Genetic Relatedness in Prunus Genus Revealed by Inter-simple Sequence Repeat Markers

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Abstract. Inter-simple sequence repeat (ISSR) markers were used to study the genetic diversity and phylogenetic relationships among 16 genotypes from subgenus Prunus (six genotypes from section Prunophora, seven genotypes from section Armeniaca and two plumcot genotypes, and one genotype from subgenus Cerasus) in Prunus genus. From the polymerase chain reaction amplifications with 20 ISSR primers showing polymorphism among subgenera and sections, 180 polymorphic ISSR bands were detected and polymorphism ratio ranged from 57% to 100%. Based on the unweighted pair group method with arithmetic mean (UPGMA) analysis and principal coordinate analysis (PCoA) using the Jaccard coefficient, a dendrogram and three-dimensional plot were constructed including genotypes in Prunus genus. Two main groups formed in the dendrogram; one of them (Cluster I) included Cerasus, whereas Cluster II included Prunus. Cluster II also divided into three subgroups, including sections Prunophora, Armeniaca, and plumcot. Both UPGMA and the PCoA demonstrated that Armeniaca genotypes had lower genetic variation and plumcot genotypes are closer to the plums than the apricots. The ISSR-based phylogeny was generally consistent with Prunus taxonomy based on molecular evidence, suggesting the applicability of ISSR analysis for genotypic and phylogenetic studies in Prunus genus.

The genus Prunus comprises five subgenera: Prunus, Amygdalus, Cerasus, Padus, and Laurocerasus and includes ≥200 species, which are economically important as sources of fruits, nuts, oil, timber, and ornamentals (Reynolds and Salesses, 1990). The subgenus Prunus includes section Prunophora comprising plums and section Armeniaca containing apricots. Each of these sections is considered to be a single gene pool (Watkins, 1976). Plums are adapted to the cooler temperate regions, whereas apricots are grown in warmer temperature regions of the world. Plums belonging to subgenus Prunophora are considered to be important for Prunus evolution because they include more than 20 species with abundant variation in their morphology. Differences in genetic diversity between plums and apricots are much influenced by the self-(in)compatibility phenotype of these species (Halasz et al., 2007a, 2007b; Milatovic and Nikolic, 2007). Although the basic chromosome number of Prunus species is x = 8, some species within subgenus Prunophora are triploid, tetraploid, and hexaploid. According to the derivative systems of these polyploids, Prunus domestica L. (6x), one of the European plums, is considered to be derived from a natural cross between Prunus spinosa L. (4x) and Prunus cerasifera Ehrh. (2x) (Cane and Lawrance, 1952). However, Zohary (1992) hypothesized that the origin of Prunus domestica is an autopolyplid derived from Prunus cerasifera. In addition, regarding the origin of European plums, Eryomine (1991) stated that it is originated of mixed descent from many other species, including Prunus microcarpa, Prunus salicina, Prunus armeniaca, and Prunus persica. The term Japanese plum was applied originally to the other two subgenera, Amygdalus and Prunus (Reyners and Salesses, 1990).

Breeding barriers exist among subgenera possessing different ploidy levels, even within the same subgenus, but artificial or natural hybrids are generally successful, in particular between Prunophora (plums) and Armeniaca (apricots), when both parents have the same ploidy level (Okie and Weinberger, 1996). The subgenera Padus and Laurocerasus are more isolated within the genus Prunus.

The traditional taxonomic classification within the genus Prunus is mainly based on fruit morphology and has been controversial (Aradhye et al., 2004). This approach is also subject to environmental influences, mainly as a result of the long generation time and large size of the trees. Trees are also influenced by agricultural factors like rootstocks or pruning. Therefore, precise characterization and identification of species within the Prunus subgenus are important to recognize gene pools, to identify pitfalls in germplasm collections, and to develop effective conservation and management strategies. New methods based on molecular evaluations may provide further insight into the genetic structure and differentiation within Prunus (Aradhye et al., 2004). Genetic characterization of diversity and relationships at both inter- and intraspecific levels in the genus, Prunus, is limited to a few molecular phylogenetic studies using ITS and chloroplast trnL-trnF spacer sequence variation (Bortiri et al., 2001) and amplified fragment length polymorphism (Aradhye et al., 2004).

