Research Article

©*ISISnet Publishers*

Bioscience Research, 10(1): 01-07, 2013
Available online at **www.isisn.org**
Print ISSN: 1811-9506
Online ISSN: 2218-3973

Utilization of IRAP technique for plums genotypes differentiation.

S. Senková, J. Žiarovská*, M. Bežo, V. Štefúnová and K. Ražná.

Department of Genetics and Plant Breeding, Faculty of Agriculture and Food Resources, Slovak University of Agriculture in Nitra; Tr. A. Hlinku 2; 94976; **Slovak Republic**.

***Corresponding author**

As plums posses wide diversity range, findings of effective marker systems for their differentiation is still actual. IRAP markers derived from Cassandra retrotransposon were applied to distinguish local Slovak plums when compared them to european standart plum cultivars. Cass1primer created totally ten different levels and Cass2 created eighteen different levels of markers. Cass2 was also defined by the presence of 10 polymorphic levels and primer Cass1 created 4 polymorphic levels. Nei and Li coefficients of genetic similarity confirmed the highest genetic similarity within the group of plums collected in four Slovak locations and constructed dendrogram based on Nei and Li data showed, that the samples of plum (*P. domestica*, L.) genotypes collected from four Slovak locations of their natural occurrence (Pečovská Nová Ves, Lipany, Torysa and Podolínec) were assessed like genetically most similar by IRAP primers derived from retrotransposon Cassandra.

Key words: Prunus domestica, local Slovak plums, IRAP.

Plum (*Prunus domestica,* L.) is hexaploid (6n) organism with 48 chromosomes. Probably, plum developed from genus green plum (*Prunus cerasifera*, Ekh.) by an autopolyploidy. Usually green plum (*P. cerasifera*, Ekh.) occurs like a diploid organism with basic chromosomal count 16 and there is also known tetraploid and hexaploid form around Balkans and Caucasus region. Interestingly, green plum (*P. cerasifera*, Ekh.) hexaploid form and plum (*P. domestica*, L.) are very similar. (Zohary, 1992, Pandey, 2008). EURISCO database associates 20 national inventory reports in which contain 4995 plum (*Prunus domestica*, L.) accessions. (http://eurisco.ecpgr.org/).

Nowadays, molecular markers facilitate orientation in plum´s wide diversity range. RAPD, SSR and AFLP markers were used for duplicates identification and variability determination in genotypes collections. (Hend, 2009; Ayanoglu, 2007; Mnejja, 2004). Molecular markers derived from retrotransposon sequences became very favorable in molecular based analyses thanks to the retrotransposon properties. Retrotransposons are present in a high copy number, situated on the various chromosomal locations with the possibility to observe DNA polymorphism between species or inside of species. Retrotransposons are analysed for phylogenetic relationships studies, genetic variability research, or genetic mapping and genes analyses. (Kalendar et al. 1999, 2006, Zein et al., 2010). The most frequently used retrotransposon based molecular marker methods are IRAP (Inter-Retrotransposon Amplified Polymorphism) and REMAP (REtrotransposon-Microsatellite Amplified Polymorphism). Efficiency study of IRAP and REMAP molecular markers using retrotransposon primers derived from different genera was performed on 36 flax (*Linum usitatissimum*, L.) genotypes by Žiarovská et al. (2012). Two retrotransposon primers derived from 2 different sequences of *Tst1* and *Cassandra* retrotransposons. *Tst1* is 5060 bp long autonomous copia-like retrotransposon, firstly defined in potato genome. Cassandra is 615 bp long non-autonomous TRIM (Terminal-repeat Retrotransposons In Miniature) element,

initially discovered in plum (*P. domestica*, L.) genome. REMAP primers were designed by the combination of first IRAP primer came from *Cassandra* element and second ISSR prajmer. Percentage polymorphic fragments gained highest score in the case of REMAP marker (86.4 %), while IRAP markers reported values 76.9 % (IRAP marker derived from Tst1 sequence) and 68.7 % (IRAP marker derived from Cassandra sequence). Markers evaluation based on dendrogram results revealed that IRAP markers define wider genetic background and relationship between landraces and cultivars. There is possible to find cluster which reflects pedigree information in REMAP dendrogram.

The aim of the study was to compare IRAP profile of local Slovak plums to other from east and west Europe and to distinguish them when used retroransposon sequences as markers.

