GENETIC DIVERSITY OF WILD RASPBERRY GENOTYPES *(RUBUS IDAEUS* L.) IN NORTH ANATOLIA BASED ON ISSR MARKERS

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Abstract. The preliminary aim of the study before future breeding program was to reveal usability of ISSR markers to assess genetic diversity and relatedness within raspberry genotypes wildly grown in Northern Turkey. In this study, 19 wild raspberry genotypes collected from Black Sea region in Turkey were used. ISSR PCR analyses were carried on the DNA of genotypes isolated using mini CTAB extraction method. The levels of polymorphism between genotypes were determined using the UBC ISSR primers. A total of 111 bands were obtained from 15 UBC ISSR primers. Out of 111 bands, 101 bands were polymorphic. The number of bands obtained per primer ranged between 3 and 10, the average number of bands were determined as 7.4. The average number of polymorphic bands per primer was 6.73. Similarities and differences between genotypes have been studied at the molecular level. The data used for statistical analysis were obtained by the evaluation of ISSR bands. Similarity coefficient and UPGMA dendrogram were built using the Basic Coordinates Analysis. According to the dendrogram, the genotypes have been divided in two main groups, one small and other large group. While the small group was only comprised of three (A8, A10 and A14) genotypes, the large group included the rest of the genotypes studied.
Keywords: *molecular, polymorphism, dendrogram, raspberry, Turkey*

Introduction

Anatolia is one of the gene resources of the world with its natural flora of hundreds of wild plant forms, and wild forms of strawberry is also very common in most parts of the Turkey, especially in Black Sea coast and on the mountains through the sea cost. The wild ancestors of many plants which now provide staples for mankind still grow this region. Turkey is also the home of wild form of many soft fruits such as raspberry, blackberry. These species are mainly originated from the foothills of the Mountains on Black Sea coast. The wild forms have so much contribution to improve new cultivars. That is why it is so important to describe the wild forms and to establish collection gardens with them (Davis, 1982; Jennings, 1988).

As an important commercial fruit crop, red raspberries (*Rubus idaeus* L.: Rosaceae) are widely grown in all temperate regions of the world and are consumed by local people. Many of the most important modern commercial red raspberry cultivars derive from hybrids or selections from the wild. Black Sea region in northern Turkey is one of the important germplasm centers of this species (Davis, 1982). They grow naturally in high elevations and require moist, rich soils. The plants are usually seen as small, spatially discrete populations over 1000 m altitude, mostly along roadsides, in woodland clearings. *Rubus idaeus* has been an important source of genes for breeding new raspberry varieties (Alice et al., 2001; Marshal et al., 2001). In the development of improved varieties, the main qualities selected for are hardiness, productivity, disease resistance, fruit size, and

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firmness. Scientists have also started using *R. idaeus* as a model plant to develop and apply genetic techniques in the study of perennials, particularly members of the family *Rosaceae* (Graham et al., 1996).

Very few recent studies have attempted to quantify the genetic variation present in raspberry germplasm in Turkey. Ercisli et al. (2008) examined genetic diversity in 11 wild selections with one red raspberry using Amplified Fragment Length Polymorphism (AFLP) markers. There are also several reports on genetic diversity in raspberries from all over the worlds and modern molecular marker techniques are essential in effectively evaluating this germplasm collection as a source of useful genetic material for raspberry breeding programs (Howell et al., 1994; Chwedorzewska et al., 2002; Lund et al., 2003).

The development of molecular biology has resulted in DNA based marker procedures that should lead to a greater understanding of relationships between species and more accurate taxonomic classification. These techniques should also allowed more effective understanding of crop plant's genetic architecture and utilization of genetic diversity by breeders and identification of species and cultivars by means other than morphological characteristics (Graham et al., 1996).