Choice of the marker system to use for a particular application depends on its ease of use and the particular objectives of the investigation (Rafalski et al., 1996). Recently, inter-simple sequence repeat (ISSR) markers have emerged as an alternative system with the reliability and several advantages over random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and simple sequence repeat (SSR). ISSR is a simple and quick method that combines most of the advantages of SSRs and AFLPs to the universality of RAPDs. The major limitations of RAPD, AFLP, and SSR methods are low reproducibility of RAPD and high cost of AFLP while flaming sequences have to be known to develop species-specific primers for SSR polymorphism. ISSR overcomes most of these limitations (Reddy et al., 2002). The main disadvantages of ISSR are the dominant nature and lower multiplex ratio. This method has been used in several fruit crops such as olive (Terzopoulos et al., 2005), pistachio (Kafkas et al., 2006), plum (Lisek
markers for fingerprinting a set of
Therefore, in the present study, we used ISSR
bromide staining. DNA was diluted to 5
mated by comparing band intensity with
Concentration of extracted DNA was esti-
minor modifications (Kafkas et al., 2005).
extracted from leaf tissue by the CTAB
reaction procedure.

Materials and Methods

Plant materials. For molecular analysis,
totally 16 genotypes from genus Prunus (six
genotypes from section Prunophora, seven
genotypes from section Armeniaca and two
plumcot genotypes, and one genotype from
subgenus Cerasus) were used (Table 1). The
genotypes were found together in a national
germplasm collection at the Fruit Research
Institute of Ministry of Agriculture in the
Malatya province of Turkey.

DNA extraction and polymerase chain
reaction procedure. Genomic DNA was
extracted from leaf tissue by the CTAB
method of Doyle and Doyle (1987) with
minor modifications (Kafkas et al., 2005).
Concentration of extracted DNA was esti-
mated by comparing band intensity with λ
DNA of known concentrations after 0.8%
agarose gel electrophoresis and ethidium
bromide staining. DNA was diluted to 5
ng μL⁻¹ for ISSR reactions.

Polymerase chain reaction (PCR) mix-
tures had a total volume of 25 μL containing
20 ng of DNA template; 0.2 μM primer; 100
μM each of dATP, dGTP, dCTP and dTTP; 1
unit of Taq DNA polymerase; 2 mM MgCl₂;
75 mM Tris-HCl; pH 8.8, 20 mM (NH₄)₂SO₄;
and 0.01% Tween 20. PCR amplifications
were performed in a gradient thermal cycler
(Eppendorf, Hamburg, Germany) with the
following temperature profile: a predenatura-
step of 3 min at 94 °C followed by 40
cycles of denaturation at 94 °C for 60 s;
annealing at 48 to 54 °C (depending on
primer) for 60 s; and extension at 72 °C for
120 s. A final extension was allowed for 7
min at 72 °C. ISSR amplification products
were analyzed by gel electrophoresis in 1.8%
agarose in 1× TBE buffer, stained with
ethidium bromide, and photographed under
ultraviolet light.

Initially, 60 ISSR primers [University of
British Columbia, Vancouver, Canada (set
#9)] were tested with six Prunus genotypes
for PCR amplification. Based on assuming
the maximum number of reproducible and
distinctly scorable polymorphic bands, 20
ISSR primers were selected for the charac-
terization of 16 Prunus genotypes. The
annealing temperatures of ISSR primers
determined by Kafkas et al. (2006) were used,
and they are given in Table 1 with their
sequences.

Data analysis. The ability of the most
informative primer pairs to differentiate
between the genotypes was assessed by
calculating their resolving power (Rp)
according to Prevost and Wilkinson (1999)
using the formula Rp = ∑ Ib, where Ib = 1 –
(2x (0.5 – p)), and p is the proportion of
the 16 genotypes containing the 1 band. The
polymorphism information content (PIC)
of each marker was calculated using PIC = 1 –
∑ Pi² where Pi is the band frequency of the ith
allele (Smith et al., 1997). Jaccard’s similarity
coefficients (Sneath and Sokal, 1973)
were calculated for all pairwise comparisons
among the 16 Prunus genotypes.

Two dendrograms were generated using
NTSYSpc version 2.11V (Exeter Software,
Setauket, NY) (Rohlf, 2004): unweighted
pair group method of arithmetic average
cluster analysis (UPGMA) and principal
coordinate analyze (PCoA) based on the total
number of amplified ISSR fragments. In
PCoA, the genotypes were plotted on first
three dimensions using the G3D procedure
of the SAS program (SAS Institute Inc., 1990).
For the first dendrogram, the bootstrap values
were calculated with 1000 replicates using
PAUP software (Swofford, 1998). The
representativeness of the dendrogram was eval-
uated by estimating cophenetic correlation
for the dendrogram and comparing it with the
similarity matrix using Mantel’s matrix cor-
respondence test (Mantel, 1967). The result
of this test is a cophenetic correlation coef-
ficient, r, indicating how well the dendro-
gram represents similarity data.

