UDC 575:630
DOI: 10.2298/GENSR1203619C
Original scientific paper

DEHYDROGENASE ISOENZYME POLYMORPHISM IN GENUS *Prunus*, SUBGENUS *Cerasus*

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Čolić S., V. Rakonjac, M. Fortić. Akšić, D.Nikolić, V. Ognjanov, and D. Rahović (2012): *Dehydrogenase isoenzyme polymorphism in genus prunus, subgenus cerasus.* - Genetika, Vol 44, No. 3,619 -632.

Dehydrogenase polymorphism was studied in 36 sour cherry (*Prunus cerasus L.*), sweet cherry (*Prunus avuim* L.), mahaleb (*Prunus mahaleb* L.), ground cherry (*Prunus fruticosa* Pall.), duke cherry (*Prunus gondounii* Redh.), Japanese flowering cherry (*Prunus serrulata* Lindl.) and four iterspecific hybrids (standard cherry rootstocks 'Gisela 5', 'Gisela 6', 'Max Ma' and 'Colt'). Inner bark of one-year-old shoots, in dormant stage, was used for enzyme extraction. Vertical PAGE was used for isoenzyme analysis: alcohol dehydrogenase (ADH), formate dehydrogenase (FDH),

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glutamate dehydrogenase (GDH), isocitrate dehydrogenaze (IDH), malate dehydrogenase (MDH), phosphogluconate dehydrogenase (PGD), and shikimate dehydrogenase (SDH). All studied systems were polymorphic at 10 loci: *Adh -1* (3 genotypes) and *Adh-2* (5 genotypes), *Fdh-1* (2 genotypes), *Gdh-1* (3 genotypes), *Idh-1* (4 genotypes) i *Idh -2* (5 genotypes), *Mdh-1* (3 genotypes), *Pgd*-1 (4 genotypes), *Sdh-1* (1 genotype) i *Sdh-2* (3 genotypes). Cluster analysis was used to construct dendrogram on which four groups of similar genotypes were separated. Obtained results indicate that studied enzyme systems can be used for determination of genus *Prunus*, subgenus *Cerasus*. Among studied enzyme systems ADH, IDH and SDH were the most polymorphic and most useful to identify genetic variability. Polymorphism of FDH and GDH in genus *Prunus*, subgenus *Cerasus* was described first time in this work. First results for dehydrogenase variability of Oblačinska indicate that polymorphism of loci *Idh-2* and *Sdh-2* can be useful for discrimination of different clones.

Key words: cluster analysis. dehydrogenase, electrophoresis, polymorphism, *Prunus* spp

INTRODUCTION

Knowledge of the genetic diversity and relationships among the cultivated and wild species of *Cerasus* subgenus is important for recognizing gene pools, identifying pitfalls in germplasm collections and developing effective conservation and management strategies (KHADIVI-KHUB *et al.*, 2012). Characterisation and identification of species and cultivars in genus *Prunus* within *Cerasus* subgenus has been mostly based on morphological traits (PEREZ-SANCHEZ *et al*., 2008, PEREZ *et al*., 2010, SHAHI-GHARAHLAR *et al*., 2010).

Isoenzyme were among the first mmolecular markers applied in horticultural science, because they allow the identification of plants in early stages of development and are not affected by the environmental conditions. Isoenzyme analysis is important technique in *Prunus* genetic and breeding for identification of cultivars (AGARWAL *et al*., 2001, MILATOVIĆ *et al*., 2009, NIKOLIĆ *et al*., 2010), detection of phylogenetic relationships among species (DAEIL, 2004), also for analysis of genetic variability in native populations (GAŠIĆ *et al*., 2001, ČOLIĆ *et al.*, 2010) and construction of gene linkage maps (CLARKE *et al*., 2009). Although molecular DNK based markers were dominantly used in the last decade, studies of isoenzyme polymorphism conducted in the last twenty years in genus *Prunus*, subgenus *Cerasus* verify that this technique was efficient to detect polymorphism. According to DAEIL (2004) MDH and GPI were the most polymorphic and most valuable to identify genetic relationships among the taxa in subgenus *Cerasus*. Some genotypes with identical morphological characters and previously treated as one cultivar can be separated on the basis of isoenzyme genotype. Recently, CORTS *et al*. (2008) used extracts from young leaves of nine sweet and eight sour cherry varieties for analysis of five isoenzyme systems in order to characterize these varieties and

detect problems of synonymies and homonymies that frequently present and found that PGM and PGI had highest discrimination power.

