Leaf peroxidase isozyme polymorphism of wild apple

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Abstract. The main aims of the study were to reveal the isozyme resemblances in the leaf peroxidase of wild apple and to define the traits related to the identification of *Malus sylvestris* Mill. The results of the study are based on leaf isozyme analysis of seven progenies selected according to the specific features of mother trees at their natural sites from the mixed forests of south-western and central Lithuania. The patterns of peroxidase isozymes were obtained by electrophoresis. The presence or absence of isoforms has been applied to compare peroxidase patterns of progenies. Species-specific peroxidase markers of *Malus sylvestris* Mill. were found. The progenies of crab apple trees had the higher proportion of identical siblings in comparison to the progenies of non-crab apple trees; autogamy or even apomixis might be the factor in wild apple if considering the resemblance of some siblings in the progenies confirmed by the simultaneous comparison of their peroxidase isozyme patterns. The phenotypic characterization of rapidly growing reproductive structures (e.g. fruit characters) may be useful for the identification of *Malus sylvestris* Mill. independent of the remaining characters of wild apple trees.

Key words: isozyme resemblances, leaf peroxidase, *Malus sylvestris* Mill., phenotype

INTRODUCTION

Malus sylvestris Mill., the apple species native to Europe, is one of the most endangered tree species in Lithuania; those that remain are very scattered. Therefore, the construction of a gene bank that can be used as a new interbreeding population is seen as a necessary step for its future conservation (Coart et al., 2003). However, this implies the discrimination between 'genuine' wild and cultivated apple trees, which is not straightforward because of the presence of phenotypic intermediates in the forests. The molecular markers applied in the majority of studies concerning hybridization between crops and wild relatives provided a valuable approach to ascertain the identity of putative wild apples. In the past, isoenzyme analyses were a common method for apple cultivar identification (Stephan et al., 2003). Previously, this method had failed to distinguish *M. sylvestris* (in the past represented only by a few single trees), *M. pumila*, *M. orientalis*, *M. asiatica* and *M. sieversii* from each other or those closely related wild species from *M.* × *domestica*. The degree of hairiness of leaves was shown to be of use as a first indication of the origin of an apple tree [*M.* × *domestica*: felted leaf surface, *M. sylvestris*: sparsely hairy (on veins) in spring and hairless in autumn; Remmy & Gruber, 1993; Wagner, 1998; see Wagner (1996) for a review on morphological discrimination between wild and cultivated apple trees] but the resolution of assignment of individuals to the wild and/or cultivated gene pool reached by molecular markers is much higher (Coart, 2003): both AFLP and microsatellite markers revealed a very clear differentiation among the wild gene pool, edible and ornamental apple cultivars, despite the fact that individuals derived from edible cultivars were found in the wild. The correlation between other phenotypic parameters, easy to evaluate in the field, and molecular markers has yet to be studied, including fruit characteristics (form, colour, taste). The main morphological characters that discriminate between wild and cultivated apple are the hairiness of inferior leaf surfaces, presence of thorns on twigs and form, colour and taste of the fruits (Wagner, 1996).

All levels of plant organization are both highly integrated in and attuned to the environment (Niklas, 1994). Metabolic processes are controlled by enzymes, influenced by the environment and used to react in response to it (Delavega, 1996). A range of environmental parameters can also influence the degree of seed set. The seed is one of the forms of dormancy maintaining the survival of the species through adverse environmental conditions that are not conducive to active growth (Osborne, 1981). According to Jefferson (1994), the ability to survive under local stresses can be fixed by apomixis.

Information about the molecular resemblances in the offspring is important to confirm the existence of identical siblings and the possibility of asexual reproduction due to local stresses in the over-utilized forests. Asexual reproduction leads to whole populations being made up of one or a few identical clones (White, 1978).

