AFLP-BASED GENETIC RELATIONSHIPS IN WILD AND CULTIVATED RED RASPBERRY GENOTYPES (RUBUS IDAEUS L.)

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ABSTRACT
The genus Rubus contains a large number of highly variably and heterogeneous species, which occur in all parts of the world except the desert regions. Turkey has notable wild grown Rubus populations and these fruits are a traditional part of Turkish diet. The leaves and fruits of wild Rubus forms are used for medicinal purposes in Turkey as well. In the present study we attempted to characterize 11 wild selections and 1 well known red raspberry cultivar, Heritage by using fluorescent dyed AFLP markers and capillary electrophoresis. Four primer combinations generated a total of 259 bands, 182 (69.0%) of which were polymorphic. The higher genetic similarity was found between AH2 and AH3 genotypes. UPGMA clustering of the accessions showed seven major groups. The cultivar Heritage independently formed its own group. AFLP profiles suggesting that the wild genotypes might be derived from seedlings rather than through clonally offshoot propagation.

Keywords: Rubus, red raspberry, AFLP, genetic diversity

Introduction
During the last century, intensified cultivation with modern agricultural technologies has caused a reduction in the genetic diversity of plant species including fruits (20). Instead, biodiversity in natural ecosystems should be prized, because it potentially affords many new foods (11, 26). Currently only 150 edible plants have been cultivated on a large scale and many minor crops are neglected. In some specific cases, genetic erosion of their gene pools has become so severe that they are often regarded lost crops. Less known crops are found in numerous agricultural ecosystems and only survive in marginal areas (22). The World Food and Agriculture Organization (FAO) emphasized the need to preserve new plant resources to broaden the biological diversity. Moreover, more recently there has been increased interest in wild grown plants because of their special genetic structure which may be linked to their medicinal value (10).

Rubus is accepted as one of the most diverse genera in plant kingdom which comprises over 400 species and subdivided into 12 subgenera (15). However, only a few subgenera of Rubus such as raspberries, blackberries, arctic fruits and flowering raspberries have been domesticated and utilized in breeding programs (15). The most important raspberries are the European red raspberry, R. idaeus L. subsp. idaeus, the North American red raspberry R. idaeus subsp. strigosus Michx and the black raspberry, R. occidentalis L. Rubus subgen. Idaeobatus is distributed principally in Asia but also in East and South Africa, Europe and North America. In contrast, subgen. Eubatus is mainly distributed in South America, Europe and North America (13, 15). The members of subgenus Idaeobatus sp. are distinguished by the ability of their mature fruits to separate from the receptacle. The subgenus is particularly well represented in the northern hemisphere. The place of origin of red raspberry (Rubus idaeus) has been postulated to be the Ida Mountains of Turkey (15). Turkey is one of the natural habitat centers of the Rubus genus and nearly all Rubus plants are widely distributed globally as wild in Turkey. Particularly Northeastern part of the country including Black Sea Region has extensive wild Rubus populations which are scattered throughout the region. Red raspberries are less known or underutilized plants in Turkey and show great diversity of forms and fruiting characters in their natural habitats in the country. The wild raspberry populations could be potential source of genetic material for breeding new cultivars. Moreover little is known about the genetic similarity of these wild types and how similar they are to the standard cultivars (9).

The identification of red raspberry fruits even wild materials has been traditionally carried out by morphological, agronomic and chemical traits (5, 7, 14, 16). Although these methods are efficient, they present practical drawback because of the effect of environmental fluctuations on the expression of most morphological traits. With the advent of molecular techniques, several types of DNA markers have been used in genetic diversity assessment in Rubus. The first technique to be used in Rubus was randomly amplified polymorphic DNA (RAPD) because of its simplicity, low cost and high multiplex ratio (12), followed by RFLPs and microsatellites (21), including wild red raspberries (1). The recently described by Vos et al. (27) amplified fragment length polymorphism (AFLP) has been used in Rubus genomic research.

The AFLP technique for typing genomic DNA is based on the selective PCR amplification of restriction fragments from total digests of genomic DNA. It combines the reliability of the RFLP technique with the power and ease of the PCR-based techniques and it is suitable for DNA of any origin or complexity (4, 29). Currently, the AFLP technique is one of the
most reliable and effective techniques used in genetic diversity assessment of fruit species (2, 3, 8).