MATERIALS AND METHODS

Biological material: Nine plum (*P. domestica*, L.) genotypes were used in analysis. They can be divided into two main groups. European standard plum cultivars (Gabrovská, Chrudimská, Švestka domácí, Čačanská lepotice and Anna spätch) belong to the first group and all local Slovak plums to the second one. Their green leaf material was kindly provided as a technical isolate of research station: Výzkumný a šlechtitelský ústav ovocnářský Holovousy s.r.o., Czech Republic. The samples of four next plum (*P. domestica*, L.) genotypes were collected from four Slovak locations of their natural occurrence (Pečovská Nová Ves, Lipany, Torysa and Podolínec). A detailed characteristic is listed in table 1. Genomic DNA was isolated using the isolation kit Invisorb® Spin Plant Mini Kit (Invitek), following the manufacturer´s manual. The obtained DNA was quantified by fluorometer (Qubit TM). IRAP technique was used for monitoring DNA polymorphism of nine plum genotypes.

IRAP Primers design: Cass1 primer was derived from 3´end of LTR (Long Terminal Repeat) plum retrotransposon *Cassandra* (NCBI accession number: AY860314). Primer orientation and primer sequence were identical with 5´- 3´ direction of 3´LTR sequence. Cass2 primer was designed based on 5´end of LTR plum retrotransposon *Cassandra.* IRAP2 was identical with 5´- 3´ direction of 5´LTR sequence. According retrotransposon properties, each primer anneal two times. Detailed primer characteristics are mentioned in table 2.

PCR conditions - PCR products were amplified in optimized conditions for each primer and for each parameter. Amplification was performed in BIOTAQ PCR kit (BIOLINE) within the conditions listed in table 3.

Settings of thermal cycler: PCR products amplification time and temperature profile of Cass1 was following: Initial denaturation step took 1 minute at 94 °C; thirty-two cycles of denaturation, primer annealing and DNA elongation steps were performed in these conditions, denaturation took 1 minute at 94 °C, primer annealing took 1 minute at 54 °C and DNA elongation took 3 minutes at 72 °C. Final DNA synthesis took 10 minutes at 72 °C. While PCR finished reactions were cooled 5 minutes at 4 °C. PCR products amplification time and temperature profile of Cass2 was following: Initial denaturation step took 3 minutes at 95 °C; thirty-two cycles of denaturation, primer annealing and DNA elongation steps were performed in these conditions, denaturation took 40 seconds at 95 °C, primer annealing took 40 seconds at 61 °C and DNA elongation took 2 minutes at 72 °C. Final DNA synthesis took 5 minutes at 72 °C. While PCR finished reactions were cooled 5 minutes at 4 °C.

PCR products electrophoretic separation and data analysis: Electrophoretic separation was performed in 1.7% agarose gel (Applichem) and 1 × TBE solution. Samples were colored by Gel Red 10 000 × (Biotium) chemistry. Electrophoresis took 4 hours at constant voltage (60V) and variable current. Electrophoreograms were obtained by Electrophoresis Documentation and Analysis KODAK EDAS 290 system. Individual fragment size was evaluated by comparing to 250 bp DNA Ladder (Invitrogen). Electrophoreogram evaluation was realized visually in MS Excel program. DNA bands were appointed like present (1) or absent (0). These observations were converted into a binary matrix. Nei, Li (1979) similarity indexes were calculated according SI_{NL} = 2× common bands count in A and B lane / (bands count in lane A + bands count in lane B).

Table 1. A detailed characteristic of tested plum (*Prunus domestica*, L.) genotypes.

Genotype	Type	Geographical origin	Pedigree
Gabrovská	cultivar	Bulgaria	Kjustendilska × Montfortska
Chrudimská	cultivar	Czech Republic	Random seedling
Švestka domácí	cultivar	Czech Republic	Random seedling
Čačanská lepotice	cultivar	Former Yugoslavia	Wahgheimova × Požegača
Anna spätch	cultivar	Hungary	Random seedling
Plum P.N.V.	unknown	Slovakia – Pečovská N. Ves	unknown
Plum T.	unknown	Slovakia – Torysa	unknown
Plum P.	unknown	Slovakia – Podolínec	unknown
Plum L.	unknown	Slovakia – Lipany	unknown

Table 2. IRAP primers characteristics

Primer	Sequence	Melting temperature	Annealing temperature	Primer length
Cass1	ACGGCGGAGCCGATCCCGGGATGTGACA	68,7 °C	54 °C	28 nt
Cass2	TCTCCGTTGGTCGATGTGGGATGTTACA	61,4 °C	61 °C	28 nt

