Acta fytotechn zootechn, 25, 2022(2): 122–129
http://www.acta.fapz.uniag.sk

Original Paper

Analyse of iPBS lenght polymorphism in selected group of *Vitis vinifera*, L. varieties

Jana Žiarovská[1]*, Adam Kováčik[1], Silvia Farkasová[1], Martina Fixelová[2], Jozef Sabo[3], Miroslava Kačániová[3]

[1]*Slovak University of Agriculture in Nitra, Faculty of Agrobiology and Food Resources, Institute of Plant and Environmental sciences, Slovak Republic*
[2]*Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Institute of Food sciences, Slovak Republic*
[3]*Slovak University of Agriculture in Nitra, Faculty of Agrobiology and Food Resources, Institute of Horticulture, Slovak Republic*

Article Details: Received: 2022-02-08 | Accepted: 2022-04-01 | Available online: 2022-06-30

https://doi.org/10.15414/afz.2022.25.02.122-129

Here, the specific natural variability of iPBS (Inter Primer Binding Sites Polymorphism) fingerprints in thirteen varieties of *Vitis vinifera*, L. was performed. All of the analysed biological material was collected in the vineyard of Sabo vinery for describing of the existing genetic polymorphism. Young leaves from a total of thirteen grapevine varieties were obtained in the in the Small Carpathians wine region of Slovakia, Vrbové. Genetic length polymorphism was studied by iPBS markers. A dendrogram of genetic similarity of generated fingerprints was constucted by UPGMA (Unweighted Pair Group Method with Arithmetic mean) and the Jaccard coefficient of genetic similarity was used for the analyse of 13 *Vitis vinifera*, L varieties. The generated dendrogram is separated into three major clusters at the genetic dissimilarity of 0.58. Cluster 1 is composed of two red varieties – Alibernet and Cabernet Sauvignon. Cluster 2 was further subdivided into two sub-clusters, where the larger one include all white varieties – Pinot Blanc, Müller-Thurgau, Welschriesling, Irsai Oliver, Grüner Veltliner, Pálava, Weisser Riesling, Sauvignon Blanc and Feteasca Regala. The second subcluster is comprised from two red varieties – Blaufränkisch and Dornfelder. The analysis prooved the the iPBS technique is an effective retrotransposon based markers to evaluate the variability of the genome in the germplasm of *Vitis vinifera* L. cultivated varieties.

Keywords: *Vitis vinifera* L., iPBS, DNA markers, polymorphism, genetic distance

1 Introduction

Vitis vinifera ssp. sativa is one of the oldest and most important fruit crops in the world. Its history began 65 million years ago and actually, the cultivation of grapevines is widespread worldwide and represents the most frequently grown species of the world's vineyards with the portion of about 98% (Giannuzzi et al., 2011).

For many years, the grapevine has been domesticated to its current form from a wild vine (*Vitis vinifera* ssp. sylvestris) belonging to the flax plants, which most often occur near river banks. The decline in the occurrence of wild vines, recorded gradually since the 19th century, is associated with anthropogenic activity in naturally occurring habitats (Terral et al., 2009).

From an evolutionary point of view, it has been shown that the primary domestication of the grapevine involved primary specific genes. Zhou et al. (2017) compared selected varieties of grapevine with wild ones. In domesticated varieties, a high presence of genes involved in carbohydrate metabolism, flower development and especially in responses to stress conditions has been demonstrated, compared to wild varieties, in which the occurrence of stress resistance genes has been limited. Currently, the availability of sequences of the annotated grapevine genome allows the identification of the expression of many proteins, thus contributing to the overall effectiveness of genomic and proteomic studies aimed at the grapevine study (Grimplet et al., 2014).

The analysis of genetic diversity and relationships among individual biological species at the different level is still

***Corresponding Author:** Jana Žiarovská, Slovak University of Agriculture in Nitra, Faculty of Agrobiology and Food Resources, Institute of Plant and Environmental sciences, Tr. Andreja Hlinku 2, 949 76, Nitra, Slovak Republic

an actual topis of many biological science disciplines. The classic strategies of decribing of the plant genetic variability belong to many interdisciplinary fields of physiology, anatomy, embryology or morphology. The listed approaches are supplemented by chemical composition analysis, characteristics of macromolecules and alloenzymes (Sharma et al., 2010).