Inter simple sequence repeats (ISSRs) have been shown to provide a powerful, rapid, simple, reproducible and inexpensive means to assess genetic diversity and identify closely related cultivars in many species (Gonzalez et al., 2002) The ISSR technique has been extensively used for several applications in molecular taxonomy, conservation and breeding. ISSR PCR has since been applied for cultivar identification (Charters et al., 1996; Cekic et al., 2001; Haritha et al., 2015; Ayesh, 2017), to detect geneflow and introgression (Allainguillume et al., 1997; Kalyani et al., 2014) and for mapping purposes (Kojima et al., 1998) and genetic diversity analysis (Cekic and Özmen, 2016). Further, ISSR PCR is useful in fingerprint and characterization of accessions and identification of cultivar and varieties (Wolf et al., 1995; Hardik et al., 2016). Against this backdrop, the present work was taken up with an objective to assess the level of genetic variation among 19 raspberry genotypes from Black Sea region in North Anatolia based on ISSR PCR before future breeding program.

Material and methods

Plant material

For research purposes, a total of 19 different raspberry genotypes from Black Sea region in North Anatolia were used. Observed observations showed wild raspberry plants spread from 1200 m above sea level of Turkey, where the forests end and the open fields begin, and on the northern slopes facing the Black Sea, where foliage is common where water leaks from the ground. Coordinates and elevation of the place where the samples are taken are recorded by specifying with Global Positioning System (GPS) (*Table 1*) and these coordinates marked on the map (*Fig. 1*) In addition, plants and surroundings are photographed in terms of ease of identification of the region and plant sample taken in the field.

DNA extraction

Total genomic DNA was extracted according to the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). DNA sample concentration was determined using a fluorometer employing a Hoechst dye (Hoefer Inc., San Francisco, CA, USA),

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and the DNA samples were diluted to a final concentration of 10 ng/µl with 1 × TE buffer and stored at -20 °C prior to polymerase chain reaction (PCR) amplification.

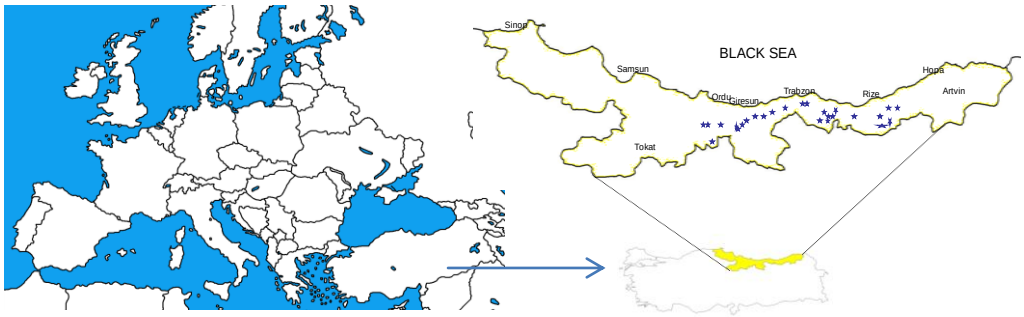

Figure 1. *The localization of Black sea coast in Turkey, where the samples were collected*

Table 1. *The latitudes, longitudes and elevations of the samples taken*

Sample ID	City	Location	Altitude (m)	Latitude	Longitude
A1	*Trabzon*	*Duzkoy*	1505	40° 50.993N	039° 25.963E
A3	*Trabzon*	*Zigana*	1999	40° 38.279N	039° 24.285E
A4	*Trabzon*	*Zigana*	2013	40° 38.409N	039° 24.362E
A5	*Trabzon*	*Sumela*	1732	40° 39.415N	039° 40.913E
A6	*Trabzon*	*Sumela*	1718	40° 39.349N	039° 40.773E
A7	*Trabzon*	*Sumela*	1714	40° 39.358N	039° 40.761E
A8	*Trabzon*	*Sumela*	1718	40° 39.473N	039° 40.730E
A9	*Trabzon*	*Uzungol*	1123	40° 36.470N	040° 23.795E
A10	*Rize*	*Ayder*	1486	40° 56.494N	041° 08.037E
A11	*Rize*	*Ayder*	1584	40° 56.143N	041° 08.252E
A12	*Giresun*	*Kumbet yaylasi*	1381	40° 34.723N	038° 26.411E
A13	*Ordu*	*Turnalik*	1495	40° 41.811N	037° 55.442E
A14	*Ordu*	*Turnalik*	1480	40° 40.582N	037° 55.763E
A15	*Ordu*	*Cambasi*	1730	40° 39.936N	037° 56.344E
A16	*Giresun*	*Bektaş yaylasi*	1623	40° 42.410N	038° 13.910E
A18	*Giresun*	*Bektas inisdibi*	1926	40° 40.133N	038° 15.615E
A19	*Giresun*	*Bektas yaylasi*	1789	40° 40.915N	038° 18.139E
A21	*Rize*	*Cagirankaya*	2043	40° 49.375N	040° 37.886E
A22	*Trabzon*	*Sisdagi*	1775	40° 51.351N	039° 09.087E

