (*Olea europaea* L.) is an important fruit in the agricultural sector of Mediterranean countries with a high economic value. It is one of the most widely cultivated plant species worldwide due to its high adaptability to diverse environmental conditions, its nutritional value, its use as table olives, its processing into oil, and its growing recognition for health benefits in recent years (Bartolini et al., 1999).

In economic terms, olive cultivation is carried out between 30° and 45° latitudes in both hemispheres. However, the rapid increase in global demand for olive oil in recent years has led to the expansion of cultivation borders in both hemispheres. The olive tree has better frost tolerance than other subtropical plant species (Larcher, 1987).

One of the most significant stress factors limiting the spread of plants is low temperatures. Under field conditions, plants face abiotic stress conditions, including biotic and cold stress. Plants can adapt to low-temperature stress by responding to physiological, biochemical, and molecular responses (Beck et al., 2007). The maximum level of frost tolerance under natural conditions is achieved in autumn when the temperature gradually drops to 0 °C. The frost damage rates of plants vary according to the rate of temperature decrease, the duration of low temperatures, the temperature before frost, and the rate of dissolution of ice crystals (Childers et al., 1995). When the temperature drops below –7 °C, olive trees may experience leaf loss and drying of thin branches, depending on the duration and severity of the cold, leading to significant yield losses (Vitagliano and Sebastiani, 2002). It has been reported that if the temperature reaches –12 °C, the plants may die (Larcher, 1970).

To determine the frost tolerance status of olive varieties, orchard observations after frost damage, viability tests (Fontanazza and Preziosi, 1969), stomadial density (Roselli et al., 1989), photosynthetic activity (Antognozzi et al., 1990), stoma size (Roselli and Venora, 1990), phenolic compounds (Roselli et al., 1992), differential thermal analysis (Martin et al., 1993), and ionic leakage (electrical conductivity) (La Porta et al., 1994; Bartolozzi and Fontanazza, 1999; Mancuso, 2000; Barranco et al., 2005; Cansev et al., 2009; Asl Moshtaghi et al., 2009) have

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been used. Due to their fast and easy application, ion leakage tests are considered the most reliable among these methods.

Frost damage in olives is observed as fruit browning, deterioration of fruit texture, blackening and shedding of leaves, bud death, branch drying, trunk bark drying or cracking, and, in later stages, tree death. This damage necessitates determining varieties with high frost tolerance suitable for regions where cold damage may occur. Elucidating the molecular mechanism associated with frost stress and identifying genes whose expression is changed by stress are crucial in laying the groundwork for breeding studies.

With recent technological advancements, molecular markers have been widely used in studies aimed at improving and protecting fruit cultivation. In fruits, as in other plants, molecular markers are employed for purposes such as genotypic identification, systematic characterization, QTL analysis, genetic mapping, marker-assisted selection (MAS), and the identification and conservation of genetic resources (Andersen and Lübberstedt, 2003; Kaçar, 2004).

One of the most effective areas in which molecular markers can be used is studying quantitative character inheritance, which has a complex genetic structure due to the multigene effect. Quantitative traits are controlled by genes at multiple loci, with the degree of influence varying across loci. These traits are also highly affected by environmental conditions, making them difficult to assess in breeding studies using classical methods. However, it is possible to identify chromosomal regions that control quantitative traits through QTL mapping based on molecular techniques (Collard and Mackill, 2008). Breeding studies using MAS can be reduced to a special phenotypic character and completed quickly and with less labor (Gupta and Rustgi, 2004). A complete understanding of the physiological, biochemical, and molecular mechanisms of tolerance in olive trees, combined with specific attention given to the signaling pathways determining the response to low temperatures, will provide new breeding tools and help select cultures with greater freezing tolerance (Petruccelli et al., 2022). An effective marker that can be used as an early selection criterion in the olive has not yet been developed. For this purpose, within the scope of the study, linkage and QTL mapping were carried out to develop a marker related to frost tolerance in olives.