Molecular DNA based markers become an essential tool in genus *Prunus* genetic studies (MARTÍNEZ-GÓMEZ *et al*., 2003). Different types of molecular markers RFLPs (BOUHADIDA *et al*., 2007), RAPDs (ZAMANI *et al*., 2012), AFLPs (TAVAUD *et al*., 2004) and SSRs (ERCISLI *et al*., 2011) have been used for the genetic characterization of germplasm and the establishment of genetic relationships between cultivars and species.

The objective of our study was to evaluate dehydrogenase isoenzyme polymorphism of six species in genus *Prunus*, subgenus *Cerasus* including cherry germplasm from the rich native flora of Serbia and four interspecific hybrids 'Gisela 5', 'Gisela 6', 'Max Ma' and 'Colt'. The goal was to establish usability of studied isoenzymes in identification of genetic diversity, relationships among genotypes so as intraspecies and intracultivar variability.

MATERIALS AND METHODS

The plant material represents 36 sour (*Prunus cerasus L.*), sweet (*Prunus avuim* L.), mahaleb (*Prunus mahaleb* L.), ground (*Prunus fruticosa* Pall.), duke (*Prunus gondounii* Redh.) and Japanese flowering cherry (*Prunus serrulata* Lindl.) genotypes. Four widely-used standard cherry rootstocks 'Gisela 5', 'Gisela 6', 'Max Ma' and 'Colt' were included. Eight 'Oblačinska' (autochthonous and heterogeneous cultivar), four wild sweet cherry, six ground and one mahaleb genotypes was selected from native flora in Serbia. Selection of genotypes was done according to observed diversity of phenology and morphological traits of tree and fruits.

Seven isoenzyme systems - alcohol dehydrogenase (ADH), formate dehydrogenase (FDH), glutamate dehydrogenase (GDH), isocitrate dehidrogenaze (IDH), malate dehydrogenase (MDH), phosphogluconate dehydrogenase (PGD) and shikimate dehydrogenase (SDH) were analyzed. Inner bark of one-year-old shoots was used for enzyme extraction. Preparation of samples was done in accordance with the protocol given by BOŠKOVIĆ *et al*. (1994) for stone fruit species. Vertical PAGE was used for isoenzyme analysis. Polyacrylamide gel containing 8% acrylamide was used for separation. Staining procedures were essentially based on the protocol for isoenzymes given by BOŠKOVIĆ *et al*. (1994). Gels were visually observed and the bands represent isoenzyme patterns, called zymograms, were analyzed. Genetic interpretations for regions attributed to polymorphic loci were proposed. Cluster analysis was done with all polymorphic loci using the UPGMA method. For cluster analysis the data of polymorphic loci were transformed into 0/1 code. Statistical analysis was conducted with the program 'Statistica' (StatSoft, Inc., Tulsa, Oklahoma, USA).

RESULTS AND DISCUSSION

All seven analyzed enzyme systems were polymorphic at 10 loci: *Adh -1* and *Adh-2, Fdh-1*, *Gdh-1*, *Idh-1* and *Idh -2*, *Mdh-1*, *Pgd*-1, *Sdh-1* and i *Sdh-2*. The patterns of polymorphic systems for each of the studied genotype was presented in the Table 1.

Tab. 1. Zymogram patterns of seven dehydrogenase systems in genus Prunus, subgenus Cerasus

