Genetic Analysis for Relationships between some Native Sour Cherry (*Prunus cerasus* L.) Using RAPD Markers

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Abstract

Prunus cerasus L. are an allotetraploid species from temperate climatic zone. The aim of this study was to characterize some native sour cherry genotypes from different orchards from Romania, based on RAPD markers. We used 14 primers RAPD to analyze a total of 14 varieties of sour cherries. After analysis, a good level of polymorphism was generated about only six primers (OPA 04, OPB10, OPA17, OPA20, OPA03 and OPB17) the rest of primers were considered monomorphic. The dendrogram separated the cultivars according to their genetic and geographic origin. The genetic similarity among the cultivars showed a good diversity between the genotypes, so we can suggest that our native cultivars of sour cherry can be considered as distinct genotypes for futures breeding programs and new cultivar identification. Results also confirm that the RAPD primers can be used for genetic studies and to evaluate the varieties for breeding programs.

Keywords: breeding, conservation, markers, RAPD, sour cherry

Introduction

Sour cherries (*Prunus cerasus* L.) belong to the *Rosaceae* family, *Prunoideae* subfamily, and *Prunus* genus, is an allotetraploid species, $2n=4x=32$, which are commonly grown in the temperate climatic zone (Anna Lisek and Elżbieta Rozpara, 2009), originated from the area that comprises Asia Minor, Iran, Iraq, and Syria (Vavilov, 1951) resulting from a natural hybridization between *Prunus avium* L. and *Prunus fruticosa* Pall. (Lezzoni et al., 1991). In our country, sour cherry reaches the northern limits (up to 60° Latitude).

In Romania the name for sour cherries is "vișina" similar with Russian name "vishnya" and also with Albanian name "vyssine", but different from Hungarian name, which is called meggy (Faust and Surányi, 1997). De Candolle (1886) mentions "so called Gypsy cherries are native in the Carpathian Basin" and also, he estimates the use of the name "vyssine" before 600 B.C.

The cultivated sour cherries "were selected from wild specimens of *Prunus cerasus* and

suspiciously distinct *Prunus acida* from around the Black and Caspian Seas". The sour cherry type is self-fertile but better crops are cross pollinated and in general, sour cherry is a good pollinizer for other varieties (Imtiyaz et al., 2017).

Sour cherries are particularly important tree species for fruit crops. The world production accounts for about 40% of sweet cherry and sour cherries together. Sour cherries are a rustic fruit species, for this reason it is met between 40° and 60° northern latitudes and grow on all continents as isolated trees in orchards of different types. Fruits are used mainly for industrialization, but also for fresh consumption, as they have complex, balanced content and rich in nutrients, mineral salts and vitamins.

Different varieties of *Prunus cerasus* (wild, traditional cultivars or domesticated species) can "paving the way for creation of new genetic variability by employing different approaches such as exploration, passport data generation, introduction, conservation (*in situ*, *ex situ*, *in vitro*)

Table 1. Varieties of *Prunus cerasus* and their geographical and genetic origin used in study

Cod used	Varieties	Geographical origin	Genetic origin
V1	Țarina	RIFG Pitesti, Romania	English Early x local variety (Vișin tufa)
V2	Rival	RIFG Pitesti, Romania	Griot Moscovski x Nana
V3	Pitic de Iași	FRDS Iași, Romania	Seedling of Plodorodnaia Miciurina
V4	Nana	FRDS Iași, Romania	Seedling of Crisana
V5	Erdu Bôtermo	FRDS Iași, Romania	Ungaria (Pandy x Nagy Angol)
V6	Kelleris	FRDS Iași, Romania	Germania, Unknown
V7	Oblacinska	FRDS Iași, Romania	Local selection from South of Serbia
V8	Ilva	FRDS Iași, Romania	Local selection
V9	Timpurii de Osoi	FRDS Iași, Romania	Local selection
V10	De Botoșani	FRDS Iași, Romania	Local selection
V11	Breznița	FRDS Iași, Romania	Local selection
V12	Engleze timpurii	FRDS Iași, Romania	Local selection
V13	Timpurii de Cluj	FRDS Cluj, Romania	Hybrid (Spaniole x <i>Pfruticosa</i>) x (English Early x <i>Pfruticosa</i>)
V14	Mocănești	FRDS Cluj, Romania	Unknown

RIFG: Research Institute for Fruit Growing; FRDS: Fruit Research & Development Station

in gene banks as well as breeding techniques namely: selection, hybridization, mutation and by various biotechnological tools" (Biswajit *et al.*, 2009).