2. Materials and methods

A cross population consisting of 104 F1 seedlings belonging to the Memecik × Uslu mapping population and their parent Memecik and Uslu varieties were used as materials in this study. Hybrids and parents were 25 years old. The orchard is located in Kemalpaşa/İzmir. All culture-related methods were done correctly in the experimental area. The temperature values of Kemalpaşa/İzmir in February between 2017 and 2019, where the frost tests were carried out, are given in Table 1.

2.1. Cell membrane injury and cold-hardiness determination via controlled freezing test

Leaves on 1-year shoots in which the product was formed in the olives were collected from hybrid individuals and parents in February between 2017 and 2019. The leaf samples were then exposed to frost stress in the laboratory.

The cell membrane injury of leaf tissues was determined using the ion leakage method developed by Arora et al. (1992). Briefly, leaf discs of 10 mm diameter were punched from the leaves, lightly rinsed in distilled water, gently blotted with paper, and placed in test tubes (one disc per tube). The tubes were placed in a glycol freezing bath (JULABO F38-ME, Seelbach, Germany). The freezing tests were kept at 11 °C, which is the critical temperature value for olives, for 2 h and performed in three replications. Subsequently, 10 mL of distilled water was added to each tube containing freezing-stressed and control (nonexposed) samples; the tubes were then shaken on a gyratory shaker (250 rpm) for 4 h at room temperature. The electrical conductivity of each sample was measured using a Selecta- CD-2005 conductivity meter (Barcelona, Spain). The electrical conductivity of each sample was measured again after the tubes were autoclaved (0.12 MPa, 120 °C, 20 min) and cooled. Proportional injury at each temperature was calculated from ion leakage data using the following equation: proportional injury = [(proportional L(t) – proportional L(c))/(1 – proportional L(c))], where proportional L(t) and proportional L(c) are percentage ion leakage data for the treatments and control samples, respectively (Arora et al., 1992). Cold-hardiness (LT_{50}) was defined as the subzero temperature at which 50% injury occurred. The obtained numerical data were processed in an Excel spreadsheet for use in the MapQTL 6 program.

2.2. Molecular analyses

DNA isolation in leaf samples from hybrid individuals and their parents was performed according to the DNA isolation protocol specified in Diversity Arrays Technology (DArT)[1]. Dominguez-Garcia et al.'s (2012) study was followed for DArT-SNP marker analyses. Accordingly, isolated DNAs were used in DArT at a 50-ng/μL concentration, and sequence analyses were performed. Data from DArT-SNP marker analyses were scored. For the data to be used in mapping, markers showing appropriate segregation (cross pollinator) were evaluated.

[1]https://help.diversityarrays.com/v1/docs/genomicdna-gdna

Table 1. February 2017–2019 temperature values.

Days	2017 (°C)	2018 (°C)	2019 (°C)
1	1.3	7.8	9.2
2	0.4	13.4	7.5
3	4.8	13.1	9.4
4	9.4	13.9	8.4
5	11.1	11.3	8.9
6	13.3	9.2	11.5
7	14.1	10.6	7.7
8	9.4	17.2	6.8
9	9.1	9.6	7.5
10	7.0	8.9	5.4
11	5.4	13.3	6.0
12	4.6	8.4	13.4
13	3.3	7.5	7.5
14	2.9	10.1	6.8
15	1.5	8.3	5.1
16	4.6	8.4	7.2
17	3.2	8.2	6.0
18	5.1	6.9	5.4
19	10.0	7.0	6.4
20	11.9	7.9	8.7
21	11.4	10.2	8.7
22	8.8	10.9	7.1
23	9.1	9.2	5.6
24	11.1	7.8	3.1
25	13.0	7.7	4.1
26	12.7	6.3	7.2
27	10.5	8.9	5.6
28	11.6	11.6	4.6

2.3. Creation of linkage groups and QTL analysis

The data obtained from DArT-SNP marker analyses were scored. Markers with lmxll (1:1) and nnxnp (1:1) expansions were evaluated by showing the appropriate segregation (cross pollinator) for the parameters to be used in the mapping. The linkage map was created using JOINMAP 5.0 (Van Ooijen, 2019) software, following the double pseudotestcross mapping strategy. In the linkage grouping made according to this strategy, linkage mapping was done separately for the mother and father. An LOD score = 5 was accepted to determine the genetic relationship between any two markers. QTLs in these linkage groups (LGs) were determined according to the MapQTL 6 (Van Ooijen, 2009). The regression model was chosen as the QTL algorithm, and loci with LOD≥3 were evaluated as QTL.

