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Original scientific paper

IRAP VARIABILITY IN *Prunus domestica (L.) Borkh* BASED ON *Cassandra* RETROTRANSPOSON POLYMORPHISM

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Plum species are reported to possess a wide genomic variability and that is why DNA markers are still actual in the characterization of its germlasm. In this study, twenty-three genotypes of European plums were assessed for the amplified length based polymorphism among the retrotransposon *Cassandra* insertions in their genomes. The obtained insertional polymorphism caused by the activity of *Cassandra* showed regional and pedigree differences in the analysed accessions of European plums. Two primers were used in analysis. The first resulted in the amplification in 203 amplicons and the 86.6 % polymorphism. Two unique fragments were obtained for the Torysa and Podolínec varieties using this primer. The second primer resulted in the amplification in 267 amplicons and 74.2 % polymorphism. Four unique fragments were obtained for the plum varieties Švestka domácí, Čačanská ranná and Elena. Hierarchical cluster analysis divided the analysed accessions into the four main clusters. To show the length polymorphism diferences of the analysed genotypes from Germany, Slovakia and former Yugoslavia more pecisely, the scattergram for them was constructed.

Key words: DNA polymorphism; European plum; retrotransposon marker; variability

INTRODUCTION

Prunus domestica (L.) Borkh (European plum, 2*n*=6*x*=48 chromosomes) grow in the temperate zone and has an edible oblong fruit. It is supposed, that plum is derived from *Prunus cerasifera* Ekh. by autoploidization in its maternal lineage (HORVATH *et al*. 2011). Green plums

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(*P. cerasifera* Ehrh.) are diploid but in the area of Balkan and Caucasus natural tetraploid and hexaploid types are distributed and both of hexaploids, *Prunus domestica (L.) Borkh* as well as *P. cerasifera* Ehrh., are reported as to be very similar (ZOHARY 1992; PANDEY *et al*. 2008).

The molecular based analysis of *Prunus domestica (L.) Borkh* provide a good orientation in plum´s wide diversity range as only a limited diversity among the plum cultivars compared to the other tree fruit species exist (ALI *et al*., 2015). The development of microsatellite based SSR markers was reported by DECROOCQ *et al.* (2004). For the allelic variation of chloroplast microsatellite loci in European plums, the same authors reported the findings, that two variants of ccmp3 cpSSR locus were detected in 15 European plum cultivars. RAPD and ISSR approaches were used by ATHANASIADIS *et al.* (2013) to analyse the degree of genetic diversity of the *Prunus domestica (L.) Borkh* and *Prunus salinica* L. collection in Greece. In total, 10 RAPD and 5 ISSRs primers were found to produce unambiguous and polymorphic bands. Both of the used approaches discriminate all the analysed genotypes, but no specific grouping was obtained in the constructed dendrogram. RAPD technique was used also by YU *et al.* (2013) to analyse a variability of Japanese plums where a very good discrimination power is reported for RAPD markers, but no specific grouping of cultivars was found. ALI *et al.* (2015) reported the AFLP markers for the genetic diversity evaluation of seven cultivars of European plum with the highest genetic distance of 0.5415 of genetic similarity index (NEI and LI, 1979). Using the nonspecific and anonymous markers for study of plant variability, the problems with reproducibility, low level of polymorphism and inter laboratory cross analyses must be faced (ŠTEFÚNOVÁ *et al*., 2015). This can be overcomed by using the specie specific markers and markers based on the retrotransposons (TREBICHALSKÝ *et al*., 2013). An IRAP based approach were developed previously (SENKOVÁ *et al*., 2012) based on the sequence data of *Prunus domestica (L.) Borkh* Cassandra retrotransposon. Cassandra is a 615 bp long non-autonomous Terminal-repeat Retrotransposons In Miniature element, that for firstly reported retrotransposon in *Prunus domestica (L.) Borkh* genome by (ANTONIUS-KLEMOLA *et al*., 2006).

DNA markers derived from retrotransposon sequences are reported often as to be very close to the ideal markers based on the retrotransposon characteristics. Retrotransposons possess the high number of copies in genomes and are situated on the various locations with the well accessibility to analyse length polymorphism between species or inside of species. Retrotransposons are often chosen in the phylogenetic relationships studies and genetic variability analyses and the most frequently used retrotransposon based molecular marker methods are IRAP (Inter-Retrotransposon Amplified Polymorphism) and REMAP (REtrotransposon-Microsatellite Amplified Polymorphism) (KALENDAR *et al*., 2006; ZEIN *et al*., 2010).