However, in apomicts, pollen formation often occurs normally in anthers generating viable reduced pollen (Nogler, 1984; Czapik, 1994). An apomict able to generate at least part of its progeny by sexuality is regarded as facultative (Carneiro et al., 2006). Progenies from facultative apomicts segregate into maternal (apomictic) and non-maternal or aberrant (derived from sexuality) classes. Apple trees are able also to produce viable autogamous seeds, but always generate the best part of its progeny by cross-fertilisation (Rejman, 1990). That allows the maintenance of a certain degree of variability among the offspring; there is considerable evidence for this statement (see Asker & Jerling, 1992).

The main aims of the study are to reveal the leaf peroxidase isozyme resemblances in wild apple and to define the traits related to the identification of *Malus sylvestris* Mill. at the local level of south-western and central Lithuania.

MATERIALS AND METHODS

The research sites of wild apples have been chosen in their typical forest habitats of south-western and central Lithuania, distinguishable for its diversity of flora (Karazija, 1988; Vaičiūnas, 2000; Balsevičius, 2001; Balevičienė & Smaliukas, 2003; Stephan et al., 2003; Table 1). The data on phenotypic traits of mother trees were obtained at their natural sites of mixed forests. The 'pure' phenotypes of the European crab apple trees (*Malus sylvestris* Mill.) and apple trees which escaped domestication (*Malus domestica* Borkh.) were determined by the complex of their specific features (Hempel & Wilhelm, 1889; Tuinyla et al., 1990; Wagner, 1995; Navasaitis et al., 2003; Borzan, 2004; Table 2).

Table 1. Distribution and forest habitats of *Malus sylvestris* Mill. in Lithuania (Karazija, 1988; Vaičiūnas, 2000; Balsevičius, 2001; Balevičienė & Smaliukas, 2003; Stephan et al., 2003).

Habitat types (NATURA 2000 Code)	Forest type series	Soil typological groups*	Distribution of habitats in Lithuania
Fennoscandian hemiboreal natural old broad-leaved deciduous and mixed-deciduous forests (9020), sub-Atlantic and medio-European oak or oak-hornbeam forests (9160)	*Carico-mixtoherbosa* *Aegopodiosa* *Aegopodiosa collina* *Oxalido-nemorosa* *Hepatico-oxalidosa* *Fliuviale-oxalidosa*	Ld, Lf Nf, Lf Nf, Lf Ld Šd, Nd Lc, Nc	Central, northern and eastern parts, scattered forests in western and south-western Lithuania

*Abbreviations of soil typological groups are explained in Table 2.

Table 2. Descriptors used in evaluation of site types and tree phenotypes of wild apple.

Soil typological group (Soil TG)	Fertile soils - normally moist (Nc) and temporarily wet (Lc) Very fertile soils - normally moist (Šd, Nd) and temporarily wet (Ld) Particularly fertile soils - normally moist (Nf) and temporarily wet (Lf)
Trunk diameter	cm (at breast height, over the bark; crosswise calliper measurement)
Tree height (H)	Total tree height (m) measured on standing tree by SUUNTO device
Crown diameter (D_{CROWN})	m (at the crown base; crosswise measurement)
Heartwood condition	By evaluation of cores (obtained by age borer at breast height) extending from bark to centre of a trunk: false heartwood is indiscernible / pale / dark / rotten
Thorn bearing	Absent / thorns / pointed shoots
Fruit shape	Flat / globose / conic / ovoid / pear-shaped (fruit cavities of stalks are shallow)
Fruit ground colour	Yellow / yellowish / greenish / green
Fruit over colour	Absent / yellow / reddish
Pattern of over colour	Absent / spots / sides
Hairiness of inferior leaf surfaces	Absent / hairy vein corners / hairy veins / hairy inferior surface

The progenies of wild apple were grown on the open ground at the Lithuanian Institute of Horticulture. With an aim to evaluate the resemblance of siblings seven progenies (108 individuals in total) of the most dissimilar mother trees (Table 3) have been selected for peroxidase analysis. The number of individuals for each progeny is given in Table 4. The choice for progenies rather than for more trees at the locations was made, because we were unable to take samples from many populations: only individually scattered trees have been found at the forests of south-western and central Lithuania.