3. Results

3.1. Cell membrane injury and cold-hardiness

Frost tests were carried out in February 2017-2018-2019 at –11 °C, which is the critical temperature value for olives, in 104 Memecik × Uslu hybrid genotypes, together with the parents of Memecik and Uslu varieties. The mean injury in Memecik and Uslu was determined to be 50.29% and 55.79%, respectively. In the frost tests performed on hybrid genotypes in February 2017, the lowest injury was statistically determined as 23.01%, whereas the highest injury was 73.77%. In the second year (February 2018) frost tests, the lowest injury was determined as 10.99%, and the highest injury was 71.95%. In the tests conducted in the third year (February 2019), the level of injury varied between 28.24% and 76.32%. The average injury values of the three-year data are 25.83% and 71.72% as the lowest and the highest, respectively.

3.2. Creating linkage groups

The amount of DNA to be used in SNP marker analysis was measured with a Qubit fluorometer (ThermoFisher Scientific, Malaysia). The isolated DNAs were prepared at a 50-ng/µL concentration and sent to the sequence for DArT-SNP analyses. The results of the DArT-SNP marker analysis showed that 21,195 SNPs were polymorphic in the Memecik × Uslu hybrid population. Of these markers, 8558 showing missing data and distortion were excluded from the data set to be mapped. Of the 12,154 markers used in the mapping, 3596 were not included in the LGs. A total of 9041 markers were used in the linkage groups created for the mother and father.

3.2.1. Linkage groups of cultivar Memecik (P1)

By conducting a linkage grouping analysis of the Memecik variety, 23 LG were obtained, which is the haploid chromosome number of olives. LG contain 4377 SNP markers. The total map length for the Memecik cultivar was determined as 2311.92 cM and the mean distance between the two markers was 0.556 cM (Table 2).

3.2.2. Linkage groups of cultivar Uslu (P2)

The data obtained from the linkage grouping of the Uslu cultivar are given in Table 3. It can be observed that 26 LG were obtained in the Uslu cultivar. The LG contained 4664 SNP markers. In the Uslu cultivar, the total LG map length was determined as 2415.89 cM and the average length between two markers was determined as 0.549 cM.

3.3. QTL analysis

The MapQTL 6 program was used for QTL analysis. The regression model was chosen as the QTL algorithm, and loci with LOD ≥ 3 were evaluated as QTL. Information on the QTL analysis performed on the phenotypic data obtained in the 3 different years in the mother-parent Memecik cultivar is given in Table 4 (ten QTLs with the highest LOD value). As seen in Table 4, the highest LOD value in the first-year QTL analysis was 2.09; in the second year, it remained at 1.61. Since these values were below the LOD ≥ 3 threshold, an effective marker related to frost tolerance could not be determined for both years. In the QTL analysis performed with the third-year data, two

Table 2. Linkage groups of the Memecik cultivar (P1).

Linkage group	Number of markers	Length (cM)	Space in the genome (%)	Average distance between two markers (cM)
1	396	191.97	8.30	0.485
2	155	126.51	5.47	0.816
3	126	85.725	3.71	0.680
4	332	107.508	4.65	0.324
5	230	153.831	6.65	0.669
6	102	44.323	1.92	0.435
7	217	92.568	4.00	0.427
8	178	121.668	5.26	0.684
9	110	69.271	3.00	0.630
10	257	121.754	5.27	0.474
11	253	125.263	5.42	0.495
12	165	101.52	4.39	0.615
13	165	88.308	3.82	0.535
14	165	108.917	4.71	0.660
15	164	60.928	2.64	0.372
16	155	93.841	4.06	0.605
17	142	94.816	4.10	0.668
18	123	45.497	1.97	0.370
19	91	81.357	3.52	0.894
20	84	52.144	2.26	0.621
21	282	155.658	6.73	0.552
22	295	110.612	4.78	0.375
23	190	77.931	3.37	0.410
Total	**4377**	**2311.92**	**100.00**	**Average: 0.556**

Table 3. Linkage groups of the Uslu cultivar (P2).