Here, a Cassandra insertion based polymorphism was analysed in the collection of twenty-three *Prunus domestica (L.) Borkh* varieties. The aim of the study was to compare Cassandra IRAP fingerprints and construct a dendrogram based on the genetic similarity indices formulated for the obtained polymorphic profiles.

MATERIAL AND METHODS

Biological material

Green leaf of 19 genotypes of *Prunus domestica (L.) Borkh* was provided by Výzkumný a šlechtitelský ústav ovocnářský Holovousy s.r.o., Czech Republic. The samples of Slovak plums were collected in locations of their natural occurrence (Table 1).

Table 1. Characteristic of analysed Prunus domestica (L.) Borkh genotypes.

Genotype	Type	Geographical origin	Pedigree
Slivka domáca PNV	genotyp	Slovakia – Pečovská N. Ves	unknown
Slivka domáca T	genotyp	Slovakia – Torysa	unknown
Slivka domáca P	genotyp	Slovakia – Podolínec	unknown
Slivka domáca L	genotyp	Slovakia – Lipany	unknown
Gabrovská	cultivar	Bulgaria	Kjustendilska × Montfortska
Chrudimská	cultivar	Czech Republic	Random seedling
Švestka domácí	cultivar	Czech Republic	Random seedling
Čačanská lepotice	cultivar	Former Yugoslavia	Wangenheimova × Požegača
Anna spätch	cultivar	Hungaria	Random seedling
Čačanská ranná	cultivar	Former Yugoslavia	Wangenheimova × Požegača
Čačanská rodná	cultivar	Former Yugoslavia	Stanley × Požegača
Elena	cultivar	Germany	Vlaška × Stanley
Hamanova švestka	cultivar	Czech Republic	unknown
Hanita	cultivar	Former Yugoslavia	President × Auerbacher
Katinka	cultivar	Germany	Ortenauer × Gerstetter
Lutzelsachsenská	cultivar	Germany	Random seedling
Presenta	cultivar	Germany	Ortenauer × President
Stanley	cultivar	USA	Agenská × Grand Duke
Tegera	cultivar	Germany	Ortenauer × Gerstetter
Valjevka	cultivar	Former Yugoslavia	Agenská × Stanley
Vlaška	cultivar	Italy	unknown
Wangenheimova	cultivar	Germany	unknown
Zimmerova	cultivar	Germany	Random seedling

DNA extraction and Cassandra IRAP primer design

Genomic DNA was isolated by Invisorb® Spin Plant Mini Kit (Invitek) following the manufacturer´s instruction. The quantity and quality of extracted DNA was proved by fluorometer (Qubit™).

PrunCassLTR1 primer was derived from the sequence of 3´end of Long Terminal Repeat of *Cassandra* stored in the NCBI under the accession number AY860314. This strategy of primer designation was used previously ŽIAROVSKÁ *et al*. (2012) and was proved in the genome analysis of common flax. PrunCassLTR2 primer was designed from the same sequence based on 5´end of *Cassandra*. According the *Cassandra* sequence characteristics, each primer anneals in two *Cassandra* regions, as is illustrated on Figure 1. A detailed characteristics of both primers are in Table 2.

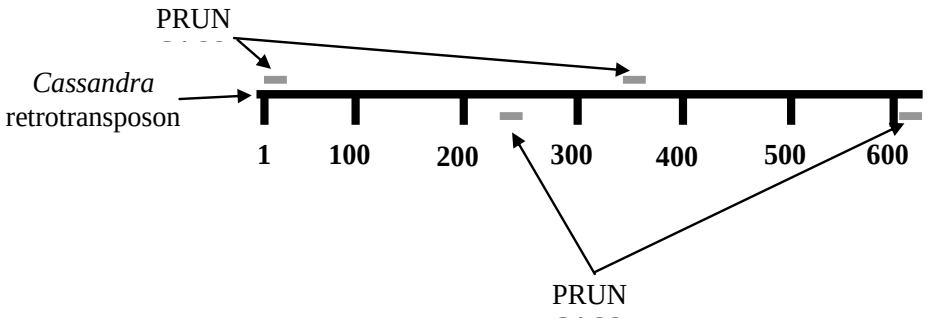

Figure 1. Graphical representation of primers annealing sites.