The first genetic maps of the grapevine were prepared by the RFLP (Restriction fragment Length Polymorphism) method (Saiki et al., 1985). Bourquin et al. (1992) analyzed 46 grapevine varieties using markers RFLP and among all found a significant polymorphism. The revolution in the molecular genetics of grapevine has been brought about by the discovery of the PCR (Polymerase Chain Reaction) technique. Later, RAPD markers were used to analysed the genetic variability of this specie, followed by AFLP (Amplification Fragment Length Polymorphism) markers. Collins and Symons (1993) reported RAPD to be effective and reproducible technique for examining and analyzing polymorphism of vine varieties. They have shown that polymorphism characteristics between *Vitis vinifera* L varieties is accessible with a single primer or using a simple mixture of two primers. Microsatellite markers became an important marker for studying the genome of grapevine. Up to date, a total of 75,185 different SSRs (Single Sequence Repeats) have been identified as the markers usable in marker studies of grapevine. The total number of developed SSR markers is high, but still represents only a small proportion of microsatellites in the genome of *Vitis vinifera* (Blondon et al., 2011).

In this study, iPBS (Inter Primer Binding Site) markers were used to analyse the whole genome length polymorphism among different grapevine varieties. iPBS markers (Figure 1) were developed as a semi-universal technique. iPBS markers are typical by their sequentional characteristics that corresponds with the natural variability of retrotransposon elements primer binding sites (Kalendar et al., 2010). All of the retrotransposons are abundant in genomes of plants conditioned iPBS markers to be applicable without a previous knowledge about the very concrete of them. They were reported to be DNA markers screening different inserted amplicons based on biotic or abiotic stress history, that the plant genomes have overcomed (Kalendar et al., 2010). Retrotransposons insertion sites were analysed by iPBS markers in many plant species such as Fagaceae species (Coutinho et al., 2018), *Paeonia anomala* L. or *Digitalis grandifolia* Mill. (Boronnikova and Kalendar, 2010), *Liparis loeselii* (Belgorudova et al., 2012) as well as in *Vitis vinifera* L. (Guo et al., 2014). Retrotransposon are also reported to be effective in high-throughput sequencing that resulted in the new types of retrotransposon-based markers (Monden et al., 2014).

The objective of this study was to analyse specific the natural variability of iPBS fingerprints in thirteen varieties of *Vitis vinifera*, L. that were collected in the vineyard of Sabo vinery and describe the existing genetic polymorphism.

2 Material and methods

2.1 Biological material

Young healthy leaves were collected in situ in the vineyard located in in the Small Carpathians wine region, Vrbové, Slovakia. Leves were transported in cold to the laboratory, they were freezed and stored until further processing. A total of 13 different grapevine varieties were used for the iPBS analyse. Four of them are red – Blaufränkisch,