No	*Cultivar/ genotype*	*Species /Interspecific hybrid*	*Zymogram patterns*						
			ADH	FDH	GDH	IDH	MDH	PGD	SDH
1	Drogans yellow	*P. avium*	2	1	1	3	1	1	1
2	Celeste	*P. avium*	2	1	1	3	1	1	1
3	Victoria	*P. avium*	2	1	1	5	1	2	1
4	Early Star	*P. avium*	2	1	1	3	1	1	1
5	Vera	*P. avium*	2	1	1	3	1	1	2
6	Sara	*P. avium*	2	1	1	6	1	2	1
7	NS KK 6/10	*P. avium*	2	1	3	2	1	2	2
8	DT X9	*P. avium*	5	1	1	5	1	1	7
9	DT X3	*P. avium*	5	1	1	5	1	1	7
10	DT X7	*P. avium*	5	1	2	5	1	1	7
11	DT K9	*P. avium*	5	1	4	11	1	1	7
12	MD	*P. serrulata*	4	1	1	4	1	3	1
13	BNS	*P. serrulata*	4	2	1	6	2	1	3
14	Amanogawa	*P. serrulata*	4	1	1	1	1	1	3
15	Lara	*P. cerasus*	1	1	1	10	3	2	1
16	Montmorency	*P. cerasus*	1	1	1	12	3	5	1
17	Rexelle	*P. cerasus*	1	1	1	10	3	2	1
18	Keleris 16	*P. cerasus*	1	1	1	12	3	5	1
19	Maynard	*P. cerasus*	3	1	3	9	3	2	2
20	Oblačinska UD 1	*P. cerasus*	1	1	1	8	2	2	2
21	Oblačinska UD 8	*P. cerasus*	1	1	1	10	2	2	1
22	Oblačinska UD 6	*P. cerasus*	1	1	1	7	4	2	2
23	Oblačinska D1 R	*P. cerasus*	1	1	1	10	2	2	2
24	Oblačinska D4 R	*P. cerasus*	1	1	1	10	2	2	2
25	Oblačinska II/10 R	*P. cerasus*	1	1	1	10	2	2	2
26	Oblačinska XI/3 R	*P. cerasus*	1	1	1	10	2	2	2
27	Oblačinska D4 RŠ	*P. cerasus*	1	1	1	10	2	2	2
28	SV 1	*P. fruticosa*	1	1	3	6	2	5	2
29	SV 2	*P. fruticosa*	1	1	3	6	2	5	2
30	SV 3	*P. fruticosa*	2	1	1	10	2	2	2

31	SV 4	*P. fruticosa*	2	1	1	10	4	5	2
32	SV 5	*P. fruticosa*	2	1	1	10	4	5	2
33	SV 7	*P. fruticosa*	2	1	1	4	2	4	2
34	Radmilovac	*P. gondouinii*	8	1	1	5	2	3	5
35	Uroš	*P. gondouinii*	9	1	1	3	2	3	5
36	TT	*P. mahaleb*	6	1	1	10	3	3	3
37	Colt	*P. avium x P. pseudocerasus*	2	1	2	2	1	2	4
38	Gisela 5	*P.cerasus x P. canescens*	1	1	3	10	4	2	1
39	Gisela 6	*P.cerasus x P. canescens*	1	1	1	7	4	2	1
40	Max Ma	*P. mahaleb x P. avium*	1	1	1	4	1	1	6

Alcohol dehydrogenase. ADH analysis resulted in zymograms (Fig. 1) with two regions of activity *Adh-1* and *Adh-2*. Contrary to SEKER (2008), who observed that *P. avium* showed polymorphism only for loci *Adh-1* we obtained variability in two regions. Wild sweet cherry was monomorphic and homozygous for both loci, while *P. avium* cultivars were monomorphic but heterozygous for loci Adh-1 and homozygous for Adh-2. Japanese flowering cherry was monomorphic for both loci. Locus *Adh-1* was homozygous, while *Adh-2* was heterozygous and showed three bands. For *P. cerasus* polymorphism was obtained for *Adh-2* loci that had *bb* and *ab* genotypes. Genotypes of *P. fruticosa* showed activity in both regions, but polymorphism only for *Adh-2* that showed homozygous genotype *aa* and heterozygous genotype *ab*. No activity for *Adh-1* and variability for presence or absence of activity in *Adh-2* recorded *for P. gonduinii.* Among all samples *P. mahaleb* had unique zymogram with genotype *bb* in loci *Adh-1* and genotype *bc* in *Adh-2* loci. Zymograms of Max Ma, Gisela 5, Gisela 6 and Colt had the same heterozygous pattern *ab* for loci *Adh-1*. Only difference that we found for loci *Adh-2* showed that Colt (homozygous *aa*) can be distinguish from other rootstocks (heterozygous *ab*). Three bands for heterozygous genotype that indicate dimeric structure of ADH found for sour, wild, sweet, flowering and ground cherry and four iterspecific hybrids but not for duke cherry and mahaleb.

