# Isoenzyme polymorphism of almond genotypes selected in the region of northern Serbia

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**Abstact**

Čolić S., Milatović D., Nikolić D., Zec G., 2010. **Isoenzyme polymorphism of almond genotypes selected in the region of northern Serbia**. Hort. Sci. (Prague), *37*: 56–61.

Isoenzyme polymorphism was studied in 20 almond (*Prunus dulcis* [Mill.] D.A. Webb) genotypes selected from seedling populations of unknown almond cultivars in the region of northern Serbia (Vojvodina). Fourteen enzyme systems were studied using the method of vertical polyacrylamide gel electrophoresis. Ten systems were polymorphic in twelve loci. This polymorphism allowed unique identification of all studied genotypes. The most useful enzyme for analysis of almond genetic variability was menadione reductase. Polymorphism identified for alkaline phosphatase, formate dehydrogenase, glutamate dehydrogenase, malic enzyme, and menadione reductase was reported for the first time in almond. Cluster analysis was used to construct a dendrogram on which five clusters with different number of genotypes could be identified.

**Keywords**: *Prunus dulcis*; cluster analysis; electrophoresis; isoenzymes

Isoenzyme variability is an abundant source of genetic markers that can be used for identification of cultivars and interspecific hybrids, early selection, monitoring of genetic diversity, and quantification of genetic relationships among populations (Byrne 1990). Isoenzymes have several advantages over traditional morphological traits because they are not influenced by environmental factors, making identification possible in early stages of development and thereby saving both time and space (Torres 1990). Also, isoenzymes can be markers for genes controlling economically important traits, so they have been used in creating genetic maps (Martínez-Gómez et al. 2003). Even though DNA markers, especially SSRs (simple sequence repeats) are becoming the predominant tool for genetic analysis, they still require expensive equipment not available in many countries. For these reasons, isoenzymes are still useful markers for genetic polymorphism identification (Daell 2004).

Isoenzyme variability of the genus *Prunus* was studied in peach (Messeguer et al. 1987; Gašić et al. 2001), apricot (Manganaris et al. 1999; Milatović et al. 2009), plum (Byrne, Littleton 1988), and cherry (Corts et al. 2008). Byrne (1990) reported high isoenzyme variability in almond, considerably higher than in other stone fruit species, which is attributed to almond self-incompatibility.

Arulsekar et al. (1986) were the first to study isoenzyme polymorphism in almond. They detected isoenzyme variability in all six studied enzyme systems: glucose phosphate isomerase (GPI), leucine aminopeptidase (LAP), aspartate amynotransferase (AAT), phosphoglucomutase (PGM), malate dehydrogenase (MDH), and 6-phosphogluconate

Supported by the Ministry of Science and Technological Development of the Republic of Serbia, Project No. TR 20103.

dehydrogenase (6PGD). Cerezo et al. (1989) observed polymorphism for catalase (CAT) and acid phosphatase (ACP). Jackson and Clarke (1991) found isocytrate dehydrogenase (IDH), 6PGD, and shikimate dehydrogenase (SDH) to be polymorphic systems in almond, having a dimeric structure. Polymorphism for aconitase (ACO) and malate dehydrogenase (MDH) in almond was observed by Arús et al. (1994). Vezvaei (2003) discovered new alleles for IDH, AAT, PGM, GPI, and SDH in a larger number of almond cultivars and six wild species originating from Iran.

The objective of this paper was to study polymorphism for 14 enzyme systems in 20 almond genotypes selected from the Vojvodina region of northern Serbia and to estimate their importance for establishing almond genetic variability.

## MATERIAL AND METHODS

Twenty almond genotypes were studied. They were sampled from large seedling populations of unknown cultivars in the region of northern Serbia-Vojvodina. These genotypes were selected based on some desirable pomological properties such as late blooming and regular cropping. Fourteen enzyme systems were analyzed: acid phosphatase (ACP), alcohol dehydrogenase (ADH), alkaline phosphatase (AKP), esterase (EST), formate dehydrogenase (FDH), glutamate dehydrogenase (GDH), glutamate oxaloacetate transaminase (GOT), malate dehydrogenase (MDH), malic enzyme (ME), menadione reductase (MNR), phosphogluconate dehydrogenase (PGD), phosphoglucomutase (PGM), peroxidase (PRX), and shikimate dehydrogenase (SDH).