Fruit Research Institutes from Romania has a long tradition of studying and conservation the autochthonous genotypes of fruits species from *Prunus* genus, so this species are very important source of genetic variability and valuable material for breeding work. But all this fruit varieties are only morphological and biochemical evaluated, and as is know this characteristics depending by environmental factors. Many results from literature based by DNA markers confirm the importance and utility of molecular analysis for cultivar identification and to evaluate the germplasm for more species. In most cases, data on genetic similarity obtained by RAPD analysis matched classifications based on morphological and agronomic traits (Casas, *et al.*, 1999).

The techniques based on molecular markers, in this case RAPD, can be used for these purposes (Sandalli *et al.*, 2005). Moreno and Trujillo (2005), used RAPD markers to discriminate cherry cultivars from the Cherry Germplasm Bank of Junta de Extremadura and to study genetic relationships among them, also to study the genetic variation among different species, and within species and to characterize *Prunus* rootstocks from different species (Casas, *et al.*, 1999), to study the patterns of random amplified polymorphic DNA (RAPD) variation in six natural populations of Ethiopian

Prunus Africana (Hailu, 2016). The results obtained by Loredana Mariniello *et al.*, (2002) using RAPD and SCAR markers can suggest a protocol to fingerprint DNA of apricot as an efficient way to control product quality and prevent fraud.

In this study we used the RAPD markers to analyze the relationships between some varieties of sour cherries from different Fruit Research Institutes from Romania. We demonstrate here that RAPD markers are useful to fingerprint and differentiate the varieties from all orchards. These results will be helpful for *Prunus* genetic resource management and conservation.

Material and Methods

Fourteen varieties of *Prunus cerasus* were analyzed in this study belonging to three different germplasm collections from different Romanian geographical area. All varieties and their geographical and genetic origin are synthesized in the next table (Tab. 1).

Genomic DNA was isolated from young leaves collected, using a CTAB (cetyltrimethylethyl ammonium bromide) extraction method (Lodhi *et al.*, 1994, improved by Pop *et al.*, 2003). DNA concentration was determined spectrophotometrically at 260 nm, with NanoDrop ND 1000. Before to continue further analysis, the DNA was diluted to the concentration of 50 ng/μl in nuclease-free water (Promega).

Fourteen random primers by Operon Technologies Inc., USA, were arbitrary chosen for

screening of all sour cherry varieties. The RAPD primers used in this study and their sequences are shown below (Tab. 2).

For optimization of PCR conditions and for initial screening were used only four varieties of sour cherry and only six primers was selected based on polymorphic bands.

DNA was amplified in 15 µl/tub reaction mixtures containing 1 µl of template DNA, 2xPCR master-mix (ready to use, from Bioline), 0,2 µM/primer and nuclease-free water (Promega). The DNA amplification was performed for 45 cycles in a thermocycler (Techne TC4000) with the following profiles: initial denaturation step of 94°C for 3 min. follow by 1 min at 93°C, 1 min. at 34°C and 1 min. at 72°C. At the end of the temperature profile a further polymerization step was carried out (72°C for 10 min).

For electrophoresis were prepared 1% agarose gels and run in 1 x TAE buffer, pH 8 (40 mM Tris,

20 mM acetic acid, 1 mM EDTA) for 1,30 hours at constant voltage of 80V, stained with ethidium bromide, 5 µl/100 ml/gel. A 100 base pair ladder (100 bp DNA Step Ladder, Fermentas) was loaded on marginal lanes to estimate the amplification band size.

The gels was visualized under UV light (UVP BioImaging Systems). The electrophoresis patterns were analyzed using TotalLab 120. For data matrix, bands were scored as present (1) or absent (0). The level of polymorphism of the primer (number of polymorphic band per total bands) and the number of banding patterns, were calculated. Genetic distance between varieties was calculated using Jaccard (1908) coefficient of similarity. Cluster analysis was obtained using the PAST software and a dendrogram was generated based on genetic distance.

