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Molecular Characterization and Assessment of Population Structure of Hulled Wheats

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ABSTRACT
Analysis of genetic diversity among genotypes and differentiation among populations are crucial for determination of conservation strategies and one of the best plant breeding approaches. In this study, ISSR markers were used to determine genetic variation and population structure in 23 hulled wheats. Together with control durum wheat and bread wheat registered cultivars, 32 wheat genotypes were analyzed with 14 ISSR markers located throughout the wheat genome. Number of alleles per locus ranged from 3 to 13 and the polymorphism information content (PIC) value ranged from 0.27 for the UBC-852 to 0.37 for the UBC-824 with an average of 0.33. High levels of polymorphism ratio (100%) were observed for ISSR primers. Mean number of polymorphic alleles (N), expected heterozygosity (He), PIC, number of effective allele (Ne), Shanon's information index (I) and genetic variation (F_{ST}) were determined as 10.21, 0.42, 0.33, 1.78, 0.61 and 0.63, respectively. UPGMA analysis based on dice genetic similarity ranged between 0.981 and 0.112 showing the high genetic diversity among hulled wheat genotypes. Results showed that the ISSR markers provided reliable and reproducible fingerprinting profiles for assessment of population structure and genetic diversity of hulled wheat genotypes. These molecular variations obtained from present study can be used in parent choosing for breeding studies.

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Kavuzlu Buğdayların Moleküler Karakterizasyonu ve Popülasyon Yapısının Değerlendirilmesi

ÖZET
Genotipler arasındaki genetik çeşitliliğin ve popülasyonlar arasındaki farklılaşmanın analizi, koruma stratejilerinin belirlenmesi ve en iyi bitki ıslah yaklaşımlarından biri için çok önemlidir. 23 kavuzlu buğdayda genetik varyasyonu ve popülasyon yapısını belirlemek için ISSR markörleri kullanılmıştır. Kontrol makarnalık buğday ve ekmeklik buğday tescilli çeşitleriyle birlikte, 14 ISSR markörü ile 32 buğday genotipi analiz edildi. Lokus başına alel sayısı 3 ile 13 arasında ve polimorfizm bilgi içeriği (PIC) değeri UBC-852 için 0.27 ile UBC-824 için 0.37 arasında ve ortalama 0.33 olarak belirlenmiştir. ISSR primerleri için yüksek düzeyde polimorfizm oranı (%100) gözlenmiştir. Ortalama polimorfik alel sayısı (N), beklenen heterozigotluk (He), PIC, etkili allel sayısı (Ne), Shanon bilgi indeksi (I) ve genetik varyasyon (F_{ST}) sırasıyla 10.21, 0.42, 0.33, 1.78, 0.61 ve 0,63 olarak belirlenmiştir. Dice genetik benzerliğine dayanan UPGMA analizi, kavuzlu buğday genotipleri arasındaki yüksek genetik çeşitliliği göstermiş olup 0.981 ile 0.112 arasında değişmiştir. Sonuçlar, ISSR markörlerinin kavuzlu buğday genotiplerinin popülasyon yapısı ve genetik çeşitliliğinin değerlendirilmesi için güvenilir ve tekrarlanabilir parmak izi profilleri sağladığını göstermiştir. Mevcut çalışmadan elde edilen bu moleküler varyasyonlar, ıslah çalışmaları için ebeveyn seçiminde kullanılabilir.

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INTRODUCTION

Hulled wheats are one of the first domesticated crop species. The grains belong to emmer (*Tiriticum dicoccon* L.) and einkorn (*T. monoccocum* L.) wheats discovered in Cayonu excavation dated back to 6500-7000 B.C. in Turkey (Harlan, 1998). Hulled wheats contain all ploidy levels such as diploid, tetraploid and hexaploid. They are known as transitional positions between wild and cultivated forms of wheat during the evolutionary process. Diploid hulled wheat, *T. monoccocum* var. *monoccocum*, is considered as a new genetic resource to improve *T. durum* and *T. aestivum* that contain a genome in their genetic structures (Heun et al., 1997). Currently, admixture of emmer and einkorn populations is generally cultivated in rural regions of Kastamonu, Sinop and Cankırı provinces of Turkey. However, the cultivated hulled wheat acreage has been decreasing (Karagoz, 1996). A small number of Turkish einkorn and emmer wheat together with durum and bread wheat landraces was characterized (Karagoz and Zencirci, 2005).