In order to explore the genetic diversity of the wild raspberry plants and to compare them with Heritage cultivar, individual plants in wild populations and cv. Heritage were sampled and examined using four AFLP markers.

Materials and Methods

Plant Material

The genotypes were selected from 2 different locations (Uzundere and Ispir) in Northeast Anatolian region in Turkey. The distance between locations was around 60 km. The leaf samples of eleven previously selected wild growing red raspberry genotypes were collected from Uzundere (AH1, AH2, AH3, AH4, AH5 genotypes) and Ispir (AH6, AH7, AH8, AH9, AH10, AH11 genotypes). In addition, well known red raspberry cultivar, namely Heritage found in the raspberry experimental area in Oltu district was also included in this study. In Uzundere district, the locations of plants were close to each other and all wild plants were found within 16 km² area. However in Ispir region, genotypes were found far away from each other, up to 50 km.

DNA isolation and fluorescent AFLP analysis

Genomic DNA was extracted from freeze-dried leaf tissue (~50 mg) using the DNeasy system (Qiagen, Valencia, CA, USA). For DNA extraction a modified CTAB method was used (6). The AFLP analysis was done as described in Vos et al. (26). In short, the purified genomic DNA extracts (~0.5 µg per sample) were digested with the restriction enzymes EcoRI and MseI. Two adapters with known sequences were then ligated to the sticky ends of the digested DNA fragments. The resulting product was diluted (20-fold) and subsequently pre-amplified by PCR with primers. PCR amplifications were programmed with the following temperature profile: one cycle of 2 min at 94°C, followed by 23 cycles of 30 sec at 94°C, 30 sec at 65°C, and 2 min at 72°C, followed by 23 cycles of 30 sec at 94°C, 30 sec at 56°C, and 2 min at 72°C. The PCR was terminated with a final incubation step of 30 min at 60°C. Among the initially tested 40 EcoRI/MseI primer combinations four primer combinations yielding more polymorphisms were selected for evaluation of the genetic diversity among the raspberry genotypes originating from 2 different districts in Anatolia (Table 1). AFLP analysis was conducted on an ABI 3130 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA) with the data collection software 3.0 (ABI). AFLP fragment analysis was performed with GeneScan Analysis Software 4.0 (ABI) and the data were assembled in binary format. Fragments were resolved using capillary electrophoresis.

Data analysis

For the genetic relationship analysis, Jaccard’s similarity coefficients (25) were calculated for all pair-wise comparisons among the 12 raspberry genotypes. Two dendrograms were generated using NTSYSpc version 2.11V (Exeter Software, Setauket, NY) (23) based on the unweighted pair-group method of arithmetic average cluster analysis (UPGMA) and Principal Coordinate Analysis (PCoA). Mantel’s matrix correspondence test (18) was used to evaluate the cophenetic correlation between the UPGMA dendrogram and similarity matrix. The result of this test is a cophenetic correlation coefficient, r, indicating how well the UPGMA dendrogram represents similarity data. In PCoA analysis, the genotypes were plotted on first three dimensions using the G3D procedure of SAS program (24).

Results and Discussion

Polymorphism of AFLP markers

The AFLP analysis of 12 raspberry genotypes originating from 2 different districts in Anatolian region of Turkey (Uzundere and Ispir) with four primer combinations generated a total of 259 fragments of which 182 were polymorphic, corresponding to 69% level of polymorphism (Table 1). The observed high level of polymorphism suggests that the wild genotypes may have originated from seedlings rather than through clonally offshoot propagation.

<table>
<thead>
<tr>
<th>Enzyme-primer combinations</th>
<th>Number of total bands (no.)</th>
<th>Number of Polymorphic bands (no.)</th>
<th>Polymorphism (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-AAG/M-CAA</td>
<td>71</td>
<td>55</td>
<td>77</td>
</tr>
<tr>
<td>E-AAC/M-CAC</td>
<td>65</td>
<td>41</td>
<td>63</td>
</tr>
<tr>
<td>E-AAC/M-CTT</td>
<td>69</td>
<td>54</td>
<td>76</td>
</tr>
<tr>
<td>E-AGG/M-CAT</td>
<td>54</td>
<td>32</td>
<td>60</td>
</tr>
<tr>
<td>Total</td>
<td>259</td>
<td>182</td>
<td>-</td>
</tr>
<tr>
<td>Mean</td>
<td>65</td>
<td>46</td>
<td>69</td>
</tr>
</tbody>
</table>