Table 2. IRAP primers characteristics

Primer	Sequence	Melting temperature	Annealing temperature	Primer length
PrunCassLTR1	ACGGCGGAGCCGATCCCGGGATGTGACA	68.7 °C	54 °C	28 nt
PrunCassLTR2	TCTCCGTTGGTCGATGTGGGATGTTACA	61.4 °C	61 °C	28 nt

PCR conditions

PCR products were amplified using the BIOTAQ PCR kit (BIOLINE) in a total volume of 15 µl. Amplification time and temperature profile of PRUNCASSLTR1 was as following: initial denaturation step - 1 minute at 94°C; 32 cycles of denaturation - 1 minute at 94°C, primer annealing - 1 minute at 54°C and DNA elongation - 3 minutes at 72°C. Final DNA synthesis - 10 minutes at 72°C. Amplification time and temperature profile of PRUNCASSLTR2 was as following: initial denaturation step - 3 minutes at 95°C; 32 cycles of denaturation - 40 seconds at 95°C, primer annealing - 40 seconds at 61°C and DNA elongation - 2 minutes at 72°C. Final DNA synthesis took 5 minutes at 72°C. Concentration of individual PCR reagents is listed in the Table 3.

Table 3. Concentration list of individual PCR reagents

PCR reagents / IRAP primer	PRUNCASSLTR1	PRUNCASSLTR2
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

PCR products electrophoretic separation and data analysis

Agarose electrophoresis was performed in 1.7% gel (Applichem) and 1 × TBE. Gels were stained by Gel Red 10 000 × (Biotium). Electrophoreograms were analysed 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 and 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). Dendrogam and scattergram were constructed based on UPGMA analysis using the SYNTAX software.

RESULTS AND DISCUSSION

In this study, two IRAP primers for plum *Cassandra* retrotransposon were used for the analysis of 23 *Prunus domestica (L.) Borkh* genotypes to obtain the length polymorphism fingerprint patterns. The nucleotide sequence of plum *Cassandra* TRIM element was prooved to be a specific one in the genome of *Prunus domestica (L.) Borkh* (ŽIAROVSKÁ *et al*., 2015). Both of the primers used in this study were derived from this same sequence and are similar in designation of the primer orientation and length. Nevertheless a significant difference was obtained in the results of PrunCassLTR1 and PrunCassLTR2 primer efficiency and primer usage.

Primer PrunCassLTR1 that was derived from the 3´end of *Cassandra* long terminal repeat region resulted in the amplification in 203 amplicons (Table 4) for the analysed 23 genotypes of *Prunus domestica (L.) Borkh* This means the average of 18.5 fragments for the individual sample. From the all amplicons, 176 of them was polymorphic what is 86.6 %. Two unique fragments were obtained for the Torysa and Podolínec varieties. The range of amplified fragments were from 250 up to the 1300 bp (Figure 2) and were separated in the 19 levels from which 15 were polymorphic (Table 5). Cassandra IRAP primer derived from the 3´end of common flax *Cassandra* was used for genetic similarity evaluation in the ŽIAROVSKÁ *et al*. (2009) study about interspecific primer transferability and genetic polymorphism testing among 36 flax (*Linum usitatissimum* L.) genotypes. *Cassandra* IRAP resulted in 16 levels in 36 genotypes flax collection where 11 were polymorphic. The percentage of polymorphic fragments were 68.7%.

Table 4. Structure of amplified product using the IRAP based on Cassandra

Primer	Total bands count	Total polymorphic bands count	Total monomorphic bands count	Percentage of polymorphic bands
PrunCassLTR1	203	176	27	86.6 %
PrunCassLTR2	267	198	69	74.2 %

Primer PrunCassLTR2 that was derived from the 5´end of *Cassandra* long terminal repeat region resulted in the amplification in 267 amplicons (Table 4) for the analysed 23 genotypes of *Prunus domestica (L.) Borkh*. This means the average of 11.6 fragments for the individual sample. From the all amplicons, 198 of them was polymorphic what is 74.2 %. Four unique fragments were obtained for the plum varieties Švestka domácí, Čačanská ranná and

Elena. The range of amplified fragments were from 250 up to the 1500 bp (Figure 3) and were separated in the 21 levels from which 18 were polymorphic (Table 5).