Genetic diversity is a cornerstone for any plant breeding program (Khush, 2002). Genetic diversity studies conducted with molecular markers are advantageous because they are not affected from environmental factors and genetic variation can be estimated using a small amount of DNA (Prasad et al., 2000). Different molecular markers such as RAPD, SSR, AFLP and ISSR have been used for genetic characterization of diverse cereal species containing wheat accessions. These molecular markers had been used in wheat for detecting genetic variation, genotype identification, and genetic mapping (Najaphy et al., 2011; Abou-Deif et al., 2013). Inter simple sequence repeats (ISSRs) have become broadly used for different goals in plant genetic researches (Karaca and Izbirak, 2008). The ISSR marker is one of the universal DNA markers amplified regions among microsatellite sequences by the polymerase chain reaction (PCR) (Gupta et al., 1994). The technique is commonly used in studies of cultivar identification, genetic diversity, genetic mapping, evolution and molecular ecology (Yang et al., 1996). Najaphy et al. (2012) showed that ISSR markers together with agronomic and morphological characters of wheat can be used to determine molecular variation in wheat genotypes. The first aim of this study was to evaluate genetic diversity in hulled wheat land races collected from Kastamonu province of Turkey by ISSR markers. The second aim was to evaluate the informativeness of ISSR markers for detecting molecular variation in hulled wheat.

MATERIAL and METHOD

Plant Material

Twenty three (23) hulled wheat populations used in this study were collected in different parts of Kastamonu province. Also, four registered durum (*Tiriticum durum* L.) wheat cultivars named as Kızıltan-91, Ç-1252, Sarıçanak-98, Y.popülasyon and five registered bread wheat (*Tiriticum aestivum* L.) cultivars named as Doğankent-1, Kıraç-66, İkizce-96, Bayraktar-2000, Bezostaja-1 were used in this study. Seeds of hulled wheat which were pre-tested in field conditions was planted in viols with durum and bread wheat cultivars. Plants germinated from seed were used as genotypes for molecular identification.

DNA Extraction

Wheat seeds were grown in plastic container in room temperature. Fresh leaves belonging to each wheat genotype were harvested for DNA isolation. DNA extraction was carried out using the CTAB method modified by Gulsen et al. (2005). Genomic DNA was suspended with 50 µl 1x TE (Tris Edta) buffer for stock solution and stored at -80 ^{o}C till use. DNA concentration of each genotype was measured through both agarose gel (1%) and NanoDrop (BioSpec-nano Shimadzu Biotech). Final DNA concentration was adjusted to 5 ng μL^{-1} to be used in ISSR-PCR amplification and then DNA samples were stored at -20 °C until use.

Molecular Analyzes

Fifty-four ISSR markers were firstly screened for consistency and their ability to produce polymorphism in wheat using DNA of randomly selected eight wheat genotypes. From 54 ISSR primers, 14 were selected for PCR amplification due to their polymorphic, clear, strong and reproductive bands (Table 1). PCR volume for each ISSR primer to characterize the thirty two wheat genotypes was adjusted to 15 µl total reaction volume including; 1.8 µl 10X PCR buffer, 1.5 µl $MgCl_2$ (25 mM), 6.5 µl dH_2O, 2 µl of 2 mM dNTP, 1.3 µl of 0.6 mM primer, 0.4 µl Taq polymerase (5 U μl^{-1}) and 1.5 µl of 20 ng μl^{-1} DNA. Amplified PCR products were separated on 2% agarose gel and agarose gel was stained with ethidium bromide. Then, it was photographed under UV light and ISSR band polymorphism visually evaluated. A 100 bp DNA

ladder was used in order to estimate molecular weight of ISSR bands.