Table 3. The data of mature wild apple trees from the forest habitats of south-western and central Lithuania (mother trees, progenies of which were examined with the aid of peroxidase analysis, are in bold).

Tree No.	Soil TG	Thorn bearing	Fruit shape	Fruit ground colour	Fruit over colour	Pattern of over colour
26	Nd	Pointed shoots	Flat	Yellow	Absent	-
27	Nd	Pointed shoots	Flat	Yellowish	Absent	-
23	**Nc**	**Pointed shoots**	**Pear-shaped**	**Green**	**Absent**	**-**
22	**Lf**	**Thorns**	**Flat**	**Yellowish**	**Reddish**	**Side**
14	Ld	Thorns	Flat	Green	Absent	-
25	Ld	Thorns	Globose	Yellow	Absent	-
3	**Nc**	**Thorns**	**Globose**	**Yellowish**	**Absent**	**-**
8	Ld	Thorns	Globose	Yellowish	Absent	-
15	Ld	Thorns	Conic	Yellowish	Absent	-
11	Nd	Thorns	Ovoid	Yellowish	Absent	-
17	Nd	Absent	Flat	Yellowish	Yellow	Side
19	Nf	Absent	Globose	Yellowish	Yellow	Both sides
13	**Lc**	**Absent**	**Globose**	**Yellowish**	**Absent**	**-**
4	**Ld**	**Absent**	**Globose**	**Yellowish**	**Absent**	**-**
6*	Nd	Absent	Globose	Yellowish	Yellow	Side
1	**Ld**	**Absent**	**Globose-torose**	**Yellowish**	**Reddish**	**Spots**
7	Nd	Absent	Globose	Greenish	Yellow	Side
9	Ld	Absent	Globose	Greenish	Reddish	Spots
16	Ld	Absent	Globose	Green	Absent	-
20	Ld	Absent	Globose	Green	Absent	-
10	Šd	Absent	Conic	Greenish	Absent	-
5	Ld	Absent	Ovoid	Yellowish	Absent	-
18	**Šd**	**Absent**	**Pear-shaped**	**Yellow**	**Absent**	**-**

*Apple tree with felted hairy leaves.

In the field the observation of non-segregation of maternal phenotypic characteristics is an indication of apomixis (Carneiro et al., 2006). In this case, identity with the mother plant means the homogeneity of the progeny and the offspring will be identical clones of each other (Jankiewicz & Orlikowska 1990; Shen et al., 2003). Furthermore, asexual reproduction leads to whole populations being made up of one or a few clones (White, 1978).

The results of the study are based on leaf peroxidase (EC 1.11.1.7.) analysis of wild apple's siblings. The fresh leaves of third-year siblings were collected in orchards in morning during the middle of July 2004. It has been determined that the number of peroxidase bands changes during the vegetation period in apple cultivars (Gelvonauskis & Šikšnianienė, 2001) and the highest number and well detectable bands of leaf peroxidase are obtained in July.

Leaf samples (0.25 g) were homogenized in Tris-Glycine buffer (pH 8.3). To distinguish isozyme ‘runs’ blue dye of bromphenol was added. Electrophoresis was performed in vertical polyacrylamide gels (Davis, 1964). Polymerization of concentrating gels was initiated with the use of an ultraviolet lamp. After that the electrophoresis gels were stained for peroxidase in a solution of o-dianisidine, benzcatechine, supplemented with hydrogen peroxide (Jaaska, 1972). The relative mobility of peroxidase (PRX) isozymes was determined from the 'runs' in polyacrylamide gels as $Rf = r_i R^{-1}$, where r_i – the distance from the starting point of a 'run' to the position of an isozyme, R – the distance from the starting point of a 'run' to the blue band of bromphenol at the bottom of the gels.