Number of total bands, number of polymorphic bands and polymorphism ratio of 12 raspberry genotypes generated by 4 AFLP primer combinations.
The number of total bands produced by each primer combination ranged from 54 (E_{AGG}/M_{CAT}) to 71 (E_{AAG}/M_{CAA}) with an average of 65 bands. The percentage of polymorphism varied considerably among the primer combinations. For example, the highest ratio of polymorphism was generated by E_{AAG}/M_{CAA} (77%) while the E_{AGG}/M_{CAT} combination yielded the lowest polymorphism (60%). This is an indication that AFLP is a powerful marker system and can be successfully used for differentiation of all raspberry genotypes used in this study. AFLP markers have been previously used in the analysis of Rubus populations (17, 19). All these studies showed that the polymorphism ratio was between 24-91% among Rubus genotypes. Although our accessions represent only 11 wild and 1 cultivated genotypes from Anatolia region, the level of polymorphism was comparable to those obtained in some previous studies (17, 19). This is an indication of the high degree of polymorphism among the genotypes tested. Kollmann et al. (17) concluded that the level of genetic variability in Rubus is determined by the plant propagation system used and they have demonstrated that the cross-pollination among Rubus plants has also an effect on the observed variability. This type of crossbreeding influences seed and fruit quality positively, through increasing the taxonomic proximity.

The AFLP marker system has been used in the studies of a number of fruit tree species in recent years (3, 8, 28). It has been postulated that the complexity of the AFLP profiles is determined by the primers, restriction enzymes applied in the analysis, as well as the composition of the genomic DNA. The AFLP markers can generate large number of polymorphisms which can be effectively used in both inter-and intra species identification.

Patterns of genetic diversity

The dendrogram produced by Jaccard’s coefficient and the UPGMA clustering method show one main cluster, subdivided on two groups (Fig. 1). Group 1 includes all wild raspberries (AH1, AH2, AH3, AH4, AH5) sampled from Uzundere district within 16 km² area. The genotypes in this group showed very high similarity index to each other and among them AH2 and AH3 genotypes possessed the highest similarity level (0.94).

The second group comprising the sampled from Ispir district wild genotypes (AH8, AH6, AH7, AH10, AH11 and cv ‘Heritage’ respectively) showed the lowest genetic similarity. The cv Heritage is clearly distinguished from wild accessions, showing high level of genetic diversity.

The genetic diversity of the raspberry genotypes from the Ispir region was significantly higher than that of the Uzundere. This could be explained by the larger distances (up to 50 km) among genotypes found within Ispir district which was only up to 4 km in Uzundere district. Fig. 2 shows PCoA analysis of 12 raspberry genotypes based on AFLP data set. The PCA analysis confirms the distribution of the grouping produced by the UPGMA analysis. Based on PCoA analysis, three separate groups were identified (Fig. 2).

The information collected and analyzed on wild raspberry genotypes has improved greatly the knowledge of the genetic variation and differentiation among wild and cultivated raspberry. This study clearly showed that the wild genotypes selected from both locations could be discriminated and none of them was judged to be similar to the cv. Heritage using AFLP markers. The similar situation was observed in UK among wild red raspberry population and cv. Glen Clova by using RAPD data (13).

The present contribution shows that AFLP is a promising approach to help in raspberry breeding programmes aiming at the development of varieties directed to agro-industrial applications while keeping biodiversity at a high level. It points to the need for a more extensive study to be developed all over raspberry growing areas between quantitative traits.
and molecular markers for a better and efficient conservation. To our knowledge, this is the first study to use AFLP markers for examining the genetic variability and relationships of wild grown red raspberries in Turkey.

Conclusions
1. Generally all *Rubus* accessions were successfully genotyped and accessing the genetic distances among them. Used in our study high polymorphic AFLP markers allowed local differentiation among genotypes origin from two districts (Uzundere and Ispir).
2. The evaluation on genetic diversity of wild *Rubus* species by DNA methods will be great benefit for creation of new breading programmes.

REFERENCES