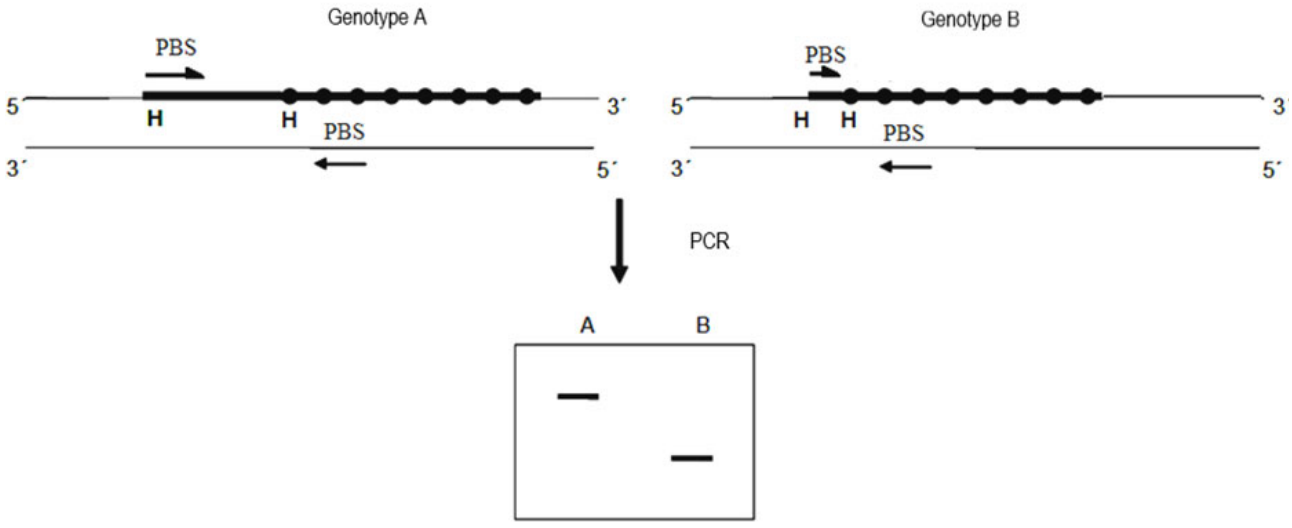

Figure 1 Principle of iPBS technique

Figure 2 Vinery Sabo

Dornfelder, Alibernet and Cabernet Sauvignon. Nine of the analysed varieties are white – Grüner Veltliner, Feteasca Regala, Welschriesling, Pálava, Sauvignon Blanc, Pinot Blanc, Weisser Riesling, Müller-Thurgau and Irsai Oliver.

2.2 DNA isolation

A total genomic DNA was obtained by the isolation method following the manufacturers instructions of the commertial kit – GeneJET Plant Genomic DNA Purification Mini Kit (ThermoScientific). A mixture of 2 leaves for each analysed variety was used to prepare a starting material for the DNA extraction. Quantity and quality of the isolated DNA was prooved at first by measuring in NanoPhotometer® P-Class (Implen) and second by PCR reaction using the universal ITS (Internal Transcribed Spacers) primers to proove its functionality.

2.3 PCR and iPBS performing

First, a mixed DNA was prepared for the purposes of selection of iPBS markers – 2 µl per variety was mixed together and subsequently diluted for following concentrations – 1/10, 1/25 and 1/50. Then, all the different DNA concentrations were used in the PCRs to amplify individual iPBS markers and selection of the most abundant for final analysis. PCR reactions were prepared in 10 µl volume with DreamTaq Green PCR Master Mix (2X) (ThermoScientific) and the thermal reaction was realized using the Agilent Technologies SureCycler 8800. PCR program has the following parameters: 95 °C – 3 min, 35 cycles of 95 °C – 30 s, 55 °C – 40 s, 72 °C – 2 min and final 72 °C – 5 min. Based on the results of the primers selection, primers 1826, 1833, 1845, 1846, 1854, 1868, 1882, 1899, 2079 and 2274 were used for iPBS analysis of *Vitis vinifera* L. varieties and the DNA dilution of 1/50 was used in the PCRs.

2.4 Data processing

Initial electrophoreotic separation of obtained iPBS fingerprints was performed in 3% agarose gel (stained by GelRed) to screen them. Then, higher resolution separation of iPBS amplicons for the purpose of data processing, PAGE electrophoresis was performed (10% PAGE; 5X TBE buffer). The gel was stained in by GelRed for 1 hour and analysed under the UV light (Biometra, wavelength 312 nm).