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. Analysis of isoenzymes was carried out by the vertical polyacrylamide gel electrophoresis method. Gels containing 8% acrylamide were used for separating all enzymes, except for GOT, GDH, and ME where 6% gels were used. Electrophoresis was performed at +4°C and consisted of three phases. Prior to sample loading, pre-electrophoresis was done for 45 min at 100 V. Thereafter, samples of 25 µl of enzyme extracts were loaded. The second phase lasted 45 min at 100 V. The third phase was carried out at 400 V for 3 to 4 h, depending on the mobility of bands of particular enzymes. For a majority of enzymes staining was performed in accordance with the protocol given by Bošković et al. (1994), and for FDH, GOT, MNR, and PGM in accordance with Wendel and Weeden (1989).

Genetic interpretation for regions attributed to polymorphic loci was proposed. Isoenzyme alleles and loci were labeled in accordance with recommendations given by Weeden (1988) and Tobutt (1993). Data obtained by analysis of polymorphic loci were transformed into the 0/1 code and processed by the method of UPGMA (unweighted pair group average) cluster analysis, and a dendrogram was constructed. Statistical analysis was carried out using the 'Statistica' program (StatSoft, Inc., Tulsa, Oklahoma, USA).

## RESULTS AND DISCUSSION

From 14 analyzed enzyme systems in the studied material, 10 systems showed polymorphism, while variability was not established for 4 systems: ADH, MDH, PGD, and PRX. Analysis of ADH performed by Friend and Carter (1989), Cerezo et al. (1989), and Mowrey et al. (1990) also demonstrated that in almond this is a monomorphic enzyme system. Nor have Byrne (1990) or Mowrey et al. (1990) established variability for PRX, while ALTUBE et al. (2003) found a number of polymorphic zones. Unlike our results, other authors observed variability for MDH (Byrne 1990; Cerezo, Socias I Company 1992) and for PGD (Arulsekar et al. 1986; Cerezo et al. 1989; Friend, Carter 1989; Cerezo, Socias I Company 1992; Arús et al. 1994). Different results are most likely due to the use of other tissues for extraction (leaf) and other types of gel (starch) compared to our study.

Analysis of isoenzyme activity detected polymorphism for the following systems: ACP, AKP, EST, FDH, GDH, GOT, ME, MNR, PGM, and SDH. Table 1 summarizes the data obtained by analyzing 20 almond genotypes for 12 polymorphic isoenzyme loci.

**Acid phosphatase (ACP)**. Three regions of activity were observed. Polymorphism was detected in the zone close to the anode (*Acp*-2) with three phenotypes (*aa, bb*, and *ab*). Zymograms obtained by Hauagge et al. (1987) revealed two monomorphic zones of activity, while Cerezo et al. (1989) and Mowrey et al. (1990) observed ACP polymorphism in almond.