Table 3. PCR concentrations

PCR reagents / IRAP primer	Cass1	Cass2
dNTP (mM)	0,3	0,3
$MgCl_2$ (mM)	3	3,3
primer (mM)	0,75	0,4
DNA template (ng per reaction)	20	20
Buffer	10×	10×
BIOTAQ polymerase (U)	1	1

RESULTS AND DISCUSSION

The sequence of plum Cassandra retrotransposon is considered characteristic for plum (*Prunus domestica*, L.) species. Both of these primers Cass1 and Cass2 were derived from this same retrotransposon and had very similar design (primer orientation and length). Nevertheless, there was observed significant difference between Cass1 and Cass2 primer efficiency and primer usage already in the case of nine genotypes collection. Antonius-Klemola et al. (2006) came to similar results, too. They designed 4 IRAP markers derived from the same LTR sequence of TRIM element Cassandra in apple genome. Primers K004, K005, K008 and K009 had the same orientation and originated from the same LTR, they were diverse in primer length 21 – 26 bp. By electrophoreogram´s comparison authors found out that primer K004 created six levels, K005 created eight levels, K008 created thirteen levels and K009 created fifteen levels. Accordingly, the differences were observed in the polymorphic levels count, where K004 was characterized by presence of two polymorphic levels, K005 was characterized by presence of five polymorphic levels, K008 was characterized by presence of eight polymorphic levels and K009 was characterized by presence of ten polymorphic levels. Cass1 created totally ten levels and Cass2 created eighteen levels. Cass2 was also defined by the presence of 10 polymorphic levels as primer K009. Primer Cass1 created 4 polymorphic levels as it is shown in the table 4. Interestingly, Cass2 primer was able to create two unique present bands and also two unique absent bands. This event was not observed in electrophoreogram gained after electrophoresis of Cass1 PCR products.

Cass1 primer under the name, P-Frodo2-02, was used for genetic similarity evaluation in the Žiarovská et al. (2012) study about interspecific primer transferability. From plum Cassandra retrotransposon derived IRAP primer was used for genetic

Table 4. Cass1 and Cass2 Electrophoreograms evaluation – levels calculation

Primer	Total level count	Polymorphic level count	Count of level with unique present band	Count of level with unique absent band
Cass1	10	4	0	0
Cass2	18	10	2	2

polymorphism testing among 36 flax (*Linum usitatissimum*, L.) genotypes. P-Frodo2-02 created totally sixteen levels and eleven were polymorphic, in 36 genotypes flax collection. Percentage polymorphic fragments were 68.7% in the case of 36-member flax collection, in comparison to 9 plum collection, where they gained 23%. (Possible to see in the table 4) Significant difference in the same primer evaluation was caused by the incomparable size of tested sets. Primer efficacy comparison between P-Frodo2-02 and P-Tst1-01 revealed greater production of polymorphic fragments 76.9 % in the case of primer derived from autonomous Tst-1 retrotransposon. Although, IRAP markers are more frequently used for their simplicity (1 IRAP primer derived from LTR is sufficient for PCR) as REMAP markers, there are REMAP marker´s analyses, which are able to provide higher level of polymorphism. (Antonius-Klemola et al. 2006, Žiarovská et al. 2009, Branco et al. 2007).

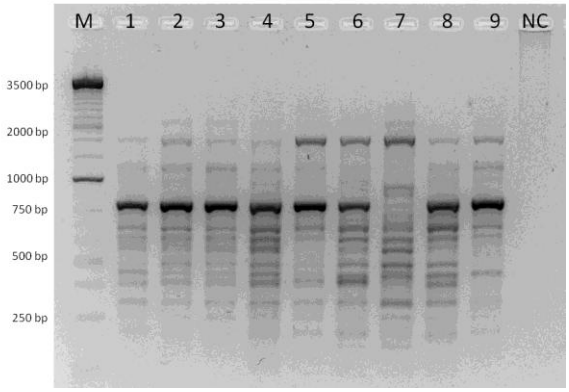

Figure 1. Electrophoreogram of Cass2 PCR products gained after electrophoretic separation. Electrophoreogram description: M... 250 bp DNA Ladder, (Invitrogen). Separated Cass2 PCR products of genotype: 1 ... Plum P.N.V. (location of collection: Pečovská Nová Ves), 2... Plum T (location of collection: Torysa), 3... Plum P (location of collection: Podolínec), 4... Plum L (location of collection: Lipany), 5... cultivar Gabrovská, 6... cultivar Chrudimská, 7... cultivar Švestka domácí, 8... cultivar Čačanská lepotice, 9... cultivar Anna spätch.