Formate dehydrogenase. Literature indicates that, so far, this system was not studied in subgenus *Cerasus*. This system was characterized with low activity and variability in one locus marked as *Fdh-1*. Two types of zymogram were present (Fig. 2). Only one sample of *P. serulatta* had heterozygous genotype *ab* with three bands characterized dimeric structure, described also in almond (ČOLIĆ *et* al., 2009). Other 39 genotypes had homozygous genotype *bb*.

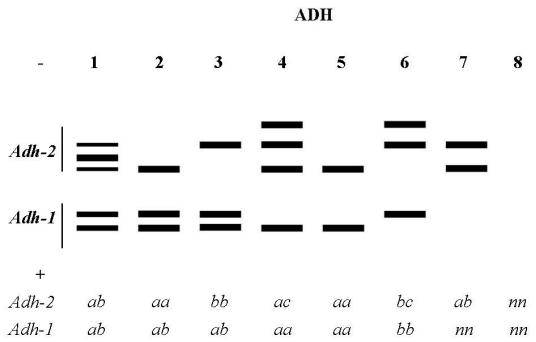

Fig. 1. Zymogrames obtained for ADH

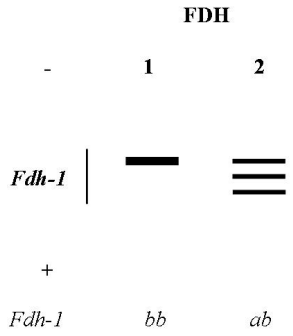

Fig. 2. Zymogrames obtained for FDH

Glutamate dehydrogenase. On GDH zymograms activity was observed in the region closer to the cathode (Fig. 3). Two alleles and three genotypes were proposed for the loci marked as *Gdh -1*. The most genotypes were *aa*, two *P. fruticosa,* one *P. avium* and one *P. cerasus* had *ab* genotype while one wild cherry and Colt had *bb* allelic constitution. One genotype of wild cherry showed no activity for GDH. Presence of seven bands in heterozygote phenotypes indicates hexameric structure reported also for almond by ČOLIĆ *et al.* (2009). The greatest polymorphism and three genotypes (*aa, ab, and bb*) for GDH showed *P. avium* This is the first report of

GDH polymorphism in subgenus *Cerasus*. GDH polymorphism was observed in the other stone fruit species such as peach (GAŠIĆ *et al*., 2001) and apricot (MILATOVIĆ *et al*., 2009).

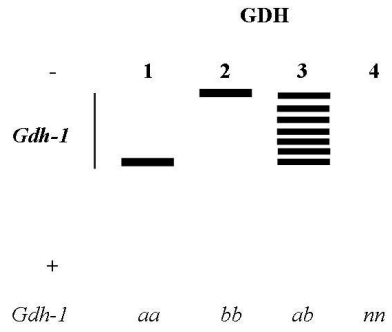

Fig. 3. Zymogrames obtained for GDH

Isocitrate dehydrogenase. IDH showed twelve different zymograms (Fig. 4) being the most polymorphic system. Both *P. avium* and *P. cerasus* showed five patterns. In accordance with previously reported results of PASHKOULOV *et al.* (2000) activity was visible in two regions. All genotypes showed activity for loci *Idh-1*, with three alleles (*a*, *b* and *c*) and four genotypes (*ab*, *bb*, *bc* and *cc*). For loci *Idh-2* we observed polymorphism and variabilty in presence or absence in activity. Three alelles and five genotypes (*aa*, *ab*, *ac, bb* and *bc*) were identified. From all observed loci in this study we found that *Idh-2* was most polymorphic. The greatest polymorphism for IDH exhibited *P. cerasus,* with discriminated five genotypes. Unique patterns observed for *P. serrulata* cv Amanogava, one sweet cherry and one "Oblačinska" genotype. Our findings about one region of activity for wild cherry and mahaleb are in accordance to results of SEKER (2008).

Malate dehydrogenase. MDH analysis resulted with observable variability in one region of activity marked as *Mdh-1* (Fig. 5), and presence of alleles *a* and *b*. Homozygous allelic constitution *aa* observed for *P. avium, P. serrulata*, Colt and Max Ma. Heterozygous allelic constitution *ab* had sour cherry, duke, ground cherry, Gisela 5 and 6. Variability was expressed in Oblačinska and *P. fruticosa.* We discriminated genotypes with two and four bands. Homozygous allelic constitution *bb* observed for *P. cerasus* and *P. mahaleb*. Contrary to our findings SEKER (2008)

reported about *Mdh-1* and *Mdh-2* loci. These differences can be attributed to different tissue for extraction (leaf), and different type of gel (starch).