Results and Discussion

Inter-simple sequence repeat polymor-
phism in Prunus. The results of ISSR finger-
printing of 16 Prunus genotypes using 20
primers are given in Table 2. From prescre-
ening assays with six Prunus primers using
60 ISSR primers, 20 ISSR markers generated
bright amplification products and polymor-
phisms and were used in further analysis.
A total of 196 reliable fragments was obtained
from 20 ISSR primers. The number of frag-
ments per primer ranged from 5 to 17 with the
average number of bands per primer being
9.8. Among the total bands, 180 fragments
were polymorphic with the average of 89%
polymorphism. The average number of poly-
meric bands per primer was 9.0 (Table 2).
According to Cao et al. (2000), 50 poly-
meric bands (loci) are sufficient for a
satisfactory classification and discrimination.
Some polymorphic bands produced by ISSR
primers seemed to be unique. If these bands
are tested in an adequate number of Prunus
genotypes in the future, the patterns can be
used to distinguish different subgenera, sec-
tions, and also cultivars or genotypes within
the sections in Prunus genus. Previously,
using 27 ISSR primers for cultivar identifi-
cations in Prunophora section, 72 polymor-
phic fragments were obtained (Lisek et al.,
2007) indicating that genetic diversity of
Prunophora genotypes is high and confirm-
ing the suitability of ISSR for the diversifi-
cation of Prunophora genotypes. It was also
previously shown that ISSR markers have
great potential to identify and establish phe-
netic relationships among plum cultivars
(Goulao et al., 2001).

Genetic relationships within and among
sections and subgenera. A dendrogram was
obtained by UPGMA method using the total
number of amplified ISSR fragments and
consisted of two main well-supported distinct
clusters corresponding to the two subgenera
Cerasus (Cluster I) and Prunus (Cluster II;
Fig. 1). The cv. Dagerigi belongs to subgenus
Cerasus formed alone like an outgroup into
Cluster I. The Cluster II was divided into
three subgroups (Prunophora, Armeniaca,
and plumcot). Within Cluster II, there was
evidence for differentiation within and
among sections or subgroups. In addition,
several significant groups with different
sections, particularly in Prunophora, are related
to the ploidy level and geographic origin of
the genotypes (Fig. 1). In the dendrogram,
Prunophora included diploid and hexaploid
plum genotypes and Armeniaca included only
diploid apricot genotypes (Fig. 1). Subgroup

Table 1. Cultivars/genotypes of Prunus assayed with intersimple sequence repeat markers in the present study.

| No. | Genotype name | Subgenus | Section | Species          |
|-----|---------------|----------|---------|-----------------|
| 1   | Stanley       | Prunus   | Prunophora | P. domestica  |
| 2   | Giant         | Prunus   | Prunophora | P. domestica  |
| 3   | Canerigi      | Prunus   | Prunophora | P. cerasifera  |
| 4   | Papaz         | Prunus   | Prunophora | P. domestica  |
| 5   | Burnmosa      | Prunus   | Prunophora | P. salicina   |
| 6   | Methley       | Prunus   | Prunophora | P. domestica  |
| 7   | Sakit 2       | Prunus   | Armeniaca | A. vulgaris    |
| 8   | Aprikoz       | Prunus   | Armeniaca | A. vulgaris    |
| 9   | Catalogou     | Prunus   | Armeniaca | A. vulgaris    |
| 10  | Hachihiholos  | Prunus   | Armeniaca | A. vulgaris    |
| 11  | Kabassi       | Prunus   | Armeniaca | A. vulgaris    |
| 12  | Zerdalino1    | Prunus   | Armeniaca | A. vulgaris    |
| 13  | Ordubat       | Prunus   | Armeniaca | A. vulgaris    |
| 14  | Inceazarigri  | Prunus   | —        | Plumcot       |
| 15  | Kayisazari    | Prunus   | —        | Plumcot       |
| 16  | Dagerigi      | Prunus   | Cerasus  | C. prostrata   |
Prunophora comprises three main plum species, namely diploid cherry plums (*Prunus cerasifera* cvs. Papaz and Canerigi), Japanese plums (*Prunus salicina* cvs. Burmosa and Methley), and hexaploid European plums (*Prunus domestica* cvs. Stanley and Giant). Interestingly, Cherry plum, Japanese plum, and European plum genotypes formed distinct single subclusters (Fig. 1). This could be resulting of different ploidy levels and origin of species. As well known, *Prunus cerasifera* and *Prunus salicina* had 2x and *Prunus domestica* 6x ploidy level. Despite some genomic similarities among diploid and hexaploid plum species, breeding barriers do exist among them. However, there are reports of successful introduction of genes from another wild diploid species into the Japanese plum, *P. salicina*, through interspecific hybridization and selection (Okie and Weinberger, 1996). Subgroup *Armeniaca* was represented by six cultivars (Sakit 2, Aprikoz, Cataloglu, Hacihaliloglu, Kabaasi, and Ordubat) and one wild form (Zerdalin1) of apricot. The results obtained in this work suggest that apricot genotypes probably share a common genetic background and show a low degree of polymorphism. The idea is supported by Hormaza (2002) who conducted SSR analysis in a wide range of apricot germplasm. There were interesting relationships among cultivars and wild form in the dendrogram related to apricot. The low chilling request table apricot cultivars, Sakit and Aprikoz, were found to be closer to each other than the other cultivars and wild form. The dried apricot cvs. Cataloglu, Hacihaliloglu, and Kabaasi were also found very close to each other. The white-flesh local apricot cultivar Ordubat had low fruit quality called wild form was to be close to wild apricot, Zerdalin1 (Fig. 1). As regarding plumcot, two genotypes (cv. Inceaz erigi and cv. Kayisi erigi) formed a separate group within the section *Prunophora*. In other words, the plumcot genotypes