The patterns of peroxidase isozymes were obtained by electrophoresis twice during the investigation. The two analyses gave the same results. The presence or absence of isoforms was determined to compare peroxidase patterns of Lithuanian wild apple progenies (classes of estimation: 1 - missing isozyme in a whole progeny, 2 - isozyme detected in the part of a progeny, 3 - isozyme detected in a whole progeny). The comparison was accomplished by applying hierarchical cluster analysis procedure of SPSS 16.0 for Windows®, which attempts to identify relatively homogeneous groups of cases (progenies, in this study) based on selected characteristics, using an algorithm that starts with each case in a separate cluster and combines clusters until only one is left. The values generated by the squared Euclidean distance measure were rescaled to 0–1 range. A dendrogram using average linkage (between-groups) was obtained to assess the cohesiveness of the clusters formed.

RESULTS

A half-number of PRX bands available for the rest of the progenies was absent in the progeny No. 23 (Table 4). In addition, the progeny failed to show PRX bands at Rf = 0.25 and Rf = 0.49. It should be noted that identical siblings (No. 0830 and No. 08103) were present in the progeny No. 23 suggesting presence of both modes of reproduction - asexual and sexual.

The analysis of PRX patterns has revealed that the only band (at Rf = 0.43: Table 4, Fig. 1) was absent in progeny No. 4, in comparison to the remaining progenies investigated. The two identical siblings (No. 0746 and 0726) were found in the progeny of mother tree No. 4 from Šilėnai forest district - the oldest (about 70 years) non-crab apple tree without false heartwood. It had some hairiness on the underneath of leaves and yellowish (greenish-yellow) globose fruit without over colour. Its fruit had 6.4 seeds on average, whereas, generally, 4 seeds per fruit were found. The absence of fruit over colour was also characteristic for the non-crab apple tree No. 13 from Balskai forest district. The PRX isozyme pattern of its progeny was comparable, as well (Table 4, Fig. 2).

Crab apple tree No. 22 was left solitary, growing at the area of latter-year cutting in the dense broad-leaved mixed forest (Lančiūnava forest district). The mother tree No. 22 had an exuberant foliage and considerable ratio of $H/D_{CROWN} = 5.2$. The primary veins of its leaves, petioles, and the over colour of its ripening fruit were purplish-red and its shoots were red-brown. The progeny of crab apple tree No. 22, like the progeny of non-crab apple tree No. 1 (see Table 3), had no band at Rf = 0.67 in comparison to the rest of the progenies (Table 4). There were two pairs of identical siblings in the progeny (No. 07121 and 07122, No. 0813 and 0827).

Unlike the rest of progenies investigated, PRX bands at Rf = 0.55 and Rf = 0.67 were present in the whole progeny No. 18 of wild non-crab from Stemplės forest district, an open space grown solitary apple tree with pear-shaped fruit (of globose-conical form; some fruits were without cavities of stalks).

Table 3. The data of mature wild apple trees from the forest habitats of south-western and central Lithuania (mother trees, progenies of which were examined with the aid of peroxidase analysis, are in bold).