ISSR band profiles were scored for each ISSR primer to create a binary format matrix. Only reliable and reproducible polymorphic bands were recorded. The ISSR binary data matrix obtained was used to calculate the dice similarity coefficient. Different genetic diversity parameters of individual ISSR markers were estimated. While effective alleles number (Ne) and Shannon's Information Index (I) was calculated using PopGene software 1.32 version, polymorphism information content (PIC) and gene diversity (He) were estimated with PowerMarker software ver. 3.25. The F_{ST} values of ISSR markers were computed by Arlequin software. Cluster analysis was conducted by complete linkage method using NTSYS-pc software version 2.02 (Rohlf, 2000). A dendrogram was constructed based on binary data of dice similarity matrix by unweighted pair group method with arithmetic average (UPGMA) cluster analysis. Principal component analysis (PCA) was also performed via this software. To assess wheat population structure and assign individuals to wheat populations, ISSR data were analyzed using a Bayesian approach in Structure v.2.3.4 (Pritchard et al., 2000). The number of supposed clusters (K) was set from two to nine. The data obtained from Structure software was then uploaded to Structure Harvester which is an online tool. The best K value (K subpopulations) was determined according to the protocol of Evanno et al. (2005). In addition, genetic diversity parameters for each population were assessed in terms of He, F_{ST}, Nm and Shanon's information index. He and F_{ST} values for populations were obtained from Structure software v.2.3.4. In addition, Nm and Shanon's information index were calculated by PopGene software 1.32 version.

RESULT and DISCUSSION

Different marker systems based on DNA had been used in wheat genetic studies such as RAPD, RFLP, AFLP, SSR, and ISSR. ISSR markers are widely polymorphic and could be used for wheat cultivars identification as many authors reported (El Maati et al., 2004; Motawei et al., 2007; Karaca and Izbirak, 2008; Sofalian et al., 2008; Carvalho et al., 2009). Furthermore, Motawei et al. (2007) also made genotypic identification in emmer and durum varieties by using ISSR primers. Fahmy et al. (2016) showed that ISSR markers could be used as fairly informative markers for gene tagging and genome mapping. We carried out molecular characterization using ISSR DNA marking technique for wheat genotypes includeing 9 emmer (*T. dicoccum*) and 14 einkorn (*T. monococcum*) and 9 registered cultivars. Fifty-four ISSR primers were tested and 14 of them were selected for the genetic diversity study (Table 1) and they were used to characterize and evaluate the genetic variation of the wheat genotypes. The selected primers generated totally 148 bands ranging from 3 to 18, with average 10.21 (95.42%) bands per primer; these values show high genetic variability (Table 2).

Table 1. Eigenvalues of first three main components of PCA
Çizelge 1. PCA'nın ilk üç ana bileşeninin değerleri

Main components	Eigenvalue	Percent	Cumulative values
1.	17.29	54.05	54.05
2.	8.32	26.02	80.07
3.	1.05	3.28	83.36