**Alkaline phosphatase (AKP)**. Several zones of activity were detected, of which one was variable

Table 1. Polymorphic loci of 20 almond genotypes from Slankamen hill

| Genotype | *Acp-2* | *Akp-2* | *Est-3* | *Fdh-1* | *Gdh-2* | *Got-1* | *Got-2* | *Me-1* | *Mnr-1* | *Pgm-1* | *Pgm-2* | *Sdh-1* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1/03 | *bb* | *bb* | *bb* | *bb* | *bb* | *bb* | *bb* | *ab* | *ab* | *bb* | *bb* | *bb* |
| 10/03 | *ab* | *ab* | *bb* | *bb* | *ac* | *bb* | *bb* | *ab* | *cc* | *bb* | *ab* | *ab* |
| 11/03 | *aa* | *aa* | *aa* | *ab* | *bb* | *bb* | *bb* | *aa* | *aa* | *bb* | *bb* | *bb* |
| 12/03 | *aa* | *aa* | *bb* | *bb* | *bc* | *bb* | *bb* | *ab* | *ab* | *ab* | *ab* | *bb* |
| 13/03 | *aa* | *aa* | *aa* | *ab* | *bc* | *bb* | *aa* | *ab* | *cc* | *bb* | *bb* | *ab* |
| 14/03 | *ab* | *ab* | *aa* | *aa* | *bb* | *bb* | *bb* | *ab* | *aa* | *bb* | *bb* | *ab* |
| 15/03 | *bb* | *bb* | *aa* | *ab* | *bb* | *bb* | *bb* | *bb* | *ab* | *bb* | *bb* | *bb* |
| 16/03 | *aa* | *aa* | *aa* | *ab* | *bb* | *bb* | *bb* | *ab* | *ab* | *bb* | *bb* | *ab* |
| 17/03 | *ab* | *ab* | *bb* | *ab* | *bb* | *ab* | *bb* | *ab* | *ac* | *bb* | *bb* | *ab* |
| 18/03 | *aa* | *aa* | *aa* | *ab* | *bb* | *bb* | *bb* | *aa* | *ab* | *bb* | *bb* | *bb* |
| 19/03 | *bb* | *bb* | *bb* | *bb* | *bb* | *bb* | *bb* | *bb* | *aa* | *bb* | *bb* | *ab* |
| 22/03 | *bb* | *bb* | *aa* | *ab* | *bb* | *bb* | *bb* | *ab* | *aa* | *bb* | *bb* | *ab* |
| 23/03 | *aa* | *aa* | *bb* | *bb* | *bc* | *ab* | *ab* | *aa* | *ab* | *bb* | *ab* | *ab* |
| 24/03 | *ab* | *ab* | *bb* | *bb* | *bb* | *bb* | *bb* | *ab* | *aa* | *bb* | *bb* | *bb* |
| 25/03 | *aa* | *aa* | *aa* | *bb* | *bb* | *bb* | *bb* | *aa* | *aa* | *bb* | *bb* | *bb* |
| 27/03 | *aa* | *aa* | *bb* | *bb* | *bc* | *bb* | *bb* | *aa* | *aa* | *bb* | *bb* | *aa* |
| 28/03 | *aa* | *aa* | *aa* | *ab* | *bb* | *bb* | *bb* | *ab* | *aa* | *ab* | *bb* | *ab* |
| 29/03 | *aa* | *aa* | *aa* | *ab* | *bc* | *bb* | *bb* | *ab* | *aa* | *ab* | *bb* | *ab* |
| A/04 | *ab* | *ab* | *aa* | *ab* | *bb* | *bb* | *bb* | *ab* | *cc* | *ab* | *ab* | *ab* |
| B/04 | *ab* | *ab* | *aa* | *bb* | *bb* | *bb* | *bb* | *ab* | *cc* | *bb* | *bb* | *bb* |

(*Akp-2*) and identical to locus *Acp-2*. Identity of *Akp-2* and *Acp-2* loci was also described in sweet cherry (Bošković, Tobutt 1998), peach (Gašić et al. 2001), and apricot (Milatović et al. 2009).

**Esterase (EST)**. A larger number of zones of activity were observed, however, only the zone marked as *Est-3* was clear enough for analysis. Presence of bands *a* and *b* and homozygous phenotypes *aa* and *bb* were detected in *Est-3*. Mamouni et al. (1998) and Altube et al. (2003) observed two polymorphic loci. Polymorphism for EST extracted from pollen is higher than that from leaf (Mamouni et al. 1998).

**Formate dehydrogenase (FDH)**. One polymorphic zone of activity close to the anode was obtained. This locus (*Fdh-1*) had two alleles (*a* and *b*) and three phenotypes (*aa, bb*, and *ab*). The zymograms show three bands in heterozygous phenotype, which is in accordance with reports by Weeden and Wendel (1989) about a dimeric structure of FDH (Fig. 1).

**Glutamate dehydrogenase (GDH)**. Two zones of activity were found, of which the anodal one was polymorphic. This locus (*Gdh-2*) had three alleles (*a, b*, and *c*) and three phenotypes were proposed: *bb, ac*, and *bc*. Even though the separation of bands was slightly weaker, seven bands were observed to occur in heterozygous phenotypes *ac* and *bc*. This indicates a hexameric structure of GDH, which is in accordance with reports by Weeden and Wendel (1989). Unlike our results, the analysis of this isoenzyme in almond pollen, performed by Mowrey et al. (1990), did not prove its variability.

**Glutamate oxaloacetate transaminase (GOT)**. Two polymorphic zones were observed in the zymograms. Both zones (*Got-1* and *Got-2*) had two alleles (*a* and *b*) each. The locus *Got-1* had two (*bb* and *ab*) and the locus *Got-2* had three (*aa, ab*, and *bb*) phenotypes. This enzyme has a dimeric structure (Weeden, Wendel 1989), which is demonstrated by the occurrence of three bands in heterozygous phenotype. Constructed zymograms are in accordance with results reported by Arulsekar et al. (1986), Arús et al. (1994), and Cerezo and Socias