2.5 Statistical analysis

GelAnalyser (free online version) software was used to prepare 1/0 matrices from reproducible iPBS fingerprints. They were selected from two independent PCRs and evaluated for the absence/ presence of individual amplified loci. Bands of the same size were evaluated as a single locus. Genetic similarity of pairwise individual varieties was calculated by Jaccard coefficient of genetic similarity (Jaccard, 1908). Dendrogram construction was based on UPGMA algorithm with the cophenetic coefficients (Lessig, 1972) calculation in SYNTAX software. Different coefficients were used to describe the effectiveness of the iPBS discrimination of the analysed set of grapevine varieties. PIC (polymorphic information content) was calculated according to De Riek et al. (2001). DP (discrimination power) was calculated according the Tessier et al. (1999). Rp (resolving power) was calculated according to Gilbert et al. (1999).

3 Results and discussion

3.1 iPBS polymorphism

First, amplicon generating screening with twenty-five iPBS primers was performed in mixed DNA (data not shown). Ten selected primers with fully clear reproducible amplicons were used in final PCRs analysis. All of them together amplified a total of 116 bands in the analysed 13 grapevine varieties (Table 1). The number of amplified bands per primer varied from 9 (1845) (Figure 3) to 15 (1882) with the average of amplicons per marker 11.6.

All of the evaluated iPBS markers resulted in clear and reproducible amplicons. The percentage of polymorphic bands varied from from 66.6% (1833) to 100% (1845) – an average at the level of 83%. The highest PIC value (0.49) was obtained for marker 1845 and the lowest (0.30) was obtained for marker 2079 with an average of 0.386 what indicates, that amplified loci are informative. An efficient discrimination of analysed varieties was confirmed by discrimination power of the used markers.

Table 1 Obtained characteristics of genetic diversity for used iPBS markers

Primer	Total levels of amplicons	Polymorphic levels of amplicons	PIC	PD	Rp
1826	10	9	0.38	0.87	3.22
1833	12	8	0.42	0.84	2.69
1845	9	9	0.49	0.85	5.26
1846	13	11	0.38	0.91	1.97
1854	10	8	0.36	0,88	3.51
1868	11	9	0.41	0.88	3.43
1882	15	13	0.44	0.84	2.26
1899	10	8	0.31	0.91	4.78
2079	12	9	0.30	0.92	3.87
2274	14	12	0.37	0.84	3.25

3.2 Genetic distance and cluster analysis

A dendrogram that shows the dissimilarity of obtained iPBS fingerprints was constructed based on the Jaccard coefficient of genetic similarity among the analysed 13 varieties of *Vitis vinifera* L. (Figure 4) using the UPGMA analysis. Analysed varieties were separated into three major clusters with the value of genetic dissimilarity of 0.58. Complete separation was obtained among analysed grapevine cultivars. Final dendrogram was constructed based on the results of individual fingerprints obtained for every iPBS marker used in the study. Cluster 1 is composed of two red varieties – Alibernet and Cabernet Sauvignon (Figure 5). Cluster 2 was further subdivided into two subclusters, where the larger one include all white varieties – Pinot Blanc, Müller-Thurgau, Welschriesling, Irsai Oliver, Grüner Veltliner, Pálava, Weisser Riesling, Sauvignon Blanc and Feteasca Regala. The second subcluster is comprised from two red varieties – Blaufränkisch and Dornfelder. The Mantel test was returned with a result of good and significant co-phenetic correlation (*r* - 0.90), that indicate a very good fit to the cluster analysis (Rohlf, 2005) and it confirms that obtained dendrogram is an effective representation of the generated iPBS amplicons.

iPBS markers were prooved for plant genomes to be a technique that can be applied for both – isolation of LTR retrotransposons as well as generate reproducible, efficient and general fingerprints by many authors (Andeden et al, 2012; Baránek et al., 2012). This marker system was reported by Kalendar et al. (2010) to be applicable for the semi-universal use for retroviruses as well as for LTR retrotransposons. This fingerprint method is highly usable to any organism, not only plants, that containes retrotransposons in its genome, because of the presence of primer binding sites that are specifically complementar to the tRNA sites. Aydin et al (2020) were reported the utility of the iPBS fingerptints to be suitable in the analysis of yeast genome variability. iPBS sequences are widely used in the whole-genome analysis of plant polymorphism and were applied previously for a wide range of plant species such as Fagaceae (Countinho et al., 2018); Liparis loeselii (Belogrudova et al., 2012); Saussurea esthonica (Gailīte et al., 2012) or Prunus armeniaca (Baránek et al., 2012); *Solanum tuberosum*, *Peonia anomala*, *Brassica rapa* or *Digitalis grandiflora* (Kalendar et al., 2010). Some of the in silico generated iPBS markers were subsequently paired with