Tree No.	Soil TG	Thorn bearing	Fruit shape	Fruit ground colour	Fruit over colour	Pattern of over colour
26	Nd	Pointed shoots	Flat	Yellow	Absent	-
27	Nd	Pointed shoots	Flat	Yellowish	Absent	-
23	**Nc**	**Pointed shoots**	**Pear-shaped**	**Green**	**Absent**	**-**
22	**Lf**	**Thorns**	**Flat**	**Yellowish**	**Reddish**	**Side**
14	Ld	Thorns	Flat	Green	Absent	-
25	Ld	Thorns	Globose	Yellow	Absent	-
3	**Nc**	**Thorns**	**Globose**	**Yellowish**	**Absent**	**-**
8	Ld	Thorns	Globose	Yellowish	Absent	-
15	Ld	Thorns	Conic	Yellowish	Absent	-
11	Nd	Thorns	Ovoid	Yellowish	Absent	-
17	Nd	Absent	Flat	Yellowish	Yellow	Side
19	Nf	Absent	Globose	Yellowish	Yellow	Both sides
13	**Lc**	**Absent**	**Globose**	**Yellowish**	**Absent**	**-**
4	**Ld**	**Absent**	**Globose**	**Yellowish**	**Absent**	**-**
6*	Nd	Absent	Globose	Yellowish	Yellow	Side
1	**Ld**	**Absent**	**Globose-torose**	**Yellowish**	**Reddish**	**Spots**
7	Nd	Absent	Globose	Greenish	Yellow	Side
9	Ld	Absent	Globose	Greenish	Reddish	Spots
16	Ld	Absent	Globose	Green	Absent	-
20	Ld	Absent	Globose	Green	Absent	-
10	Šd	Absent	Conic	Greenish	Absent	-
5	Ld	Absent	Ovoid	Yellowish	Absent	-
18	**Šd**	**Absent**	**Pear-shaped**	**Yellow**	**Absent**	**-**

*Apple tree with felted hairy leaves.

Table 4. The specificity of peroxidase isozymes in leaf extracts of Lithuanian wild apple progenies according to the values of relative mobility (V – isozyme detected in a whole progeny, N - missing isozyme in a whole progeny, empty cells - isozyme detected in the part of a progeny).

Progeny No.	Offspring/ Number of identical siblings	Rf values**											
		0.2	0.4	0.4	0.5	0.6	0.6	0.6	0.6	0.7	0.7	0.8	0.9
3*	11/0		**N**				**N**						**N**
18	20/0		**N**		**V**		**N**		**V**				
13	22/2		**N**				**N**						
4	20/2		**N**										
22*	11/2+2						**N**		**N**	**N**	**N**	**N**	**N**
23*	9/2	**N**		**N**		**V**	**N**			**N**	**N**	**N**	
1	11/0					**N**		**N**	**N**	**N**	**N**		

* Progeny of a crab apple tree.