Linkage group	Number of markers	Length (cM)	Space in the genome (%)	Average distance between two markers (cM)
1	230	110.46	4.57	0.480
2	160	89.92	3.72	0.562
3	205	114.83	4.75	0.560
4	274	169.82	7.03	0.620
5	130	98.37	4.07	0.757
6	251	102.83	4.26	0.410
7	184	133.38	5.52	0.725
8	152	124.16	5.14	0.817
9	145	69.99	2.90	0.483
10	137	86.43	3.58	0.631
11	263	137.41	5.69	0.522
12	208	64.79	2.68	0.311
13	203	110.08	4.56	0.542
14	154	101.43	4.20	0.659
15	186	77.62	3.21	0.417
16	180	89.50	3.70	0.497
17	166	108.36	4.49	0.653
18	142	47.38	1.96	0.334
19	133	52.92	2.19	0.398
20	88	47.06	1.95	0.535
21	64	71.67	2.97	1.120
22	266	54.36	2.25	0.204
23	296	113.93	4.72	0.385
24	223	104.75	4.34	0.470
25	126	79.35	3.28	0.630
26	98	55.07	2.28	0.562
Total	**4664**	**2415.89**	**100.00**	**Average: 0.549**

candidate QTL regions with LOD ≥ 3 were determined. These markers were detected in the tenth linkage group, in the region of 58.639 cM and 54.894 cM. Candidate QTLs were detected at 58.649 cM M2256 was 1.00 cM from M2265, M2383, and M4900 and had a LOD of 3.03 (Figure). The percentage of explained phenotypic variation of this marker was 14.20. QTL was detected at 54.894 cM The distance was 1.081 cM from M1388, with a LOD of 3.02 and a phenotypic variation of 14.20%.

The QTL loci data, obtained from analyses considering the 3 years of phenotypic information for cultivar Uslu, are presented in Table 5. No QTL regions related to frost tolerance with LOD values of 3 or above were detected in any of the three years. The highest LOD values for cultivar Uslu were 2.24 in the first year, 1.29 in the second year, and 2.04 in the final year.

4. Discussion

DNA marker development studies on frost tolerance in olives were conducted on 104 F1 hybrid genotypes obtained by crossing the Memecik and Uslu olive cultivars. DArT-SNP marker analyses were performed on these genotypes in comparison with the parents, and linkage mapping was initially carried out for both parents. The linkage map was created using JOINMAP 5.0 software, employing the double pseudotestcross mapping strategy.

For creating ideal linkage maps, it is important to obtain as many LG as the haploid chromosome number

Table 4. QTL loci determined by years in the Memecik cultivar.

Year	Linkage group	Location (cM)	Locus	LOD	Announced phenotypic variance (%)
2017	7	66.366	-	2.09	9.40
	5	33.995	-	1.85	8.30
	5	25.995	-	1.80	8.10
	7	65.366	-	1.77	8.00
	5	34.995	-	1.76	8.00
	5	24.809	-	1.70	7.70
	7	68.691	-	1.65	7.50
	7	59.637	M4464	1.60	7.30
	5	20.161	-	1.56	7.10
	7	70.850	-	1.46	6.70
2018	11	56.860	-	1.61	7.30
	13	25.610	-	1.52	6.90
	5	104.268	M2400	1.49	6.80
	11	62.663	M6251	1.44	6.50
	13	24.610	-	1.44	6.50
	4	23.658	M3134	1.35	6.10
	12	1.981	M4623	1.34	6.10
	12	1.981	M4761	1.34	6.10
	10	59.649	-	1.31	6.00
	4	13.087	-	1.27	5.80
2019	**10**	**58.649**	**-**	**3.03**	**14.20**
	10	**54.894**	**-**	**3.02**	**14.20**
	10	59.649	-	2.86	13.50
	10	53.894	-	2.70	12.80
	10	48.824	-	1.74	8.40
	4	10.438	-	1.72	8.40
	2	117.441	-	1.68	8.20
	11	56.860	-	1.59	7.70
	2	116.441	-	1.56	7.60
	10	44.083	-	1.56	7.60