Table 2. Sequence of intersimple sequence repeat (ISSR) primers, annealing temperatures, number of total and polymorphic bands, percentage of polymorphism, polymorphism information content, and resolving power in the DNA fingerprinting of 16 genotypes from *Prunus* genus sampled from Turkey.

| ISSR primers | Sequence (5’-3’) | Annealing temp. (°C) | Total bands (no.) | Polymorphic bands (no.) | Polymorphism (%) | Resolving power | Polymorphism information content |
|--------------|-----------------|---------------------|-------------------|------------------------|-----------------|----------------|----------------------------------|
| BC807        | (AG)8T          | 50                  | 8                 | 7                      | 88              | 1.089          | 0.674                             |
| BC812        | (GA)8A          | 50                  | 12                | 11                     | 92              | 0.670          | 0.853                             |
| BC814        | (CT)8A          | 50                  | 14                | 13                     | 93              | 0.760          | 0.817                             |
| BC815        | (CT)8G          | 52                  | 13                | 13                     | 100             | 0.875          | 0.703                             |
| BC817        | (CA)8A          | 50                  | 11                | 11                     | 100             | 0.841          | 0.754                             |
| BC818        | (CA)8G          | 52                  | 8                 | 8                      | 100             | 0.641          | 0.864                             |
| BC825        | (AC)8T          | 50                  | 15                | 15                     | 100             | 0.833          | 0.751                             |
| BC827        | (AC)8G          | 52                  | 13                | 13                     | 100             | 0.625          | 0.871                             |
| BC829        | (TG)8C          | 52                  | 6                 | 5                      | 83              | 0.854          | 0.525                             |
| BC835        | (AG)8YC         | 54                  | 10                | 8                      | 80              | 0.438          | 0.926                             |
| BC840        | (GA)8YT         | 52                  | 8                 | 7                      | 88              | 0.964          | 0.695                             |
| BC841        | (GA)8YC         | 54                  | 9                 | 8                      | 89              | 0.828          | 0.742                             |
| BC843        | (CT)8RA         | 52                  | 6                 | 6                      | 100             | 0.792          | 0.708                             |
| BC847        | (CA)8RC         | 52                  | 7                 | 4                      | 57              | 0.406          | 0.938                             |
| BC868        | (GAA)8k         | 48                  | 9                 | 9                      | 100             | 0.847          | 0.733                             |
| BC873        | (GACA)4         | 48                  | 17                | 17                     | 100             | 0.721          | 0.838                             |
| BC876        | (GATA)2 (GACA)2 | 48                 | 13                | 13                     | 100             | 0.567          | 0.879                             |
| BC888        | BDB(CA)2        | 51                  | 7                 | 5                      | 71              | 1.000          | 0.716                             |
| BC890        | VHV(GT)2        | 51                  | 5                 | 3                      | 60              | 1.542          | 0.350                             |
| BC891        | VHV(TG)2        | 51                  | 5                 | 4                      | 80              | 1.281          | 0.501                             |
| Total        |                 |                     | 196               | 180                    |                 | 89             | 0.829                             | 0.742                             |