Allele length of ISSR markers was ranged from 180 bp to 980 bp. The maximum number of alleles was observed at UBC-852 and their size ranged from 200 to 880 bp. An average of 10.8 polymorphic alleles per locus was detected for the thirty-two wheat genotypes. Primers UBC843, UBC822, UBC840, UBC823, UBC851, UBC852, UBC818, UBC815 and UBC826 were the most informative and they have 100% polymorphism ratio. With 75% polymorphism ratio, primer UBC824 showed the lowest number of bands (Table 1). These polymorphism ratios were quite high than the polymorphism rates reported by Gulbitti et al. (2007) for *T. dicoccoides*, *T. monococcum* ssp. *boeoticum*, and *T. urartu,* 32.34%, 42.63% and 27.71%, respectively. However, the polymorphism levels were lower than those obtained by Sofalian et al. (2008) using ISSR markers on 27 wheat genotypes including 18 spring landraces and 9 cultivars. Du et al. (2002) reported 87% of polymorphism in 47 hybrid wheats with 11 ISSR markers. Najaphy et al. (2011) observed that 10 ISSR primers generated 80.2% polymorphism among 30 wheat accessions. Morgante et al. (2002) showed that the polymorphism of ISSR markers depends on the microsatellite frequency and distribution throughout the genome of the species. PIC of ISSR markers recorded mean value of 0.33, with a variation ranging from 0.27 to 0.37. The lowest and the highest PIC values were obtained for primer UBC852 and UBC824, respectively. In the present study, the mean PIC value was higher than ISSR markers used by Najaphy et al. (2011). This difference is probably due to use of different genotypes and ISSR markers. The highest value of Nei's genetic diversity (He) for ISSR

primers was observed for the UBC824 (0.49) and the lowest value was observed for the UBC852 (0.33). Ne (Number of effective alleles) values ranged from 1.59 (UBC852) to 1.96 (UBC824). UBC822, UBC851, UBC818 and UBC815 presented the highest Ne values. UBC852 has the lowest Shannon's index (I) ranging from 0.51 to 0.68, with mean 0.61 (Table 1).

Table 2. Genetic diversity parameters of ISSR markers used for assessment of genetic variation of wheat genotypes

Çizelge 2. Buğday genotiplerinin genetik varyasyonunun değerlendirilmesinde kullanılan ISSR markörlerinin genetik çeşitlilik parametreleri

No	Primers	SP	N	AL	PB	He	PIC	I	Ne	F_{ST}
1	UBC–843	$(CT)_8RA$	11	810–380	100	0.42	0.33	0.61	1.75	0.59
2	UBC–853	$(CT)_8RT$	15	950–280	93.7	0.43	0.34	0.63	1.83	0.63
3	UBC–846	$(CA)_8RT$	11	950–300	91.6	0.39	0.30	0.57	1.72	0.61
4	UBC–824	$(TC)_8G$	3	920–700	75	0.49	0.37	0.68	1.96	0.70
5	UBC–815	$(CT)_8G$	12	920–180	100	0.45	0.35	0.65	1.85	0.41
6	UBC–852	$(CT)_8RA$	18	880–200	100	0.33	0.27	0.51	1.59	0.43
7	UBC–813	$(CT)_8T$	5	900–450	83.3	0.40	0.31	0.59	1.71	0.47
8	UBC–845	$(CT)_8RG$	12	980–180	92.3	0.36	0.28	0.54	1.65	0.47
9	UBC-840	$(GA)_8YT$	12	920–220	100	0.41	0.32	0.60	1.76	0.63
10	UBC–823	$(TC)_8C$	10	920–490	100	0.43	0.34	0.63	1.81	0.42
11	UBC–851	$(GT)_8YG$	8	740–250	100	0.46	0.35	0.66	1.87	0.99
12	UBC–826	$(AC)_8C$	12	850–350	100	0.41	0.33	0.61	1.72	0.68
13	UBC–818	$(CA)_8G$	8	880–230	100	0.45	0.35	0.65	1.86	0.85
14	UBC–822	$(TC)_8A$	6	920–310	100	0.46	0.36	0.66	1.88	0.98
Total			143							
Mean			10.21		95.42	0.42	0.33	0.61	1.78	0.63

SP, Sequence of primers; N, Number of polymorphic allels; AL, Allel length (bp); PB, Percentage of polymorphic bands (%); He, Nei's (1973) gene diversity index; PIC, Polymorphism information content; I, Shannon's information index; Ne, Effective alleles number; FST, Fixation index