of the species, cover the entire genome, and ensure high marker density.. In the study, 23 linkage groups for the maternal parent (Memecik) and 26 linkage groups for the paternal parent (Uslu) were determined. The number of markers included in the LG is much higher than many previous genetic maps of the olive. In another mapping study conducted on the Memecik × Uslu hybrid population, the total length of the LG was stated as 2921.90 cM for Memecik and 2543.20 cM for the Uslu variety. The average distance between two markers was 1.41 cM in Memecik and 1.38 cM in Uslu. In another study, the total number of markers was 3903 (Çetin et al., 2016). Using the same marker system (DArT-SNP), the number of markers more than doubled in the LG obtained for varieties relevant to the current study. The first linkage mapping study of the olive was done by Baldoni et al. (1999). Later, De La Rosa et al. (2003), using RAPD, AFLP, RFLP, and SSR techniques, mapped the Leccino × Dolce Agogia cross population. The researchers determined 249 markers in 39 LG for the Leccino olive cultivar and the map length as 2765 cM. For Dolce Agogia, the authors determined 236 markers in 30 LG and the map length to be 2445 cM. The mean distance between two markers for maternal and paternal cultivars was reported as 13.2 cM and 1.9 cM, respectively. Wu et al. (2004) reported 23 linkage groups for the Kalamata variety and 27 linkage groups for the Frantoio variety. The map lengths were determined as 798 cM and 759 cM, respectively; the total number of markers used in mapping was 152, and the distance between the two markers was 12.3 cM in the Frantoio cultivar and 11.5 cM in the Kalamata cultivar. The authors also stated that the olive genome length was approximately 3000 cM. In the Gemlik olive variety, 105 markers were determined in 16 LG, and the length of the linkage groups was listed as 2891 cM (İpek et al., 2008).