ISSR amplification

15 primers that produced clear and reproducible fragments were selected out of a hundred UBC primers, which were previously tested for further analyses. ISSR amplification was performed in a 20 µl volume containing 20 ng genome DNA, 1 × Taq buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.75 µM primer, 0.5 units of Taq DNA polymerase. The amplification reaction consisted of an initial denaturation step at 94 °C for 5 min, followed by 45 cycles of 45 s at 94 °C, annealing at 50 °C–56 °C for 45 s, extension at 72 °C for 90 s, and ended with extension at 72 °C for 7 min. The amplified products were resolved electrophoretically on 2.0% agarose gels run at 100 V in

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1.0 × TBE buffer, visualized by staining with ethidium bromide (0.5 μg/ml), and photographed under ultraviolet light (Charters et al., 1996; Rafalski et al., 1996) The amplifications were repeated twice and only clear repetitive bands were used in data analysis, and molecular weights were estimated using a 100 bp DNA marker (Vivantis).

Data analysis

Alleles were designated on the basis of fragment size; bands were scored as diallelic (1 = band present, 0 = band absent). To assess the genetic variation among the genotypes, the eighteen ISSR markers were analyzed using NTSYS-pc (Rohlf, 2002). The coefficients of genetic similarity for all pair-wise comparisons were computed using Jaccard's coefficient. A similarity matrix was used to create a dendrogram using the unweighted pair group method with arithmetic mean (UPGMA). The observed number of bands, polymorphic band number, polymorphic band ratio per primer, Resolving power (RP value) and Polymorphic information content (PIC) were calculated with the POPGENE software version 1.31 (Yeh et al., 1997) to estimate genetic variation. Polymorphic information content (PIC), the probability in detecting polymorphism by a primer or primer combination between two randomly drawn genotypes was calculated using the formula PIC = 1 - Σpi2, where pi is the frequency of the ith allele (Sehgal et al., 2009); Resolving power (Rp) which is the ability of each primer to detect level of variation between individuals was calculated according to Prevost and Wilkinson (1999): Rp = ΣIb where Ib (band informativeness) takes the values of: 1 – [2|0.5 – p|], where p is the proportion of individuals containing the band, and marker index (MI) for each primer was also calculated as a product of two functions - the polymorphic information content and effective multiplex ratio (EMR) (Varshney et al., 2007). Group constellations were also independently developed by using principal component analysis (PCA) to verify the grouping obtained through the dendrogram. PCA further helped in depicting the variation among the genotypes in a three-dimensional mode (McVean, 2009).

Results and discussion

ISSR polymorphism

On the basis of the number, intensity and reproducibility of ISSR bands 15 primers were selected out of a hundred UBC primers, which were previously tested (Cekic et al., 2001). Bands with the same mobility were treated as identical fragments. Weak bands with negligible intensity and smear bands were both excluded from final analysis (*Fig. 2*). Each primer was evaluated for total band number (TBN), polymorphic band number (PBN) and the ratio of polymorphism (PR = PBN/TBN × 100). The amplification of ISSR markers was consistent between repeat PCR runs. The number of polymorphic bands produced ranged from 3 (for primer 889) to 10 (for primers 881 and 888). The number of scored bands varied from four to ten with an average of 7.40 bands per primer and an average of 6.73 polymorphic bands per primer. In total, 101 bands out of 111 derived from 15 primers were polymorphic (*Table 2*). Cao et al. (2000) reported that 50 polymorphic bands were sufficient for determination of differences and similarities between genotypes. With our 101 polymorphic band obtained in our study, it appears to have exited. So that this variability allowed raspberry genotypes to be distinguished on their genetic bases. The polymorphism level with 90% was higher than