Mean: 9.8 9.0 89
occupied the basal sister position to plum species within *Prunophora*. Previously, members of plum × apricot, based on their morphological characteristics, are considered to be closer to plums than to apricots in terms of leaf, seed, external color, flesh, and taste characteristics (Guleryuz and Ercisli, 1995). This suggests that the crosses could be resulting of open pollination of apricot with plum than backcrosses with plum of these hybrids. Mehlenbacher et al. (1990) reported that the cross is generally more successful when plum is used as the female parent and are useful sources of genes for late bloom. This could be explained by possible repeated backcrossing plum–apricot hybrids with plums. However, Liu et al. (2007) reported that hybrids of plum and apricot were more similar to apricot than the plum. The difference between the two studies could be explained by the used multiple male parents, which made their genetic background rather complex.

The pattern of differentiation among the genotypes within *Prunus* suggests four gene pools corresponding to the subgenera *Prunus* and *Cerasus* and also sections *Prunophora* and *Armeniaca* in *Prunus* subgenus, within which gene flow can potentially occur as interspecific hybrids within the same ploidy level are viable with the same levels of fertility.

Genetic similarities between genotypes were estimated using the Jaccard coefficient, and the similarity coefficient matrix was established in Table 3. The average Jaccard coefficient within and between sections and subgenera indicated that similarities within sections were higher than those between subgenera. The genetic variability was lower within *Armeniaca* genotypes than within *Prunophora*. The mean genetic similarity coefficient was 0.47, indicating that genetic diversity among *Prunus* genotypes is high. The similarity values varied from 0.27 (Aprikoz-Zerdalino1) to 0.93 (Kabaasi-Dagerigi) (Table 3). The cophenetic correlation coefficient by Mantel test indicated a high correlation, $r = 0.96$, between the similarity matrix and the UPGMA dendrogram. The cophenetic correlation coefficient is considered to be a very good representation of the data matrix in the dendrogram if it is 0.90 or greater (Romesburg, 1990)

Associations among subgenera and sections were also revealed by PCoA (Fig. 2). In the three-dimensional PCoA plot, in general, similar groupings with the UPGMA dendrogram and additional information were also revealed (e.g., the plumcots were placed between apricots and plums that reflect their phylogenetic relationships). The first three principal axes accounted for 30%, 11%, and 10% of the total variation, respectively, indicating the complex multidimensional nature of ISSR variation. The three-dimensional projection of genotypes along the first three principal axes revealed the overall genetic relationships among the subgenera and sections (Fig. 2). The two subgenera, *Prunus* and *Cerasus*, produced tight clusters and exhibited considerable divergence. The sections of *Armeniaca* and *Prunophora* and plum × apricot crosses also exhibited considerable divergence. Surprisingly, the first principal axis, which accounted for most variation (30%), contributed the least to the separation of *Prunophora*. The factor loadings along the second axis (11%) contributed to the separation *Armeniaca* from the remaining section. The third axis accounting for

![Fig. 2. Three-dimensional projection of intersimple sequence repeat variation calculated by principal coordinate analysis for 16 genotypes from *Prunus* genus.](image)
only 10% of the total variation was heavily loaded to discriminate the subgenera and sections *Cerasus*, *Prunus*, *Prunophora*, and *Armeniaca*. *Cerasus* and *Prunus* appeared to be the most divergent among the subgenera within the genus. According to Watkins (1976), members of the subgenus *Cerasus* were considered to be ancient and were the first to diverge from the ancestral *Prunus*. The two multivariate approaches, UPGMA and PCoA, used in the analysis of genetic relationships within and among the sections and subgenera of *Prunus* produced generally comparable results. Nevertheless, PCoA is known to be less sensitive to distances between close neighbors but represents more accurately distances sensitive to distances between close neighbors. In conclusion, genotypes showed considerable differentiation along the sectional and subgeneric boundaries and allowed for some generalization on the genetic structure and differentiation within the genus *Prunus* by using the ISSRs. Evaluation of existing germplasm collections contributes tremendously to the understanding of overall patterns of distribution of genetic variation and allows for drawing some general conclusions. These results obtained by the ISSR analysis of *Prunus* genotypes may provide useful information for molecular identification, pedigree analysis, genetic improvement, germplasm conservation, and construction of core collections in *Prunus*.

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