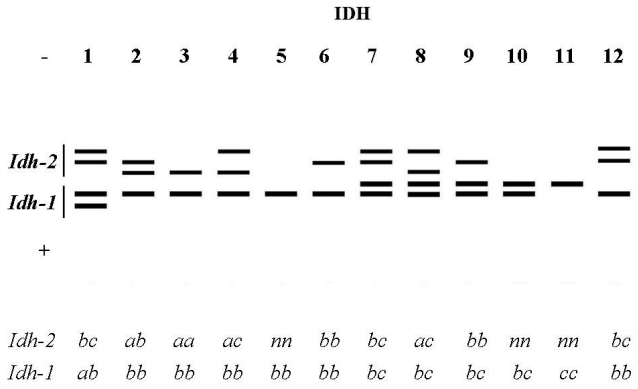

Fig. 4. Zymogrames obtained for IDH

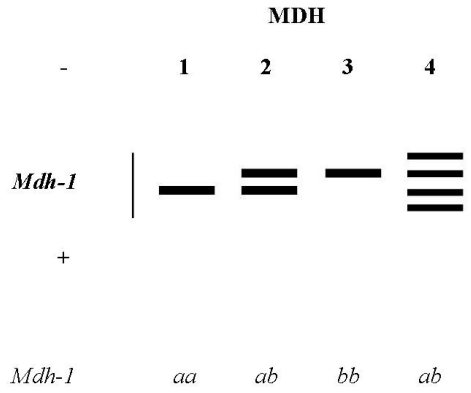

Fig. 5. Zymogrames obtained for MDH

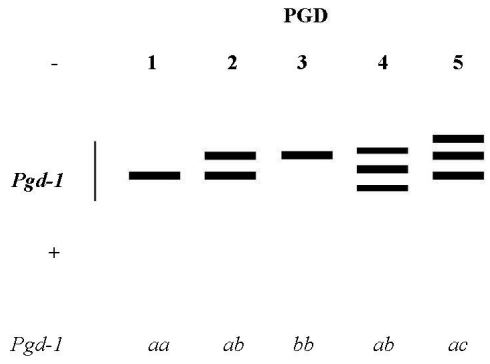

Fig. 6. Zymogrames obtained for PGD

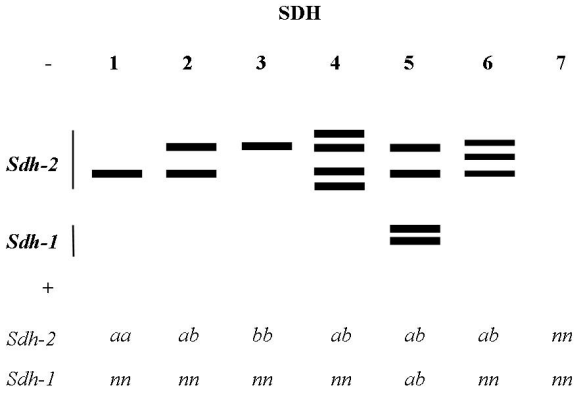

Fig. 7. Zymogrames obtained for SDH

Phosphogluconate dehydrogenase. One polymorphic region of activity was observed on PGD zymograms (Fig. 6) in the region closer to the cathode. Three alleles and four genotypes (aa, ab, ac and bb) were distinguished. The PGD system displayed five patterns. Homozygous allelic constitution *aa* obtained for wild cherry, while *bb* observed for *P. gonduinii* and *P. mahaleb*. Two allelic constitutions *aa* and *ab* obtained for sweet, *aa* and *bb* for flowering and *ab* and *ac* for ground and sour cherry. Zymograms of Gisela 5, Gisela 6 and Colt had the same heterozygous genotype *ab* while Max Ma was homozygous (*aa*). That difference can distinguish Max Ma from other rootstocks. More variability in sweet cherry than CORTS *et al*. (2008) reported can be explained by wider genetic basis of genotypes in our research. Our findings indicate dimeric structure of loci for sour and ground cherry.