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the findings with 69% polymorphism level of Ercisli et al. (2008), where 12 raspberry genotypes in eastern Turkey were analysed by AFLP markers. The average PIC and RP values were 0.25 and 0.41, ranging according to primers from 0.0729 to 0.3515, and from 0.08 to 0.57 for PIC and RP values, respectively (*Table 2*). Chesnokov and Artemyeva (2015) reported that the maximum PIC value is 0.5 for the dominant markers like ISSR, and for the markers with equal distribution in the population the PIC values are higher.

Table 2. *Band number, Polymorphic band number, Polymorphic band ratio (%), PIC (polymorphism information content) and RP (resolving power) values in raspberry genotypes*

ISSR primer code	Primer sequence and anchors	Band number	Polymorphic band number	Polymorphic band ratio (%)	PIC value	RP value
807	*AGAGAGAGAGAGAGAG**T***	10	8	80	0.1844	0.30
856	*ACACACACACACACAC**YA***	9	7	78	0.2092	0.35
881	*GGGTGGGTGGGTGGGT*	10	10	100	0.3227	0.46
888	***BDB**CACACACACACACA*	10	10	100	0.3211	0.49
889	***DBD**ACACACACACACAC*	3	3	100	0.0729	0.08
890	***VHV**GTGTGTGTGTGTGT*	9	7	78	0.2352	0.43
891	***HVH**TGTGTGTGTGTGTG*	5	4	80	0.2516	0.48
808	*AGAGAGAGAGAGAGAG**C***	7	6	86	0.2310	0.45
810	*GAGAGAGAGAGAGAGA**T***	6	6	100	0.2826	0.40
811	*GAGAGAGAGAGAGAGA**C***	6	5	83	0.3021	0.54
826	*ACACACACACACACAC**C***	4	4	100	0.2793	0.34
835	*AGAGAGAGAGAGAGAG**YC***	8	8	100	0.3515	0.57
841	*GAGAGAGAGAGAGAGA**YC***	7	7	100	0.2935	0.48
842	*GAGAGAGAGAGAGAGA**YG***	9	8	89	0.2049	0.33
844	*CTCTCTCTCTCTCTCT**RC***	8	8	100	0.2715	0.41
Average		**7.40**	**6.73**	**91.60**	**0.25**	**0.41**
Total		**111**	**101**			

Cluster analysis

Genetic similarity among varieties was estimated using dissimilarity coefficient matrix based on ISSR bands scored. Pair wise values of dissimilarity coefficients ranged from 0.57 for genotypes with the same scored bands to 0.83 for the most similar genotypes. The dendrogram was constructed based on the similarity matrix, using the unweighted pair group method with arithmetic mean (UPGMA) method (Rohlf, 1992). The 19 raspberry genotypes were divided into two main clusters (*Fig. 3*), in which three genotypes in one group and the rest of the genotypes falling under the other group. While the first major group contained only one subgroup, the second major cluster was further separated into several subgroups. The coefficient values in first subgroup ranged from 0.74-0.83. On the other hand, the similarity of the second varied 0.62 to 0.75 (*Fig. 3*).