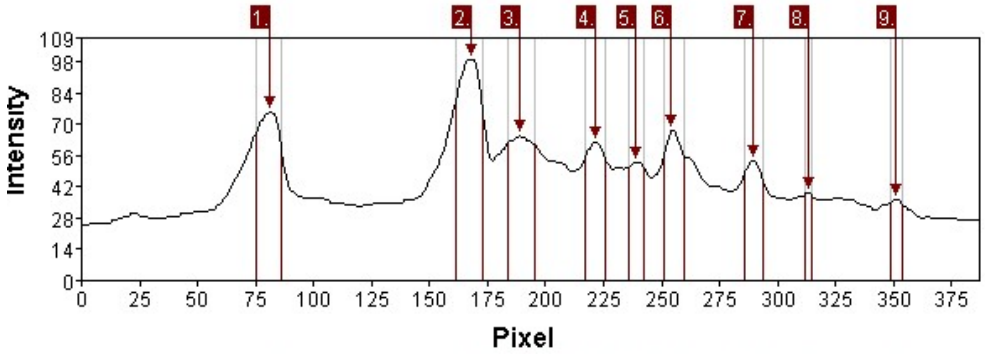

Figure 3 Fingerprint profile of Alibernet variety based on 1845 marker amplicon profile

	1	2	3	4	5	6	7	8	9	10	11	12	13
1	0.000												
2	0.385	0.000											
3	0.600	0.375	0.000										
4	0.625	0.500	0.200	0.000									
5	0.667	0.474	0.389	0.333	0.000								
6	0.714	0.476	0.400	0.350	0.409	0.000							
7	0.762	0.591	0.368	0.316	0.455	0.318	0.000						
8	0.714	0.545	0.400	0.263	0.333	0.190	0.318	0.000					
9	0.667	0.500	0.350	0.300	0.286	0.227	0.273	0.304	0.000				
10	0.682	0.522	0.455	0.409	0.318	0.400	0.375	0.333	0.217	0.000			
11	0.611	0.500	0.333	0.368	0.429	0.286	0.409	0.286	0.318	0.417	0.000		
12	0.611	0.500	0.500	0.524	0.429	0.435	0.409	0.500	0.391	0.480	0.583	0.000	
13	0.611	0.500	0.421	0.368	0.350	0.500	0.409	0.435	0.458	0.480	0.455	0.300	0.000

Figure 4 Jaccard coefficient of genetic similarity of analysed thirteen varieties of *Vitis vinifera*, L.
1 – Alibernet; 2 – Cabernet Sauvignon; 3 – Pinot Blanc; 4 – Müller-Thurgau; 5 – Welschriesling; 6 – Irsai Oliver; 7 – Grüner Veltliner; 8 – Pálava; 9 – Weisser Riesling; 10 – Sauvignon Blanc; 11 – Feteasca Regala; 12 – Blaufränkisch; 13 – Dornfelder

existing retrotransposons, such as 1882, that is linked to the *Linum usitatissimum* FL7 LTR retrotransposon (Smýkal et al., 2011). For all of these plants, this DNA based marker technique was reported to be reliable one. Not only the iPBS, but another retrotransposon based fingerprint techniques such as SSAP, RBIP, IRAP or REMAP are applied the analysis of genome polymorphism in plants (Balážová et al., 2014; Guo et al., 2014).