Results and Discussions

The DNA concentration ranged between 281.76 - 919.31 ng/µl, with purity between 2.09-2.14 nm. Good RAPD profiles were obtained with all the samples.

A total of 14 primers RAPD were tested, but only six primers amplified across all the varieties from study because the rest of them were monomorphic and had been excluded from analysis. The selection of the primers to be used in such studies is very important for the sensitivity for the determination of genetic variability (Sandalli *et al.*, 2005).

All selected primers with the number of polymorphic band, the amplified fragment size and the percentage of polymorphism are presented in Tab. 3.

In total it was amplified 388 reproducible fragments (11.8 polymorphic markers per primer).

Table 2. The RAPD primers selected for PCR amplification

Crt. no.	Primer name	Primer DNA sequences (5'-3')
1	OPA 01	CAGGCCCTTC
2	OPA 03	AGTCAGCCAC
3	OPA 04	AATCGGGCTG
4	OPA 17	GACCGCTTGT
5	OPA 18	AGGTGACCGT
6	OPA 20	GTTGCGATCC
7	OPB 08	GTCCACACGG
8	OPB 09	TGGGGGACTC
9	OPB 10	CTGCTGGGAC
10	OPB 17	AGGGAACGAG
11	OPAB 11	GTGCGCAATG
12	OPX 03	TGGCGCAGTG
13	OPE 14	TGCGGCTGAG
14	AB 11	CAATCGCCGT

Table 3. Selected primers and their amplification fragment size, total number of amplification fragments, number of polymorphic and percentage of polymorphism among the 14 varieties of sour cherry studies.

Primer name	Fragment size (min-max) (pb)	Total number of amplification products	Total number of polymorphic bands	Percentage of polymorphism
OPA04	312-1214	77	9	100
OPA20	774-1246	33	11	100
OPA17	287-1375	94	16	94
OPB10	703-1300	47	5	94
OPA03	400-1193	59	12	100
OPB17	104-638	78	16	100

Figure 1. RAPD bands profile generated by OPA17 for different varieties of sour cherry included in this study (V1-V14, see tab.1), M – molecular marker (100 bp DNA Step Ladder, Fermentas)

Figure 2. RAPD bands profile generated by OPA17 for different varieties of sour cherry included in this study (V1-V14, see tab.1), M – molecular marker (100 bp DNA Step Ladder, Fermentas)

The number of polymorphic bands ranged from five (OPB10) to sixteen (OPA17 and OPB17). The percentage of polymorphism was 100% in almost patterns, exception made OPA17 and OPB10 which have one monomorphic band per profile. Amplified DNA fragment ranged in length from 104 pb (OPB17) to 1375 pb (OPA17).

Figure 1 represents RAPD profile obtained with OPA17 primer, which amplified 17 polymorphic bands one of them was monomorphic, so only 16 were considered for analysis. OPB10 (figure 2) amplified six polymorphic bands but only five were considered for analysis, because one of them was also monomorphic. The fragment size generated of this primer was between 703 and 1300 pb, almost like OPA20. Fragments close in size were amplified by OPA04 (312-1214 pb) and OPA03 (400-1193 pb). OPB17 generated the smallest fragments (104-638 pb), but with the most of amplification products (78).

Genetic relationships among populations were further examined by PAST clustering

analysis and represented by a dendrogram, using Jaccard coefficient. RAPD primers have been able to characterize all varieties from this study based on difference in their hereditary material. This characterization can provide valuable information about genetic diversity in the released varieties.

All fourteen analyzed varieties were divided into three groups keeping a certain extent their geographical origin. Among these samples, Jaccard similarity coefficient values ranged from 0.17 to 0.68 (Tab. 4.). The lowest genetic variability was exhibited between "Ilva" and "Timpuriu de Osoi" (0.68). While value 0.17 was between cultivars "Timpuriu de Cluj" and "Țarina" with a different geographical origin (FRDS Cluj and RIFG Pitești). The dendrogram clearly shows the varieties "Rival" and "Țarina" and also "Timpuriu de Cluj" and "Mocănești" are more closely based on their geographical origin.