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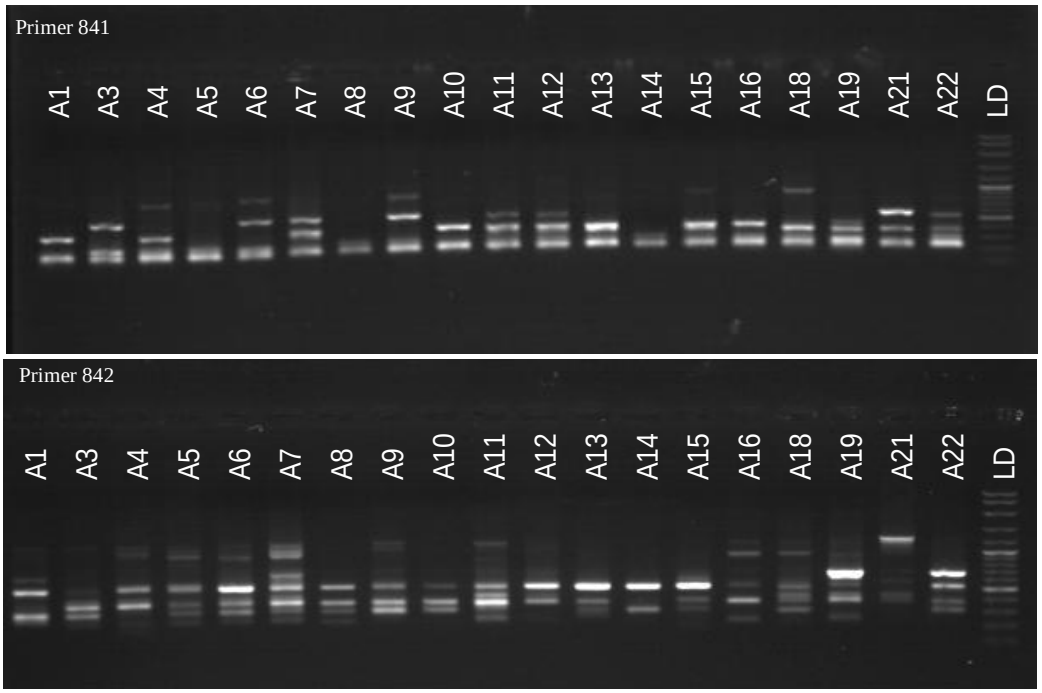

Figure 2. *Agarose gel of polymorphic DNA amplification profiles of different raspberry genotypes obtained with UBC ISSR primer 841 and 842 (LD: 100 bp LADDER -Vivantis)*

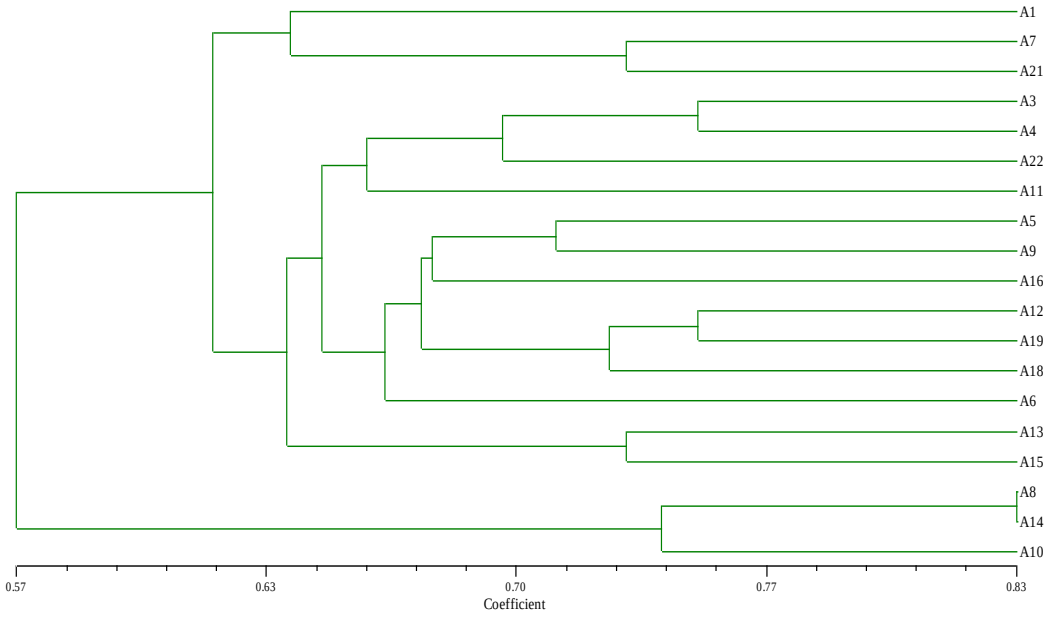

Figure 3. *Dendrogram showing genetic relationships among value of ISSR markers for raspberry genotypes*