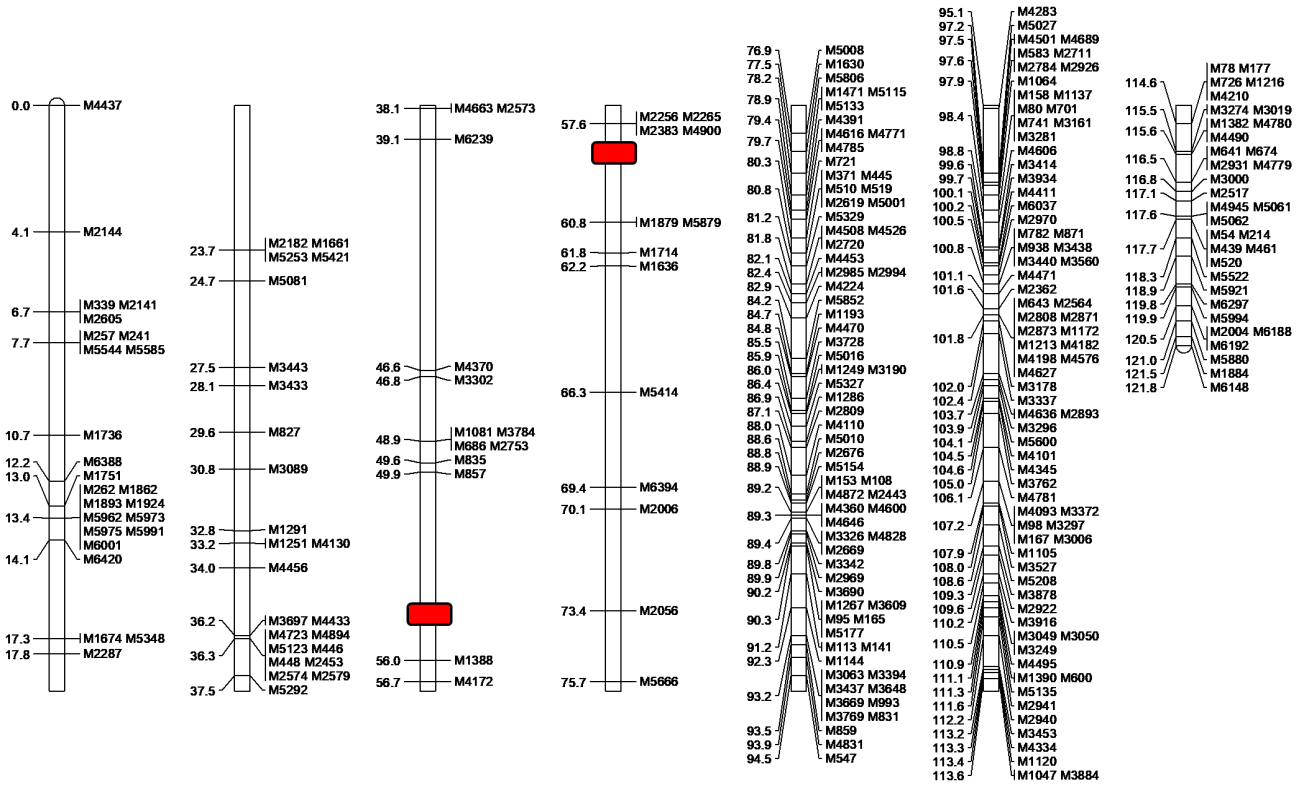

Figure. Tenth linkage group of the Memecik variety.

Table 5. QTL loci determined by years in the Uslu cultivar.

Year	Linkage group	Location (cM)	Locus	LOD	Announced phenotypic variance (%)
2017	7	114.378	-	2.24	10.00
	15	69.804	M7317	2.12	9.50
	7	132.016	-	1.77	8.00
	7	131.016	-	1.75	7.90
	7	128.043	-	1.52	6.90
	16	74.577	M7233	1.52	6.90
	7	127.043	-	1.47	6.70
	16	70.166	-	1.45	6.60
	16	73.496	M7253	1.44	6.50
	16	73.496	M7304	1.44	6.50
2018	11	82.108	M10100	1.29	5.90
	3	111.007	-	1.27	5.80
	16	70.166	-	1.18	5.40
	4	55.663	-	1.15	5.30
	16	60.515	-	1.14	5.20
	4	54.663	-	1.13	5.20
	24	6.688	-	1.09	5.00
	5	58.659	-	1.05	4.80
	7	114.378	-	1.00	4.60
	11	92.802	-	0.99	4.50

Table 5. (Continued.)

2019	11	108.592	-	2.04	9.80
	11	74.121	-	2.01	9.70
	11	87.514	-	1.94	9.30
	3	2.000	-	1.92	9.30
	11	94.507	M11675	1.88	9.10
	11	111.288	-	1.88	9.10
	11	100.827	-	1.70	8.30
	11	112.288	-	1.61	7.80
	3	11.000	-	1.52	7.40
	1	86.698	-	1.39	6.80

By mapping the Picholine Marocaine × Picholine du Languedoc hybrid population, 40 LG were identified for the mother and 38 for the father. The length of the maps was calculated as 1547.40 cM in the mother variety and 1428 cM in the father variety. The number of markers included in the LG was 170, and the distance between the two markers was 10.8 cM (El Aabidine et al., 2010). Similarly, Khadari et al. (2010) reported 36 LG for the Oliviere cultivar and 31 LG for the Arbequina cultivar. The map length was 2210.20 cM, the number of markers in the LG was 222, and the distance between two markers was 11.2 cM for the Oliviere cultivar. For the Arbequina variety, the map length was 1966.2 cM, the number of markers in the LG was 219, and the distance between two markers was 10.3 cM.

In another study (Dominguez-Garcia et al., 2012), in which 47 LG were determined for the Picual cultivar and 39 for the Arbequina cultivar, the researchers determined that the map length for the Picual was 1205.1 cM, the number of markers included in the LG was 257, and the distance between two markers was 9.64 cM; for the Arbequina variety, the map length was 1639.3 cM, the number of markers in the LG was 392, and the distance between two markers was 8.04 cM. A year later, Ben Sadok et al. (2013) formed the connection groups of Oliviere and Arbequina cultivars. On the map, 25 LG were obtained for Oliviere and 21 for Arbequina. For the Oliviere variety, the map length was 1745.3 cM, the number of markers in the LG was 212, and the distance between two markers was 8.23 cM For the Arbequina cultivar, the length of the map was determined as 1597.6 cM, the number of markers in the LG was 252, and the distance between two markers was determined as 6.34 cM.