Andeden et al., (2012) compred the results of analysis of the genetic polymorphism and variability of the different species of wild chickpeas using the ISSR and iPBS fingerprints. As a result, a total of 136 ISSR amplified

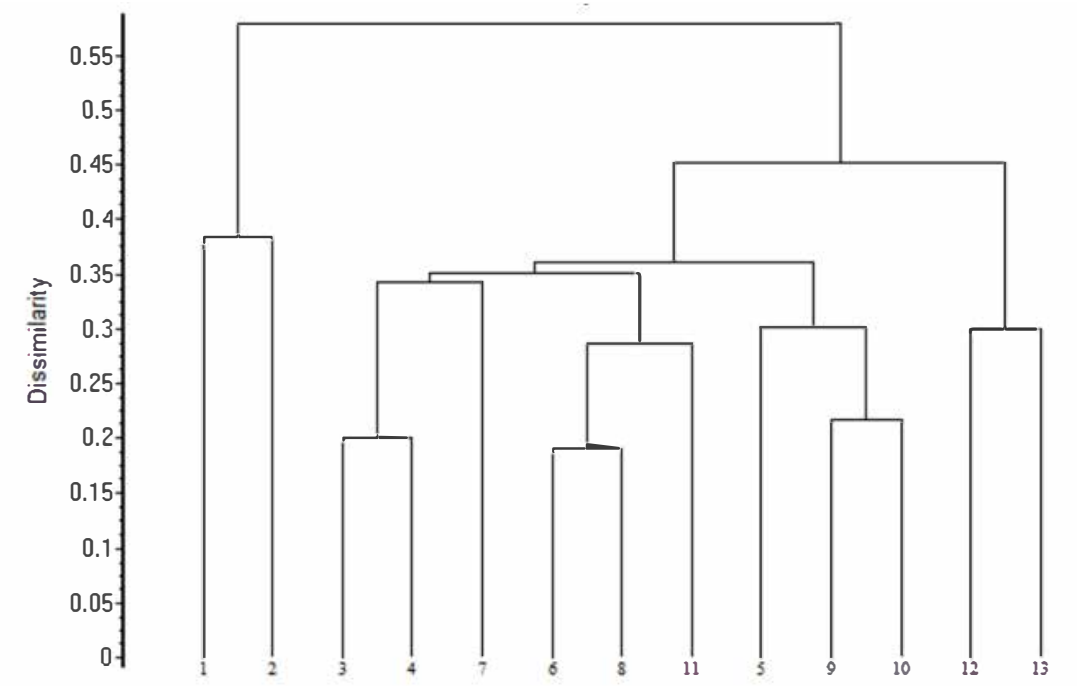

Figure 5 Dendrogram of analysed thirteen varieties of *Vitis vinifera*, L.
1 – Alibernet; 2 – Cabernet Sauvignon; 3 – Pinot Blanc; 4 – Müller-Thurgau; 5 – Welschriesling; 6 – Irsai Oliver; 7 – Grüner Veltliner; 8 – Pálava; 9 – Weisser Riesling; 10 – Sauvignon Blanc; 11 – Feteasca Regala; 12 – Blaufränkisch; 13 – Dornfelder

bands using 10 different primers were obtained. In these set of amplicons, 135 of them were analysed as polymorphic, what represents 99.3% with an average of 13.5 polymorphic fragment per primer. In the study of these authors, iPBS generated 130 amplicons with the 100% polymorphism and withe the average of 13.0 bands per primer. Both marker systems were concordant in the value of PIC – 0.91.

Actually, DNA based marker techniques are an important part of molecular breeding strategies for many plants as well as for grapevine. Genetic divergence among accessions demonstrated practical applications in grape breeding programs, as the choice of relatively divergent parents will maximize the frequency of progeny with superior characteristics (deOliveira et al., 2020). Beside the whole genomic markers, specific are developed, too. Emanuelli et al. (2014) has reported four missense mutations in the *VvDXS* gene that is tightly linked to muscat flavor. DNA markers are widely used in grapevine breeding to create forms with combined resistance genes, such as *RpV* genes (Ilnitskaya et al., 2020).