Table 5. PrunCassLTR1 and PrunCassLTR2 obtained amplicons – levels calculation

Primer	Total level count	Polymorphic level count	Count of level with unique present band
PrunCassLTR1	19	15	1
PrunCassLTR2	21	18	2

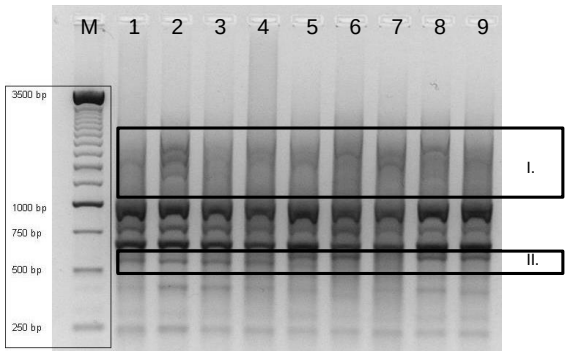

Figure 2. PRUNCASSLTR1 amplified products in agarose gel separation. The unique fragments level are in the rectangle I. and II. M - 250 bp DNA Ladder; 1 – Presenta; 2 – Elena; 3 – Stanley; 4 – Wangenheimova; 5 – Hamanova švestka; 6 –Valjevka; 7- Cacanska rana; 8 - Cacanska Lepotice; 9 – Švestka domácí.

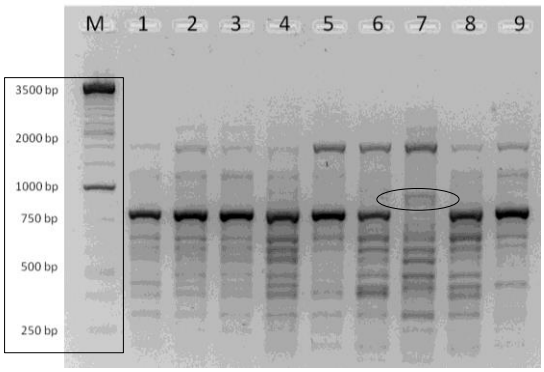

Figure 3. PrunCassLTR2 amplified products in agarose gel separation. The unique fragments amplified for Čačanská lepotice is marked in the oval. M - 250 bp DNA Ladder; 1 - plum from Pečovská Nová Ves; 2 - plum from Torysa; 3 plum from Podolínec; 4 – plum from Lipany; 5 – Gabrovská; 6 – Chrudimská; 7 – Švestka domácí; 8 - Cacanska Lepotice; 9 - Anna Spätch.

ANTONIUS-KLEMOLA *et al.* (2006) reported similar results for the apple *Cassandra* TRIM element. They designed four IRAP markers derived from the apple *Cassandra* type and they all had the same orientation and originated from the same long terminal repeats with the diverse in primer length 21 – 26 bp. By resulting electrophoreogram comparisons authors found out that the primers provided different levels of amplified products ranged from six up to the fifteen levels. Accordingly, the differences were observed in the polymorphic levels count, from two up to ten polymorphic levels. In this study, PrunCassLTR1 primer resulted in ten levels of amplified products and PrunCassLTR2 in eighteen levels. Primer PrunCassLTR2 was also defined by the presence of 10 polymorphic levels and primer PrunCassLTR1 created 4 polymorphic levels as it is shown in the Table 4. For PrunCassLTR2 primer, two unique bands were present and also two unique absent bands. This was not observed in electrophoreogram gained after electrophoresis of PrunCassLTR1 amplified products.

The hierarchical cluster analysis divided analysed European plum genotypes into the four main clusters (Figure 4). The old local genotypes from Slovakia were grouped together with the Hungarian variety Anna Spätch (cluster V.). Cluster III. that comprises from two smaller clusters contain all of the Germany genotypes with the only exception – cultivar Elena. This was analysed as to be te most different from all the analysed genotypes of plum together with the cultivar Stanley. Elena posses the variety Stanley in its pedigree as well as the variety Vlaška, but this one is located among the German genotypes in the cluster III.