In a study carried out on a population of 121 F1 individuals of Gemlik × Edincik hybrids, 25 combined LG were obtained. The linkage map had 5643 markers, and the average distance between two markers was 0.53 cM. The authors stated that this map contained high-density markers and could be used to determine QTL in olive breeding programs (İpek et al., 2016). However, Marchese et al. (2016) obtained 23 LG in a study in which the F2 population obtained from the self-pollination of the Koroneiki variety was mapped. It was stated that these groups had a total length of 1189.7 cM and contained 1597 SNP markers.

Mariotti et al. (2020) reported that 23 LG were obtained for both parents in the map created using Leccino × Dolce Agogia cultivars. Using SNP markers, a highly dense map containing 16,743 markers (7006 for the mother variety and 9737 for the father variety) was created. The researchers, who created linkage groups equal to the haploid chromosome number of the olive for both parents, reported that the genome length was higher than expected. They obtained maps with a length of 5680 cM for the Leccino cultivar and 3538 cM for the Dolce Agogia cultivar, and the average distance between markers was 0.810 cM for the mother variety and 0.363 cM for the father variety.

Compared to the results of other studies, this study is among those that carried out high-density genetic mapping of olives. The number of markers mapped in both parents was 9041, and the mean distance between two markers was 0.556 cM for the mother and 0.549 cM for the father. The use of DArT-SNP marker technology was quite effective in obtaining this result. Thousands of SNP markers were identified simultaneously in the population studied using DArT analysis, which resulted from developments in new-generation sequencing technologies. This technology was previously used in olives by Dominguez-Garcia et al. (2012) and Çetin et al. (2016); 1630 and 3903 DArT-SNP markers were obtained in those studies, respectively. A total of 21,195 polymorphic DArT-SNP markers were obtained.

MapQTL6 software was used for QTL analysis, and loci with LOD ≥ 3 were evaluated as QTL (Van Ooijen, 2009). In the QTL analysis performed in 3 different years in the mother-parent Memecik cultivar, QTL could not be

detected above LOD ≥ 3 in the first and second years. In the third year, two candidate QTL regions with LOD ≥ 3 were identified. These markers were detected in the region of 58.639 cM and 54.894 cM in LG10. Candidate QTL was detected at 58.649 cM. The M2256 was at a distance of 1.00 cM from the M2265, M2383, and M4900 and had a LOD of 3.03. The phenotypic variation explained by this marker was 14.20%. The QTL detected at 54.894 cM was at a distance of 1.081 cM from marker M1388, to which it was closest, and its LOD value was 3.02. The phenotypic variation explained in this marker was again 14.20%. However, in the Uslu cultivar, which is the father-parent, a QTL region above LOD ≥ 3 related to frost tolerance could not be detected during the 3 years. The presence and effectiveness of the obtained QTL regions varied over the years. Similar cases were found in studies on phenotypic data obtained in different years (Toojinda et al., 2003; Linge et al., 2015; Yu et al., 2018; Rehman et al., 2020). It is desirable to determine the same QTLs for any character in different years and environmental conditions (Mete, 2015). However, quantitative characteristics are influenced by multiple genes and reflect the phenotype together with environmental factors. Phenotypic variation is also influenced by many genes and environmental factors (Asins, 2002; Khan, 2015).

The resistance mechanism to cold stress in plants is a complex process influenced by many factors. Environmental changes, hormonal changes, gene activity, new gene products, the accumulation of soluble substances, and changes in lipid composition affect frost tolerance (Howarth and Ougham, 1993; Aslantaş, 1999). The frost damage rates of hybrid genotypes change between years, but this depends on changing environmental factors. Accordingly, the QTL regions detected in different years vary. It is thought that the most important factor affecting this situation is that each hybrid individual is a single tree, and the fluctuation in the physiological state of the trees depends on the product load. Selecting an appropriate population is crucial for successful genetic mapping. In this respect, the parents selected should be genetically different from each other and show opposite characteristics in terms of the characteristics to be mapped (Mete, 2015). When examined in terms of the contrast between the characters mapped, it was seen that the frost tolerance of the Uslu cultivar was relatively low, and the Memecik cultivar was in the moderately tolerant group. Accordingly, very few QTLs with LOD ≥ 3 were identified. The high number of chromosomes (2n = 46), the highly heterozygous structure of the olives, and the very long juvenile period in hatching populations are the most important concerns while determining quantitative trait loci (Bracci et al., 2011; Atienza et al., 2014). In addition, the hybrid populations created in the past were only obtained according to morphological characteristics, and sufficient genetic variation did not occur (Mete and Çetin, 2017). For this reason, very little QTL information exists about important properties in olives (Belaj et al., 2011; Bracci et al., 2011; Dominguez-Garcia et al., 2012; Ben Sadok et al., 2013).