Evidence of relationships for most of the cultivars according to their geographic origin was found (Figure 3). First group is constituted only by

Table 4. Jaccard similarity between all varieties of sour cherry included in this study

-	Tarina	Rival	Pitic_de_IS	Nana	Erdy_Boterma	Keleris	Oblacinska	Ilva	Timpuriu_de_Osoi	De_Botosani	Breznita	Timpurii_engl.	Timpuriu_de_Cj	Mocanesti
Tarina	-	0.29	0.36	0.38	0.38	0.35	0.36	0.28	0.30	0.30	0.32	0.29	0.17	0.18
Rival	0.29	-	0.50	0.41	0.37	0.30	0.31	0.53	0.34	0.39	0.37	0.37	0.26	0.20
Pitic_de_IS	0.36	0.50	-	0.59	0.64	0.50	0.45	0.44	0.42	0.46	0.44	0.55	0.31	0.29
Nana	0.38	0.41	0.59	-	0.50	0.60	0.51	0.43	0.41	0.41	0.50	0.54	0.30	0.32
Erdy_Boterma	0.38	0.37	0.64	0.50	-	0.39	0.40	0.33	0.34	0.29	0.36	0.43	0.33	0.22
Keleris	0.35	0.30	0.50	0.60	0.39	-	0.54	0.39	0.40	0.35	0.49	0.56	0.30	0.32
Oblacinska	0.36	0.31	0.45	0.51	0.40	0.54	-	0.44	0.50	0.36	0.44	0.55	0.34	0.32
Ilva	0.28	0.53	0.44	0.43	0.33	0.39	0.44	-	0.68	0.53	0.46	0.54	0.36	0.27
Timpuriu_de_Osoi	0.30	0.34	0.42	0.41	0.34	0.40	0.50	0.68	-	0.61	0.49	0.57	0.34	0.32
De_Botosani	0.30	0.39	0.46	0.41	0.29	0.35	0.36	0.53	0.61	-	0.61	0.49	0.22	0.33
Breznita	0.32	0.37	0.44	0.50	0.36	0.49	0.44	0.46	0.49	0.61	-	0.58	0.30	0.43
Timpurii_engl.	0.29	0.37	0.55	0.54	0.43	0.56	0.55	0.54	0.57	0.49	0.58	-	0.33	0.43
Timpuriu_de_Cj	0.17	0.26	0.31	0.30	0.33	0.30	0.34	0.36	0.34	0.22	0.30	0.33	-	0.35
Mocanesti	0.18	0.20	0.29	0.32	0.22	0.32	0.32	0.27	0.32	0.33	0.43	0.43	0.35	-

Figure 3. Dendrogram generated using PAST software, based on the genetic relationships between *Prunus cerasus* varieties and the similarity calculated using Jaccard coefficient

five varieties of sour cherry all are local selection from FRDS Iași: "Ilva", "Timpuriu de Osoi", "De Botoșani", "Breznița" and "Engleze timpurii". "Timpuriu de Cluj" appear to belong first group, because one of the genitor of this hybrid from FRDS Cluj is "Engleze timpurii" (see tab.1).

The second cluster includes four cultivars: two are from FRDS Iași collection "Pitic de Iași" and "Erdy Bötermo" and two are from RIFG Pitești.

The similarity coefficient shows a low genetic variability between these two varieties (0.64). But between "Rival" and "Tarina" which are both from RIFG Pitești the similarity coefficient it was higher (0.29).

The similarity coefficient between "Nana" and "Keleris" are more low (0.60) then "Nana" and "Oblacinska" (0.51). This explain how is arranged

the varieties from the third group. All this varieties of sour cherry are from FRDS Iași.

All varieties of sour cherry which was from different geographical collections have been identified using only six RAPD primers. Only seven RAPD primers were used to quantify levels of genetic diversity within six populations and genetic differentiation among populations of *Prunus africana* in Ethiopia (Hailu Atnafu, 2016) and to characterize *Prunus* rootstocks from different species from the breeding program at Aula Dei Experimental Station (Zaragoza, Spain) (Casas *et al.*, 1999).

Conclusions

A closer genetic similarity was found in all varieties analyzed which were from the same geographical origin showed by Jaccard's similarity coefficient. RAPD primers have the ability to identify and to group sour cherry cultivars according to their local origin and in one case according to their genetic origin. RAPD demonstrate again that are suitable for easy, quick and inexpensive identification sour cherry varieties.

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