Another cluster where the provenience and the pedigree can be found is the cluster VI. and VII. – here, the genotypes from former Yugoslavia are localized. Cultivars Cacanska rana, Cacanska rodna and Cacanska lepotica possess the cultivar Požegača in their pedigrees. Nei and Li genetic distance coefficient for the cultivars Cacanská rana and Cacanska rodna has the value of 0,167 and for the cultivars Cacanská rodna and Cacanská lepotica has the value 0,182.

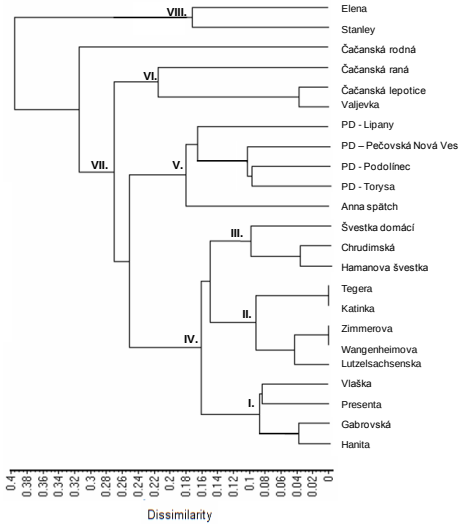

Figure 4. Dendrogram for the Cassandra IRAP analysis of the plum genotypes evaluated in this study.

Both of the primers were proved for the assessment of genetic relatedness among the plum genotypes using the Nei and Li coefficients of genetic similarity. The parentage lineage of

three full-sib European plum cultivars - Cacanska najbolja, Cacanska rana and Cacanska lepotica was obtained by DECROOCQ *et al.* (2004) by the analysis SSR and four chloroplastic microsatellite loci, too. The average value for analysed genotypes are in the range from 0.75 to 0.82 for primer PrunCassLTR1 and in the range from 0.32 to 0.64 for PrunCassLTR2 primer. PrunCassLTR1 primer showed to be less polymorphism in this set of genotypes comparing it to the primer PrunCassLTR2.

Evaluating the accessions that belong to the International reference cultivars (Cacanska rodna, Hanita and Stanley), a low genetic distance was observed among them with the average of 0,266. This suppose the *Cassandra* IRAP as an functional marker system for European plum gene pool evaluation, as the results of SSR analysis performed by SEHIC *et al*. (2015) grouped all this into a one cluster different from Balsgård and Norwegian plum accessions. To show the length polymorphism diferences of the analysed genotypes from Germany, Slovakia and former Yugoslavia more precisely, the scattergram for them was constructed (Figure 5). Here, the grouping from the dendrogram was more tight for them. All the varieties, that were not grouped directly with the others with the same provenience in the dendrogram (Elena and Hanita), stay separated from the main cluster in the scattergram, too. KALENDAR *et al*. (2000) reported that retrotransposon distribution patterns can show eco-geographical gradients due to the underlying effect of environment and stress on retrotransposon activation.

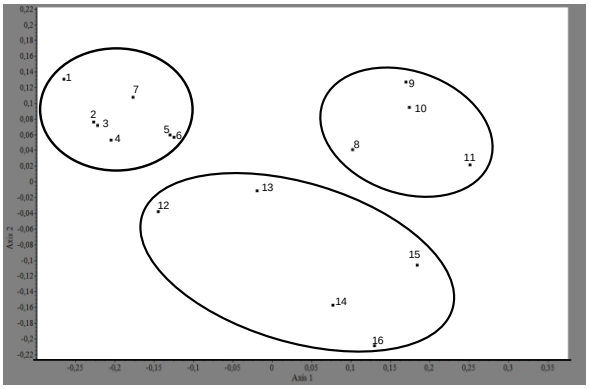

Figure 5. Scattergram of German, Slovak and former Yugoslavian genotypes of analysed plums. 1 – Elena; 2 – Katinka; 3 – Tegera; 4 – Presenta; 5 – Wagenheimova; 6 – Zimmerova; 7 – Lutzelsachsenska; 8 – PD_Lipany; 9 – PD_Torysa; 10 – PD_Podolínec; 11 – PD_Pečovská Nová Ves; 12 – Hanita; 13 – Čacanská rodná; 14 – Čačanská raná; 15– Čačanská Lepotice; 16 - Valjevka