In the first QTL study conducted on olives by Ben Sadok et al. (2013), in which the parameters affecting yield were examined, 35 candidate QTLs were determined, and it was stated that the olive yield was under mixed genetic and environmental control. In another study on the determination of QTLs in olives by Atienza et al. (2014), tree strength and several fruit characteristics were examined; 22 candidate QTL regions were identified for Arbequina and five candidate QTL regions for Picual. The phenotypic variation explained for these quantitative trait loci regarding oil content, moisture content, fruit pulp ratio, fruit weight, and stem diameter varied between 12.70% and 30.40%.

In a study carried out on the Gemlik variety, three candidate QTLs were determined for fruit seed weight, one for fruit weight, and seven candidate QTLs affecting the amount of fatty acids (LOD > 2) (İpek et al., 2008). Çetin et al. (2016) identified 39 candidate QTL regions for olive fruit ripening (LOD > 2) and 23 candidate QTL regions for fruit flesh firmness (LOD > 3). Four candidate QTL regions were identified for the separation of seeds from fruit flesh that provided similar results in two different years. It was reported that these QTLs were identified in LG1 of the Uslu variety and that it was easy to distinguish fruit flesh from seed (LOD > 2).

Hernández et al. (2017) identified 32 candidate QTLs associated with oleic and linoleic acid synthesis in olive oil. As a result of a mapping study in which high-density LG were created, a functional marker for self-incompatibility was developed for the first time in olives (Mariotti et al., 2020).

Two genome maps of olives have been published to date. The first genome sequencing was done by Cruz et al. (2016), and, in that study, the sequence information of 1.31 Gb of the estimated 1.38 Gb in the olive genome was completed. Researchers have determined that 56,349 different protein-coding genes result from RNA sequencing from leaf, root, and fruit tissues at various stages. A year after this study was conducted, genome mapping in wild olives was published, and it was determined that there are more than 50,000 protein-coding genes in the olive genome (Unver et al., 2017).

5. Conclusion

In the study, a linkage mapping with high marker saturation was performed and a LG haploid chromosome number of 23 was determined for the maternal parent (Memecik) and

26 for the paternal parent (Uslu). In the linkage mapping, there were 4377 markers in the Memecik variety and 4664 markers in the Uslu variety. The total number of markers mapped in both parents was 9041. The total length of LG was determined as 2311.92 cM for the Memecik cultivar and 2415.89 cM for the Uslu cultivar.

In the Memecik cultivar, two candidate QTL regions with LOD ≥ 3 associated with frost tolerance were identified. These markers were detected as 58.639 cM and 54.894 cM in LG10. Candidate QTLs were detected at 58.649 cM. The M2256 was at a distance of 1.00 cM from M2265, M2383, and M4900 and had a LOD of 3.03. The phenotypic variation explained by this marker was 14.20%. The QTL detected at 54.894 cM was at a distance of 1.081 cM from marker M1388, to which it was closest, and its LOD value was 3.02. The phenotypic variation explained in this marker was again 14.20%. In the Uslu cultivar, which is the father-parent, a QTL region above LOD ≥ 3 related to frost tolerance could not be detected in the study's 3-year period.

Breeding studies to develop olive varieties with high frost tolerance will be possible by comprehensively investigating the molecular mechanisms of olives' response to low temperatures. This study developed important QTL markers for MAS in olive cultivars with high frost tolerance.

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Conflict of Interest
The authors declare that they have no conflict of interest.

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