In order to investigate genetic relationships among emmer and einkorn wheat genotypes cluster analysis were performed as based on dice similarity coefficients. Dice similarity matrix based on ISSR markers was used to classify the wheat genotypes. The mean value of dice similarity coefficient was 0.553 and genetic similarity ranged from 11% to 98% for all wheat genotypes based on ISSR analysis. The similarity values clearly revealed significant differences among the hulled wheat genotypes (Kastamonu=KST). In terms of genetic similarity, KST18, KST10 and KST7 genotypes were the closest genotypes with 0.981 similarity coefficient. On the contrary, some genotypes showed low genetic similarity, such as KST12-KST13 (0.1157) and KST10-KST13 (0.1148). KST6-KST13 with 0.112 dice coefficient had the lowest genetic similarity (Figure 1). Furthermore, KST6-KST10 with 0.9541 similarity coefficient and KST6-KST16 with 0.9009 similarity coefficient were determined to be the closest to each other (Figure 2). The dendrogram resulting from UPGMA cluster analysis showed that two main clusters (A and B clusters) were classified into thirty-two wheat genotypes (Figure 1). Cluster A included generally *T. dicoccum* genotypes, but cluster B consists of mainly *T. monococcum* genotypes. The first main group (A) was divided into two sub groups and the first sub-group (A1) consisted of emmer wheats (*T. dicoccum* L.) population excluding only one emmer wheat genotype (Figure 2). On the other hand, the second sub-group of cluster A (A2) comprises tetraploid and hexaploid registered cultivars. The durum wheat genotypes were clustered much closer to the crustacean tetraploid subgroups (A1) than the hexaploid varieties (Figure 1). Based on ISSR dendrogram, the first group (PopA) includes 11 genotypes (KST10, KST8, KST7, KST6, KST16, KST1, KST3, KST12, KST14, KST4 and KST11) along with 9 registered cultivars. The second main group B was also formed mainly diploid hulled wheats (*T. monoccocum* var. *monoccocum*). The group B includes KST15, KST2, KST20, KST23, KST21, KST5, KST8, KST13, KST9, KST22, KST19 and KST17. Among the registered cultivars, İkizce-96 and Bayraktar-2000 grouped close to each other in the UPGMA dendrogram. The UPGMA dendrogram obtained in present study distinguished the wheat genotypes according to their ploidy level (tetraploid and diploid). Gurcan et al (2017) demonstrated that SSR markers effectively grouped hulled wheats (emmer and einkorn) according to their ploidy level. Our results show that the ISSR marker system is effective to mainly distinguish wheat genotypes, consistently with their ploidy levels. Einkorn (*T. monococcum*) and emmer (*T. diccoccum*) wheat generally were classified separately. Also Motawei et al. reported that ISSR and RAPD primers divided

wheat genotypes into two main groups based on their pedigrees. Carvalho et al. (2009) stated that ISSR primers group wheat genotypes based on their ploidy levels. Their results are consistent with this results.