Group constellations were also independently developed by using principal component analysis (PCA) to verify the grouping obtained through the dendrogram. PCA further helped in depicting the variation among the genotypes (Asif et al., 2015). The principle component analysis roughly also put the genotypes into two different

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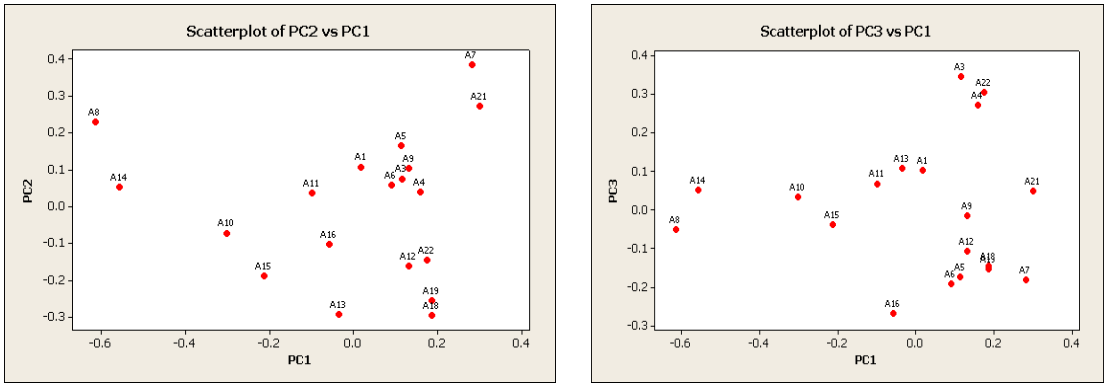
clusters as parallel to the dendrogram of the raspberry genotypes. The small main group consists of the A8, A10 and A14 genotypes which form a different cluster in the PC1-PC2 and PC1-PC3 planes (*Fig. 4a, b*). Although, A8 and A14 were found to be 100% similar in same group of dendrogram, these two genotypes were still apart from each other by the different dimensional view of PCA analysis. On the other hand, while A18 and A19 were differentiated around 26% level in the dendrogram, PCA analysis put these two genotypes very close each other in both PC1-PC2 and PC1-PC3 dimensions (*Fig. 4a, b*).



a **b**

Figure 4. *The principle component analysis with three components of raspberry genotypes according to 15 ISSR primers*

Conclusion

Screening of the 19 genotypes of different raspberry genotypes revealed that banding profiles obtained with fifteen ISSR primers were enough to distinguish the diversity among the wild raspberry genotypes. The fifteen ISSR primers we used on 19 raspberry genotypes resulted 101 polymorphic bands, which is much higher than the reports of Cao et al. (2000), where 50 polymorphic bands were sufficient for determination of differences and similarities between genotypes. The results indicated that the ISSR technique is effective to develop genotype specific banding patterns valuable for genotype identification. Since ISSR PCR technique does not require previous DNA sequence information and uses very small quantity of DNA, it is considered as one of the most widely used techniques for genotype identification and genetic diversity studies. The study also revealed that the dendrogram created using UPGMA method and the PCA analysis were complementary, and were useful to use together in diversity studies.

Although no scientific data was recorded regarding the morphological characters of genotypes, the observation of morphologies of genotypes during in collection parcel did not reveal obvious correlation with morphological characters. This work was the first attempt to use ISSR markers to assess genetic diversity and relatedness within raspberry genotypes wildly grown in Norhern Turkey, and the information generated herein may be useful for the improvement of this crop and could serve a promising future for breeding programs in our genetic collection. Further studies examining new molecular markers correlating morphological and pomological characters could explain more completely the diversity within this species.

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