Further evaluation of primers was carried out on their own assessment of genetic relatedness among the plum genotypes, using the Nei, Li coefficients of genetic similarity. Different values acquired studied genotypes, for primer Cass1 (0.75 to 1.00) and for Cass2 primer 0.32 to 0.643, as shown in Table 6 and 7. Implying that, Cass1 primer evaluated the entire set of genotypes genetically more similar to each other as primer Cass2. Cass1 and Cass2 Nei, Li coefficients of genetic similarity confirmed the highest genetic similarity within the group of plums collected from 4 Slovak locations (plum PNV, plum T, plum P and plum L). Mentioned genotypes (G1, G2, G3 and G4, compare with Table no. 6) reached the coefficients from 0.941 to 1.00. According this evaluation as completely identical genotypes were labeled G1 and G4, G2 and G3. Nei, Li coefficients in the case of Cass1 marker evaluation also marked cultivars Čačanska lepotica (G8) and Anna spätch (G9) like identical. Analysis of Cass2 marker also showed the highest values of Nei, Li coefficients inside of G1, G2, G3 and G4 group. In contrary with analysis of IRAP1 marker, there was not found any difference between G1, G2, G3 and G4 based on Nei, Li coefficients according Cass2 assessment. (Table 7) Genotypes G5 (cultivar Gabrovská) and G2 (plum T) or G3 (plum P) were assessed as at least genetically similar based on Nei, Li coefficients of genetic similarity evaluation of Cass1 marker. Nei, Li genetic similarity analysis marked as at least similar genotypes G7 (švestka domácí) and G5 (Gabrovská), also genotypes G9 (Anna spätch) and G7 (švestka domácí).

Constructed dendrogram based on Nei and Li data showed, that the samples of plum (*P. domestica*, L.) genotypes collected from four Slovak locations of their natural occurrence (Pečovská Nová Ves, Lipany, Torysa and Podolínec) were assessed like genetically most similar by testing both IRAP primers (figure 2). IRAP primers derived from LTR sequences of plum retrotransposon Cassandra seems to be suitable for genetic

Table 5. Structure of produced fragments.

Primer	Total bands count	Total polymorphic bands count	Total monomorphic bands count	Percentage of polymorphic bands
Cass1	70	16	54	23 %
Cass2	122	50	72	41%

Table 6. Nei and Li coefficients of similarity based on Cass1 primer.

	G1	G2	G3	G4	G5	G6	G7	G8	G9
G1	1,000	0,941	0,941	1,000	0,800	0,800	0,857	0,875	0,875
G2		1,000	1,000	0,941	0,750	0,750	0,800	0,824	0,824
G3			1,000	0,941	0,750	0,750	0,800	0,824	0,824
G4				1,000	0,800	0,800	0,857	0,875	0,875
G5					1,000	1,000	0,923	0,933	0,933
G6						1,000	0,923	0,933	0,933
G7							1,000	0,857	0,857
G8								1,000	1,000
G9									1,000

Table description: G1 ... Plum P.N.V. (location of collection: Pečovská Nová Ves), G2... Plum T (location of collection: Torysa), G3... Plum P (location of collection: Podolínec), G4... Plum L (location of collection: Lipany), G5... cultivar Gabrovská, G6... cultivar Chrudimská, G7... cultivar Švestka domácí, G8... cultivar Čačanská lepotice, G9... cultivar Anna spätch.

Table 7. Nei and Li coefficients of similarity based on Cass2 primer.

	G1	G2	G3	G4	G5	G6	G7	G8	G9
G1	1,000	0,643	0,643	0,643	0,480	0,600	0,429	0,571	0,560
G2		1,000	0,643	0,643	0,480	0,600	0,429	0,571	0,560
G3			1,000	0,643	0,480	0,600	0,429	0,571	0,560
G4				1,000	0,480	0,600	0,429	0,571	0,560
G5					1,000	0,444	0,320	0,480	0,545
G6						1,000	0,467	0,600	0,519
G7							1,000	0,429	0,320
G8								1,000	0,560
G9									1,000

Table description: G1 ... Plum P.N.V. (location of collection: Pečovská Nová Ves), G2... Plum T (location of collection: Torysa), G3... Plum P (location of collection: Podolínec), G4... Plum L (location of collection: Lipany), G5... cultivar Gabrovská, G6... cultivar Chrudimská, G7... cultivar Švestka domácí, G8... cultivar Čačanská lepotice, G9... cultivar Anna spätch.