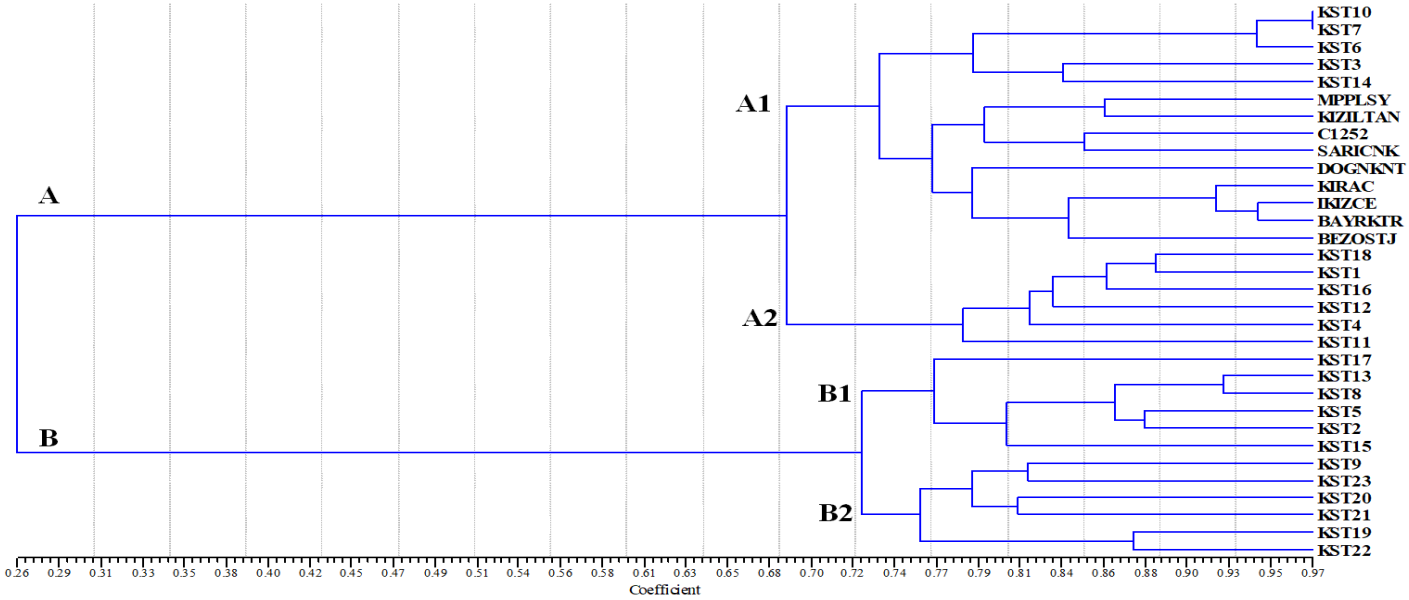

Figure 1. UPGMA dendrogram generated by using dice similarity index
Şekil 1. Dice benzerlik indeksi kullanılarak oluşturulan UPGMA dendrogramı