The genomic analysis of *Vitis vinifera* L. reported, that the retrotransposons are approximately 17–24% of the whole genome of this specie (Velasco et al., 2007). A set of 33 clones and control genotypes of grapevines were used in the study of genetic analysis of russian Vitis collection (Milovanov et al., 2019). In this study, four iPBS primers (2228, 2230, 2237, 2415) were selected to be suitable to generate fingerprints data from the 30 tested. The length of the generated fragments resulted in the range from 300 up to the 6000 bp and a unique banding pattern was found for control varieties and clones.

Retrotransposons are reported to have an partial role in the mechanisms of generating of genetic diversity and are very efficient for fingerprints technique development thanks their high abundance in genomes of the plants and due to their specific pattern of distribution (Buti et al., 2009). Different PCR fingerprints marker techniques are known and used to generate insertional polymorphisms of LTR retrotransposons, such as inter retrotransposon amplified polymorphism, retrotransposon based insertional polymorphism or retrotransposon-microsatellite amplified polymorphism (Kalendar et al., 2019). In the case of *Vitis vinifera* L., these techniques have been used in genetic diversity assessment for both, varieties and clones distinguishing (Castro et al., 2012).

In our study, 567 iPBS bands were gererated as a total, when 10 different iPBS primers used. The average numbers of iPBS amplicons are comparable with the study of Baránek et al. (2012), but is less than in the case of study of Gailite and Rungis, (2012) where a different separating strategy was used to analyse the iPBS amplicons. Results obtained in this study indicated that this technique is qualified to detected the principal polymorphism (83%) when comparing it to the results of Chinese grape varieties using different types of DNA based markers.

To evaluate the genetic analysis of the performance of the iPBS markers used in the study, the calculated values of polymorphic information content, discrimination power and resolving power were analysed. The PIC values are standartly used to describe the ability of a specific primer to discriminate the selected group of accessions and to evaluate the efficiency of generated polymorphic amplicons in revealing genetic diversity among the varieties. In our study, the PIC values varied among the markers and ranged from 0.30 to 0.49 that indicate the usefulness of the used iPBS markers and their ability to distinguish the analysed grapevine varieties, as the PIC for this type of DNA marker technique is of maximum of 0.5 (De Riek et al., 2001). The average polymorphic information content values (0.386) obtained in this study is higher than those that is reported for Portuguese grapevine varieties by Castro et al. (2012) with SSAP (0.2867), AFLP (0.2996) and REMAP (0.2860) marker techniques. Rp coefficient considers the number of polymorphic amplicons in a generated fingerprints and represents the informative value of individual polymorphic amplicons (Salunkhe et al., 2013). It is reported in literature to be more informative than polymorphic information content to describe the discrimination ability of primers in a diversity study (Mangini et al., 2010). The resolving power values ranged from 1.97 to 4.98 (Table 1) in this study, what is higher than in the case of SSR marker results for wheat (Salunkhe et al., 2013). The values of PIC, DP and Rp values obtained in our study are in concordance with those that are reported for grapevine varieties.

4 Conclusions

In this study, we have successfuly characterized the polymorphism of generated iPBS fingerprints was in group of 13 grapevine varieties that were obtained from the vineyard of Sabo vinery (Slovak Republic). The analysis confirmed that the iPBS technique is easilly applicable and informativeness for DNA based markers evaluation and assesment of the genetic diversity in *Vitis vinifera* L. cultivated varieties. The dendrogram constructed on UPGMA algorithm has divided 13 analyzed accessions into three main clusters and all of the analysed grapevine varieties were separated completely in this dendrogram. This study results consider the iPBS technique to be sufficient for whole genome polymorphic analysis of the genome of *Vitis vinifera* L. that will be useful in the

characterization of its diversity, germplasm assessment and management and the information generated by these technique can be used in breeding and conservation of this specie.

Acknowledgments

This work has been supported by the grants of APVV-19-0180 Aplikácia molekulárno-biologických metód pri vývoji referenčných materiálov určených na detekciu falšovania a hodnotenia kvality potravín (50%) and by the Operational programm Integrated Infrastructure within the project: Demand-driven research for the sustainable and inovative food, Drive4SIFood 313011V336, cofinanced by the European Regional Development Fund (50%).

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