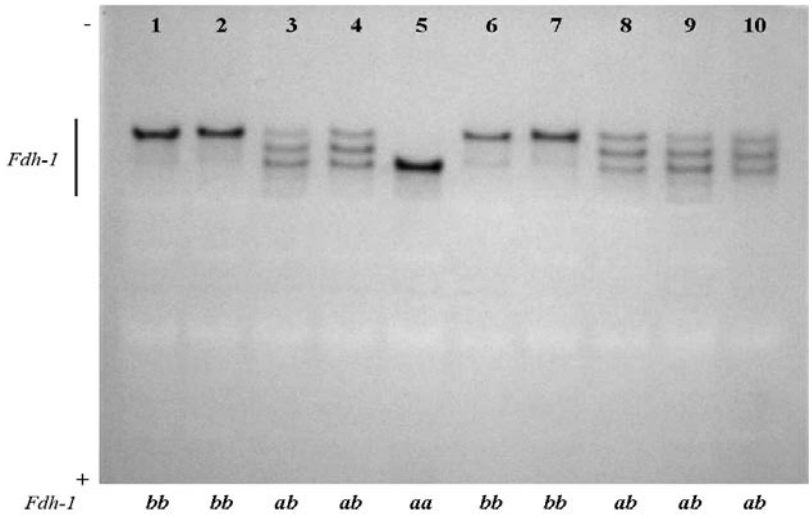


Fig. 1. FDH (formate dehydrogenase) zymograms of analyzed almond genotypes with proposed genetic interpretation: 12/03 (1), 23/03 (2), 29/03 (3), 11/03 (4), 14/03 (5), 27/03 (6), 19/03 (7), B/04 (8), 22/03 (9), 18/03 (10)

I Company (1992). The largest polymorphism was observed by Vezvaei (2003) in an Iranian population, which is connected to the fact that this region is the primary gene center for almond.

**Malic enzyme (ME)**. This enzyme exhibited activity in one polymorphic locus (*Me-1*) with two alleles (*a* and *b*) and three phenotypes (*aa, bb*, and *ab*). Separation of bands was possible due to a longer electrophoretic procedure and a decrease of gel concentration. A dimeric structure of this enzyme is observed in the zymograms. Previous studies (Friend, Carter 1989; Mowrey et al. 1990) carried out by leaf extractions and by using starch gel did not show variability for ME in almond.

**Menadione reductase (MNR)**. According to Friend and Carter (1989) this is a monomorphic system. In our study variability was observed for the first time. There were three regions of activity, of which the central one (*Mnr-1*) was polymorphic. The locus *Mnr-1* had alleles *a*, *b*, and *c.* Four different phenotypes (*aa*, *ab*, *ac*, and *cc*) were obtained (Fig. 2).

**Phosphoglucomutase (PGM)**. Two zones of activity, closer to the cathode, were observed in the zymograms. Both loci (*Pgm-1* and *Pgm-2*) had two bands (*a* and *b*) and two phenotypes (*bb* and *ab*), which is in accordance with reports by Hauagge et al. (1987). Mowrey et al. (1990) observed four alleles in the locus *Pgm-1.* The highest polymorphism was detected by Vezvaei (2003) for *Pgm-2* (presence of a new allele *f*) in the Iranian population of almond.

**Shikimate dehydrogenase (SDH)**. One polymorphic locus (*Sdh-1*) with two alleles (*a* and *b*) and three phenotypes (*aa*, *bb*, and *ab*) were revealed. The same results were achieved by Cerezo and Socias I Company (1992) as well as Vezvaei (2003).

Genetic relationships among genotypes were established using UPGMA cluster analysis of isoenzyme loci. A dendrogram was constructed, where