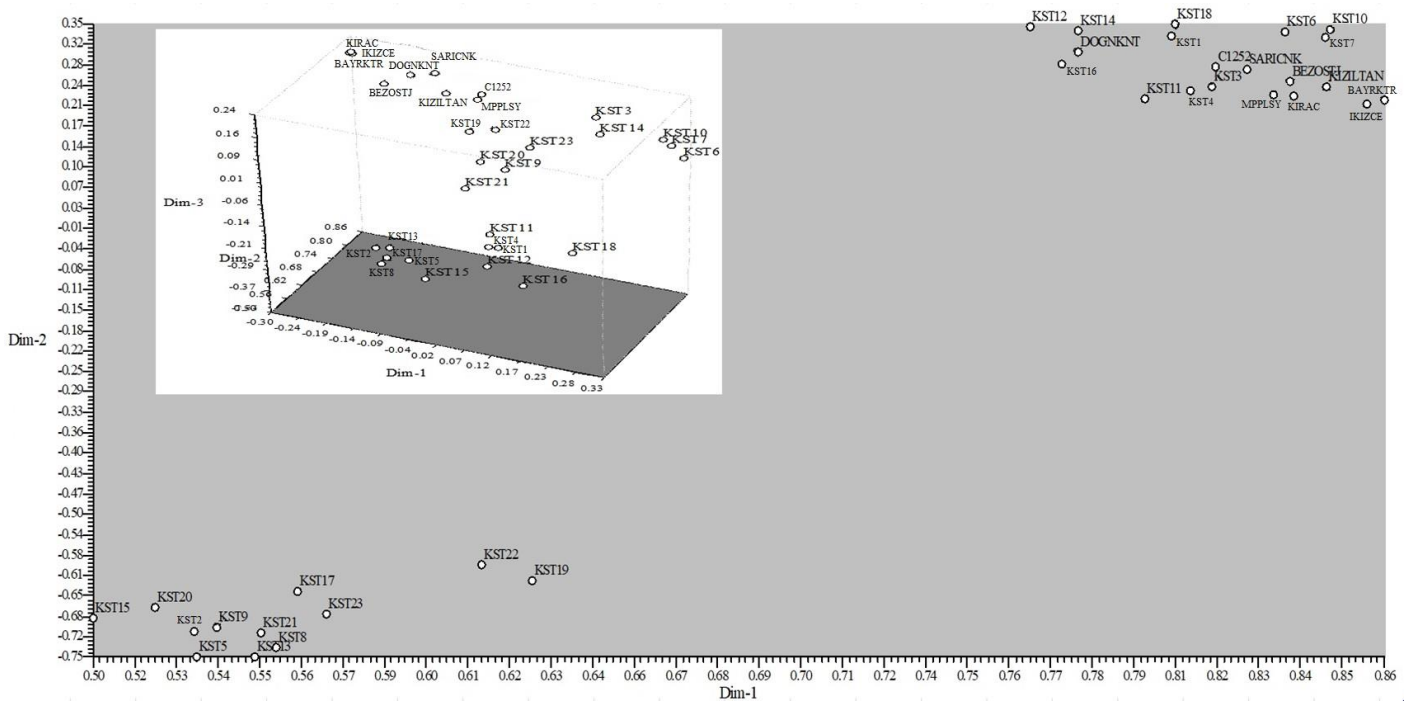

Figure 2. Principal component analysis based on ISSR data of 32 wheat genotypes
Şekil 2. 32 buğday genotipinin ISSR verilerine dayalı temel bileşen analizi

In this study, the principal component analysis (PCA) has also been performed to reveal the genetic diversity. Two and three-dimensional graphics were created by using NTSYS-pc software version 2.02. The results of PCA are demonstrated in Figure 2 and showed that all wheat genotypes are classified genetically into two groups similarly to UPGMA dendrogram. Overall, *T. monococcum* (einkorn) and *T. dicoccum* (emmer) were fairly separated by the PCA. First group in PCA include einkorn wheat genotypes except for KST17 (emmer). Second group consists of emmer, bread and durum wheat genotypes. The cumulative sum of the eigenvalues of first three divisions for two and three-dimensional graphs in

basic components analysis explains 83.36 of total variation. First component of PCA with Eigenvalues was explained 54.05 of molecular variation. Principal Component Analysis (PCA) based on molecular data was performed to reveal the genetic difference between genotypes. According to the PCA result, the additive sum of the first three main components was determined as 83.36% (Table 1).

Analysis of genetic structure of 32 genotypes using bayesian approach in Structure software explained to population structure in wheat. Analysis of ISSR data produced the highest log likelihood scores when number of populations was set at two, which was consistent with clustering based on genetic distance. All the wheat genotypes were classified into two populations as PopA and PopB. Wheat genotypes are represented by vertical columns colored red and green (Figure 3).