Shikimate dehydrogenase. On SDH zymograms (Fig. 7) two regions with bands was observed, but activity for loci *Sdh-1* was showed only for *P. gonduinii*. That difference can distinguish *P. gonduinii* from other taxa in this research. For wild cherry we observed no activity for this system, while sweet cherry showed greatest polymorphism with two alleles (*a* and *b*) and two types of zymograms. For *Sdh-2* two allelic constitution obtained for sour and flowering cherry (*aa* and *ab*, *aa* and *bb,* respectively*).* Intraspecific variability was detected for Oblačinska sour cherry, where we distinguish *aa* and *ab* genotypes. Diversity of Oblačinska sour cherry clones on the morphological, chemical and pomological level has been approved in research of NIKOLIĆ *et al*. (2005) and RAKONJAC *et al*. (2010). No intraspecific variability for SDH and only *ab* allelic constitution was detected within the *P. fruticosa* genotypes. Unique four band pattern for SDH represented Colt. Two unique bands were probably incorporated from *P. pseudocerasus* genome. Dimeric structure of loci *Sdh-2* was recorded for Max Ma.

Tab. 2. Polymorphic loci obtained for seven isoenzymatic systems

Genotype	Species/Hybrid	No of polymorphic loci	Polyimorphic loci
Wild cherry	*P. avium*	4	*Gdh-1, Idh-2, Pgd-1, Sdh-2*
Sweet cherry	*P. avium*	2	*Gdh-1, Idh-1*
Flowering cherry	*P. serrulata*	6	*Fdh-1, Gdh-1, Idh-1, Idh-2, Pgd-1, Sdh-2*
Sour cherry	*P. cerasus*	6	*Adh-2, Gdh-1, Idh-1, Idh-2, Pgd-1, Sdh-1*
Oblačinska	*P. cerasus*	1	*Idh-2, Sdh-2*
Ground cherry	*P. fruticosa*	5	*Adh-2, Gdh-1, Idh-1, Idh-2, Pgd-1*
Duke cherry	*P. gonduinii*	2	*Adh-2, Idh-2*

Greatest dehydrogenase variability showed *P. serrulata* and *P. cerasus* that had six polymorphic loci (Tab. 2). Of 40 studied cherry genotypes, nineteen showed unique zymogrames and can be distinguished from other genotypes. Also, on the basis of unique polymorphism in one locus species can be discriminate mutually. Among ten polymorphic loci *Fdh-1* was unique for *P. serrulata* and locus *Sdh-1* for *P. cerasus*.

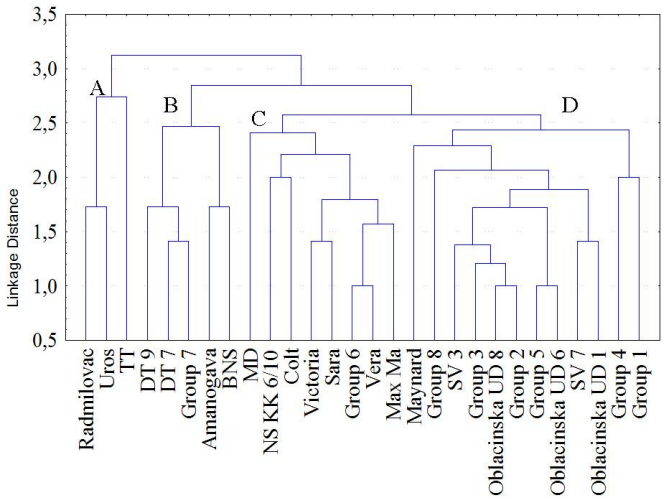

Fig. 8. Dendrogram of 40 analyzed genotypes generated from the isoenzyme data by UPGMA cluster analysis
Group 1- SV1, SV2; *Group 2*- Oblačinska višnja: D1 R, D4 R, II/10 R, XI/3 R, D4 RŠ; *Group 3*- Lara, Rexelle; *Group 4*- Montmorency, Keleris 16; *Group 5*- Gisela 5, Gisela 6; *Group 6*- Drogans yellow, Celeste, Early Star; *Group 7*- DT X9, DT X3; *Group 8*-SV4, SV5