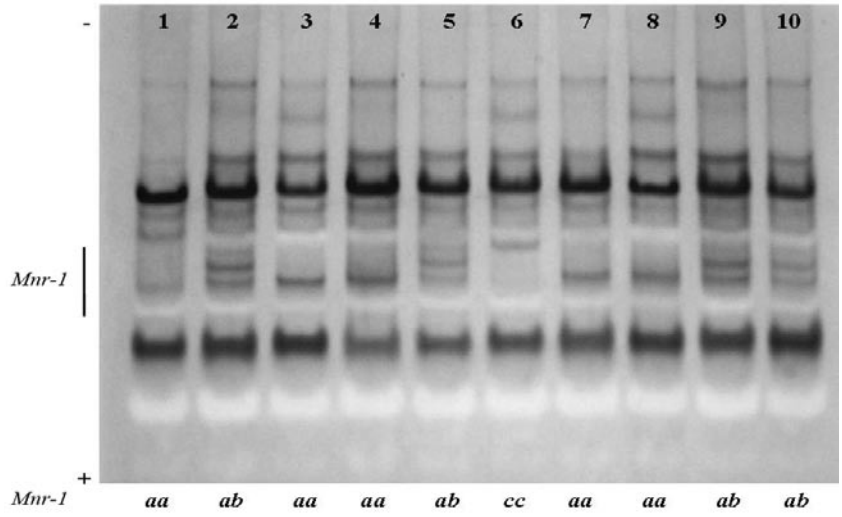


Fig. 2. MNR (menadione reductase) zymograms of analyzed almond genotypes with proposed genetic interpretation: 28/03 (1), 12/03 (2), 27/03 (3), 14/03 (4), 1/03 (5), 10/03 (6), 19/03 (7), 22/03 (8), 16/03 (9), 15/03 (10)

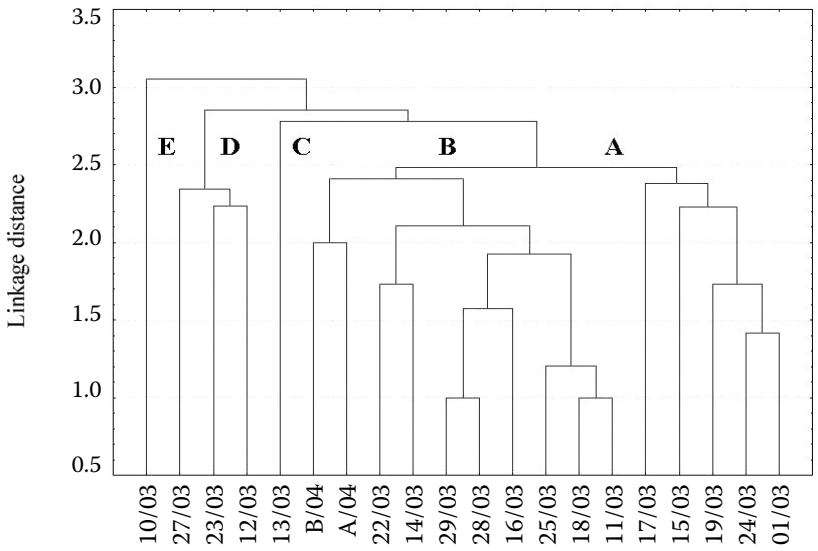


Fig. 3. Dendrogram of 20 analyzed almond genotypes generated from the isoenzyme data by UPGMA cluster analysis

five clusters with different numbers of genotypes were observed (Fig. 3). Cluster A contained five genotypes of which 24/03 and 01/03 are genetically most closely related (d = 1.4). Cluster B was the largest and placed 10 genotypes into three groups. In this cluster genotypes 28/03 and 29/03 and 11/03 and 18/03 were genetically most closely related (d = 1). Cluster C comprised only one genotype. Cluster D consisted of three genotypes, genetically fairly distant. Only one genotype (10/03) genetically most distant (d = 3.1) from the rest of genotypes made up cluster E.

The studied almond genotypes are characterized by high genetic variability. Each of the 20 studied genotypes is characterized by a unique isoenzyme profile. Based on characteristic phenotype for only one enzyme locus, the following genotypes could be determined: 14/03 (*Fdh-1 aa*), 13/03 (*Got-2 aa*), 17/03 (*Mnr-1 ac*), and 27/03 (*Sdh-1 aa*). The highest variability was established for MNR, which makes it a good genetic marker.

High genetic variability in almond cultivars and wild species was also established using DNA markers such as Random Amplification of Polymorphic DNA (RAPD) (Bartolozzi et al. 1998; Mir Ali, Nabulsi 2003), Amplified Fragment Length Polymorphism (AFLP) (Sorkheh et al. 2007), and Simple Sequence Repeats (SSR) (Xu et al. 2004; Xie et al. 2006). Although analysis of isoenzymes detects smaller numbers of loci and therefore lower polymorphism, our results showed a sufficient level of variability to distinguish all studied almond genotypes.

Twelve polymorphic loci and 26 alleles were identified in this study. Five new loci, previously not described in the literature, were proposed: *Akp-2, Fdh-1, Gdh-1, Me-1*, and *Mnr-1*. This indicates that isoenzyme polymorphism in almond is higher than previously found.

**Acknowledgements**

The authors gratefully acknowledge the Ministry of Science and Technological Development of the Republic of Serbia for their financial support of this research, as part of the project TR 20103 "New cultivars, selections and growing technologies as factors of fruit production intensification".

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Received for publication September 7, 2009
Accepted after corrections March 22, 2009

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