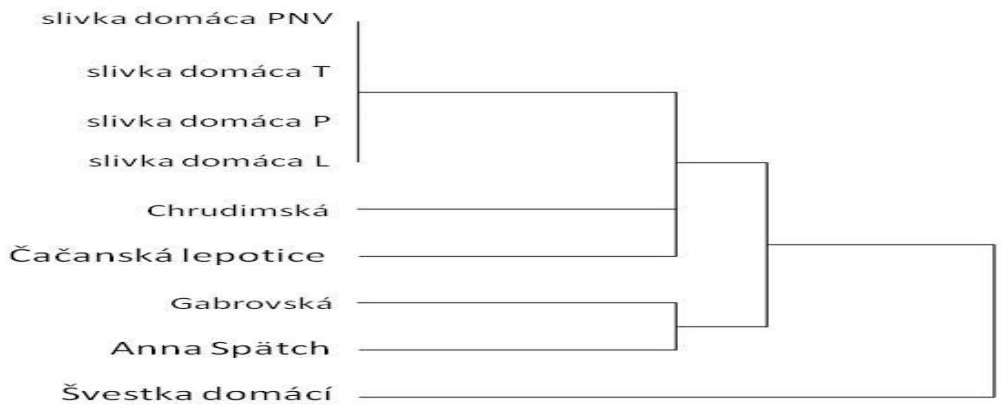

Figure 2. Dendrogram of tested plum genotypes based on IRAP markers.

diversity evaluation among plum (*P. domestica*, L.) genotypes collection.

Conclusions

Analyses of IRAP based polymorphism is a very efficient tool for describing of differences among plums. A total of 28 fragment levels among nine *P. domestica* accessions were scored with only two Cassandra IRAP primers and the number of polymorphic levels was 14. Genotypes that are showed as most related within a dendrogram displays different Cassandra insertion pattern and all of them are local Slovak plums.

Acknowledgement: This work was supported by the project KEGA 001SPU/4-2012 and scientific instruments were obtained thanks to ECOVA and ECOVA+ projects.

REFERENCES

Antonius-Klemola K, Kalendar R, Schulman AH, 2006. TRIM retrotransposons occur in apple and are polymorphic between varieties but not spots. Theoretical and Applied Genetics, vol. 112, 2006, pp. 999–1008.

Branco CJS, Vieira EA, Malone G, Kopp MM, Malone E, Bernardes A, Mistura CC, Carvalho FIF, Oliveira CA, 2007. IRAP and REMAP assessments of genetic similarity in rice. Journal of Applied Genetics, vol. 48, 2007, no. 2, pp. 107–113.

Kalendar R, Schulman AH, 2006. IRAP and REMAP for retrotransposon-based genotyping and fingerprinting. Nature Protocols, vol. 1, 2006, no.5, pp. 2478-2484.

Kalendar R, Grob T, Regina M, Suoniemi A, Schulman A. 1998. IRAP and REMAP: two new retrotransposon-based DNA fingerprinting techniques. Theoretical and Applied Genetics, vol. 98, 1998, no. 5, pp. 704-711.

Mnejja M, Garcia-Mas J, Howad W, Badenes ML, Arús P, 2004. Simple-sequence repeat (SSR) markers of Japanese plum (*Prunus salicina*, Lindl.) are highly polymorphic and transferable to peach and almond. *Molecular Ecology Notes*, vol. 4, 2004, p. 163 – 166.

Nei M, LI WH, 1979. Mathematical model for studying genetic variation in terms of restriction andonucleases. Proceedings of the National Academy of Sciences, vol. 76, 1979, pp. 5296-5273.

Pandey A, Roshini Nayar E, Venkateswaran K, Bhandari D C, 2008. Genetic resources of Prunus (*Rosaceae*) in India. Genetic Resources and Crop Evolution, 2008, no. 55, p. 91–104.

Search results for „Prunus domestica“ In *National Inventory Reports* [online]. 2012. [cit. 2012-02-13]. Available at: <http://eurisco.ecpgr.org/>.

Zein I, Jawhar M, Arabi MIE, 2010. Efficiency of IRAP and ITS-RFLP marker systems in accessing genetic variation of *Pyrenophora graminea*. Genetics and Molecular Biology, vol. 33, 2010, no. 2, pp. 328-332.

Zohary D, 1992. Is the European plum,

Prunus domestica L., a *P. cerasifera* EHRH. × *P. spinosa L*. allo-polyploid? Euphytica, vol. 60, 1992, no. 7, p. 75-77.

Žiarovská J, Ražná K, Senková S, Štefúnová V, Bežo M, 2012. Variability of Linum usitatissimum L. based on molecular markers. Journal of Agricultural and Biological Science, vol. 7, 2012, no. 1, pp. 50-58.