Different DNA markers are still used in the studies of genetic diversity across a wide range of plant species such as random amplified polymorphic DNA (POPOVIČ *et al*., 2015; BALÁŽOVÁ *et al*., 2016a; KUŤKA HLOZÁKOVÁ *et al*., 2016), simple sequence repeat (BUHINIČEK *et al*., 2015; BALÁŽOVÁ *et al*., 2016 b,c; GÁLOVÁ *et al*., 2015; ŠARAC *et al*., 2015), amplified fragment length polymorphism (MILELLA *et al*., 2011; LABAJOVÁ *et al*., 2013), inter-simple sequence repeat (ŽIAROVSKÁ *et al*., 2013) or retrotransposon based markers (MEHMOOD *et al*., 2013; TREBICHALSKÝ *et al*., 2013). These methods are technically simple, fairly cheap and generate a relatively large number of markers per sample.

Previous studies on the variability of the European plum accessions conducted by RAPD and ISSR confirm the effectiveness of molecular markers for the diversification of plum cultivars (ILGIN *et al*., 2009). Molecular analysis of different plum accession were performed by RAPD, RAM and AFLP until now. YU *et al*. (2013) used 11 random amplified polymorphic DNA markers to identify a total of 73 European plum cultivars of different origins. The cultivars were separated by the amplification profiles of 9 of the used primers. MORILLO *et al*. (2015) reported random amplified microsatellite markers (RAM) for the analysis of 14 *Prunus domestica (L.) Borkh* varieties. Using 8 markers, the accessions were distinguished, too. ALI *et al*. (2015) performed the AFLP analysis and three of the used primer combinations produced 106 amplification products in the set of 7 European plum cultivars. In this study, the amplification patterns were the same for four of the analysed genotypes of plum, what may be a consequence of the the specific *Cassandra* insertion pattern in *Prunus domestica (L.) Borkh.* Because insertion or loss of retrotransposons affects the regulatory machinery of genes (KOBAYASHI *et al*., 2004), the connection between environmentally-activated retrotransposition and genetic differentiation could be of particular importance for genes involved in environmental adaptation and can result in a very specific amplification patterns.

The primers based on the sequences of LTR regions of European plum retrotransposon Cassandra were used for genetic diversity evaluation among *Prunus domestica (L.) Borkh* genotypes collection. The PrunCassLTR1 possess the lower discrimination power as PrunCassLTR2 primer. The wide pedigree and origin relationships were found in the constructed dendrogram of 23 different plum genotypes analysed in the study.

CONCLUSION

IRAP based polymorphism was prooved here as to be a suitable tool to describe the natural DNA variability of European plums. Cassandra retrotransposon is an effective marker to desing an IRAP primer that amplify reproducible loci. A total of 270 fragments were scored for 23 analysed accessions. Unique fragments were obtained for the varieties Švestka domácí, Čačanská ranná and Elena and specific banding profile was obtained for genotypes from Germany, Slovakia and former Yugoslavia.

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IRAP VARJABILNOST *Prunus domestica (L.) Borkh* NA OSNOVU *Cassandra* RETROTRANSPOZON POLIMORFIZMA

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Izvod

Vrste šljiva poseduju veliku genetičku varijabilnost i zato DNK marekri su još uvek aktuelni za karakterizaciju germplazme. U ovim istraživanjima, 23 genotipa Evropskih šljiva su ispitane za polimorfizam amplikovane dužine između retrotranspozon *Cassandra* insercija u njihovom genomu. Dobijen insercioni polimorfizam uzrokovan aktivnošću *Cassandra* pokazao je regionalnu i pedigre diferencijaciju u analiziranim uzorcima Evropske šljive. Dva prajmera su korišćena u analizi. Prvi je rezultirao u amplifikaciji 203 amplikona i 86.6% polimorfizmu. Dva jedinstvena fragmenta su dobijena za Torysa i Podolínec varijitete sa ovim prajmerima. Drugi prajmer je rezultirao amplifikacijom u 267 amplikonu i 74.2% polimorfizmu. Četiri jedinstvena fragmenta su dobijena za varijitete šljive Švestka domácí, Čačanská rana i Elena. Hijerahiska kladster analiza razdvojila je analizirane uzorke u četiri glavna klastera. Da bi pokazali razliku u polimorfizmu dužine analiziranih genotipova iz Nemačke, Slovačke i bivše Jugoslavije preciznije dvodimenzionalni grafikon od njih je konstruisan.

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