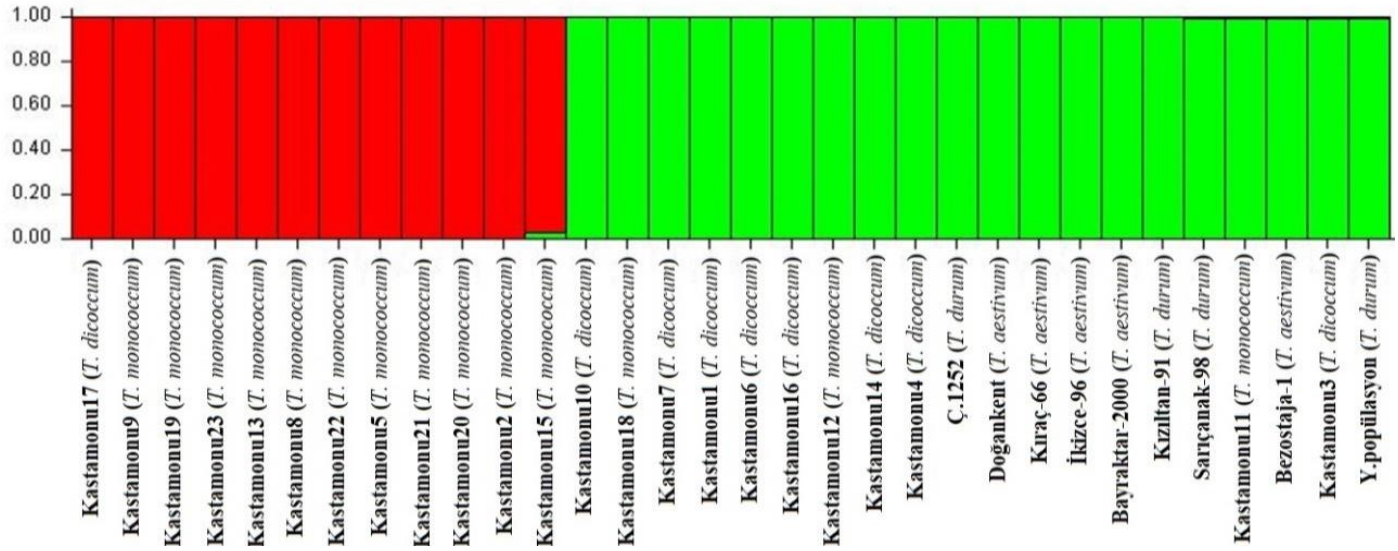

Figure 3. Population structure of 32 wheat genotypes for K=2
Şekil 3. K=2 için 32 buğday genotipinin popülasyon yapısı

T. monoccuccum and *T. diccocum* genotypes clustered in PopA and PopB, respectively. PopB includes both durum and bread wheat genotypes. According to membership coefficient, 18 genotypes were assigned into the largest subpopB (PopB) included *T. diccocum* (einkorn) genotypes mostly from Kastamonu. According to the structure program, genotypes with population membership coefficient below 80% were assumed to be hybrid, and genotypes above 80% were assumed to be pure (Gurcan et al., 2017). All wheat genotypes in this study were considered as pure genotypes since population membership coefficients based on sutrucrure analysis were above 0.80. Each wheat genotype was shown by a coloured bar according to estimated membership to each of PopA and PopB. The pairwise Fst values are statistically significant for the comparisons between subpopulations. The expected heterozygosity, FST, Nm and I were estimated to analyse the genetic structure of wheat populations by PopGene32 software. PopA (0.67) and PopB (0.63) have FST values approximately resemble to each other. The average of expected heterozygosity (He) and Shannon's Index (I) for two populations were found to be 0.1834 and 0.265 respectively. The highest and the lowest Nei's gene diversity (He) were related to PopB (0.188) and PopA (0.178), respectively. Shannon's diversity index was higher for *T. diccocum* (PopB). Average of Nm referring gene flow was found to be 0.135. The low genetic divergence was observed within subpopulations (Table 3).

Table 3. Genetic variation parameters among wheat genotypes
Çizelge 3. Buğday genotipleri arasındaki genetik varyasyon parametreleri

Subpopulation	He	F_{ST}	Nm	I
PopA	0.1786	0.6787	0.12	0.26
PopB	0.1882	0.6308	0.15	0.27
Average	0.1834	0.6547	0.135	0.265

He, Nei's (1973) gene diversity index; F_{ST}, Fixation index; Nm, Estimate of gene flow from F_{ST}; I, Shannon's information index

CONCLUSION

The present study definitely indicated that the 32 wheat genotypes could be separated with ISSR primers having a high level of polymorphism ratio. As result, the present study demonstrates that ISSR markers are useful for molecular characterization and analysis of population of hulled wheats. In addition, the ISSR markers identified can provide useful information for breeding programs to select the individuals. However, the utility of ISSR markers in separating wheats according to ploidy level should be confirmed by future cytological studies.

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Author Contribution Rates

The authors declare that they contribute equally to the article.

Conflict of Interests

Authors declare that there is no conflict of interests.

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