Application of cluster analysis on all polymorphic loci resulted in a dendrogram shown on Figure 8. Analyzed genotypes are connected on different hierarchical levels. Four clusters were identified and among them eight groups of genotypes with the same isoenzymatic profile. Dendrogram showed clear separation of Cluster A, represent *P. mahaleb* and two genotypes of *P. gonduinii*. Six genotypes that were split into subgroup of wild cherry and subgroup of flowering cherry represent cluster B includes. Ten genotypes grouped in cluster C: seven sweet cherry, one flowering cherry and rootstocks Colt and Max Ma (interspecies hybrids where *P. avium* is aone of the parents). Our results for different linkage between

Victoria, Sara, Vera and Maynard than reported by LJUBOJEVIĆ *et al*. (2012) showed that morphological variability did not always correspond with molecular characterisation. Cluster D consisted of 15 *P. fruticosa* and *P. cerasus* genotypes. Although we expected that all genotypes of Oblačinska will fall in the same group, results showed discrimination into three subgroups. Also three genotypes of Oblačinska had unique isoenzyme profile. This diversity corresponds to variability obtained for loci *Idh-2* and *Sdh-2*. Significant discrimination observed also for *P. fruticosa*, separated in two groups and two unique genotypes.

Classification into four clusters corresponds with origin of genotypes, while further discrimination into subgroups of related genotypes can not be clearly defined.

CONCLUSION

A significant erosion of genetic diversity of cereals was observed at the visited areas. A large number of landraces and old varieties of small grains, which represent the original genetic diversity, have been lost. Former remote villages and farms, during a past few decades, have become more accessible, in most of cases that caused the replacement of landraces by cultivated varieties or land has changed purpose. However, in isolated, remote villages and farms, lacking good roads or without any road network, the last traces of almost exterminated cereal biodiversity still could be found. The urgent and prompt action is required to preserve and regenerate endangered genetic diversity.

Received July30th, 2012
Accepted November 30th, 2012

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POLIMORFIZAM DEHIDROGENAZA RODA *Prunus*, PODROD *Cerasus*

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Polimorfizam dehidrogenaza proučavan je kod 36 genotipova višnje (*Prunus cerasus L.*), trešnje (*P. avuim* L.), magrive (*P. mahaleb* L.), stepske višnje (*P. fruticosa* Pall.), marele (*P. gondounii* Redh.), japanske ukrasne trešnje (*P. serrulata* Lindl.) kao i četiri interspecies hibrida koji se koriste kao standardne podloge za trešnju: Gisela 5, Gisela 6, Max Ma i Colt.. Za pripremanje enzimskog ekstrakta korišćena je unutrašnja kora jednogodišnjih grančica, prikipljenih u fazi mirovanja. Metoda vertikalne poliakrilamidne gel elektroforeze (PAGE) korišćena je za razdvajanje proteina za analizu enzimskih sistema: ADH (alkohol dehidrogenaze), FDH (format dehidrogenaze), GDH (glutamat dehidrogenaze), IDH (izocitrat dehidrogenaze), MDH (malat dehidrogenaze), PGD (fosfoglukonat dehidrogenaze) i SDH (šikimat dehidrogenaze). Polimorfizam je utvrđen za sve proučavane sisteme. Ukupno je utvrđeno 10 polimorfnih lokusa i to Adh -1 (3 genotipa) i Adh-2 (5 genotipova), Fdh-1 (2 genotipa), Gdh-1 (3 genotipa), Idh-1 (4 genotipa) i Idh -2 (5 genotipova), Mdh-1 (3 genotipa), Pgd-1 (4 genotipa), Sdh-1 (1 genotip) i Sdh-2 (3 genotipa). Primenom klaster analize dobijen je dendrogram na kome se mogu izdvojiti četiri grupe srodnih genotipova. Dobijeni rezultati ukazuju da se proučavani sistemi mogu uspešno koristiti u determinaciji roda *Prunus*, podroda *Cerasus*. Za utvrđivanje genetičke varijabilnosti najveći značaj imaju enzimski sistemi ADH, IDH i SDH. Polimrfizam FDH i GDH roda *Prunus*, podroda *Cerasus* je po prvi put opisan u ovom radu. Prvi rezultati o varijabilnosti dehidrogenaza kod Oblačinske višnje ukazuju da polimorfizam lokusa *Idh-2* i *Sdh-2* može biti koristan za identifikaciji klonova.

Primljeno 30. VII. 2012
Odobreno 30. XI. 2012