** Peroxidase band with Rf value 0.21 was detected in all the siblings.

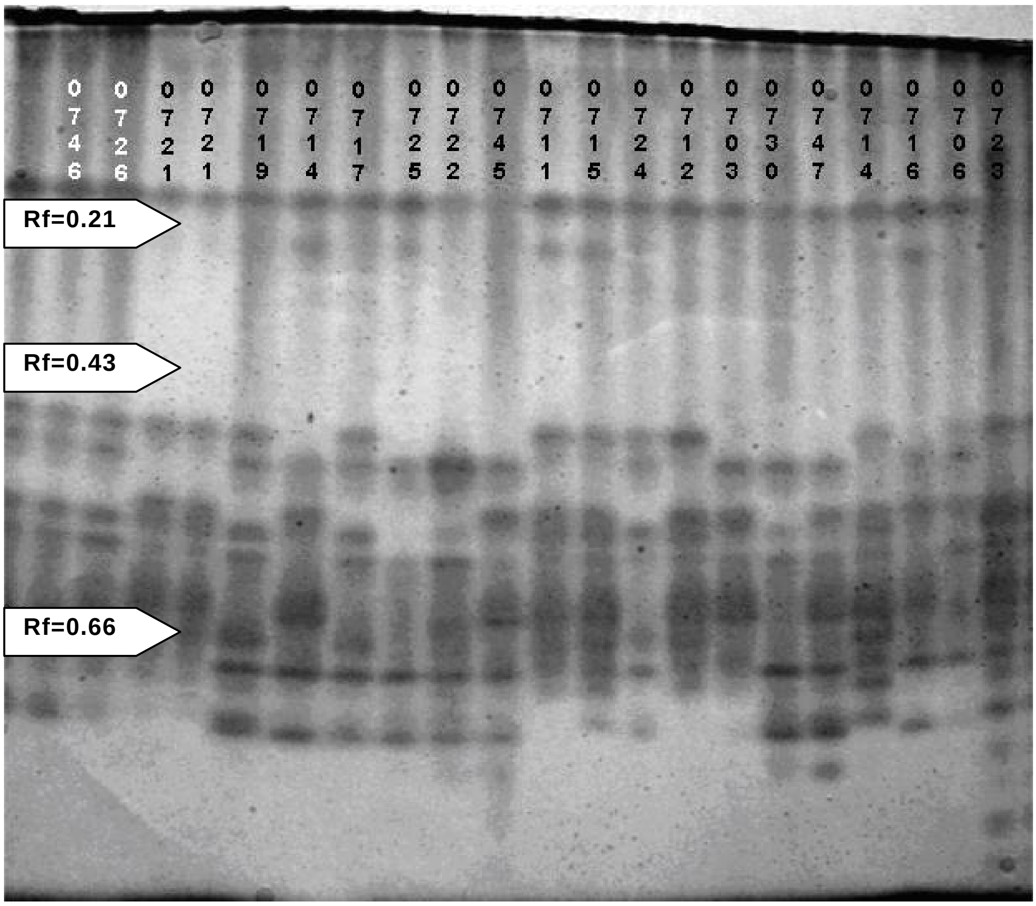
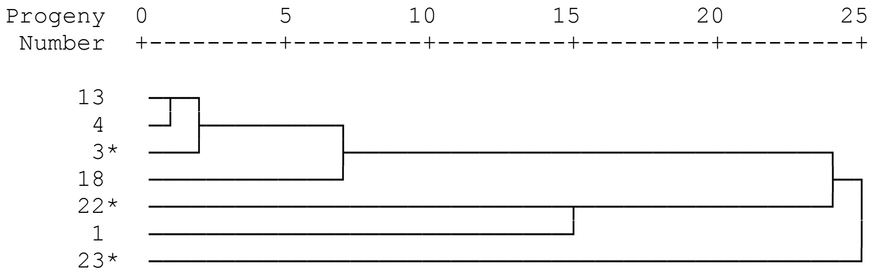


Fig. 1. The progeny of wild apple tree No. 4: peroxidase 'runs' in polyacrylamide gels (the numbers of identical siblings are in white).



* Progeny of a crab apple tree.

Fig. 2. Hierarchical cluster analysis for the interval data from the patterns of peroxidase isozymes of Lithuanian wild apple progenies: rescaled distance cluster combine.

In summary, several specific bands of peroxidase were determined for the progenies of *Malus sylvestris* Mill. as species-specific markers: present band at Rf = 0.67 and absent band at Rf = 0.43. PRX band with Rf value 0.21 was detected in all the siblings.

DISCUSSION

As the observed segregation of peroxidase bands could be interpreted in different ways (see Fig. 2), combining the information derived from different types of analysis (molecular vs. morphological) minimizes the risks related to misinterpreting the data. Hence it was the prerequisite for the investigation to presume that crab apple tree No. 23, isolated from relatives inside forest cover, on mineral soil (Šilinė forest district, Nc site), produced some apomictic or autogamous seeds. This concept is supported by the unusual fruit - pear-shaped (oblong-waisted), without over colour - each containing 0−1−2 seeds, whereas, generally, 4 seeds per fruit were found, and third-year siblings raised from the seed - 29% of the seed produced the offspring. The trunk heartwood of mother tree No. 23 was rotten. In conclusion, all that means that the reproduction of wild apple may be affected by the responses of mother trees to stresses: biotic (due to insufficient pollination, flower bud cold sensitivity, etc.) and abiotic (owing to inadequate moisture conditions at the mineral soils of Nc sites, sudden removal of surrounding stand, etc.), in the over-utilized forests of south-western and central Lithuania.

As to the phenotype of wild apple fruit, crab apple trees generally bear flat-shaped fruits that have no over colour (Table 3) typical of crab apples (Hempel & Wilhelm, 1889; Tuinyla et al., 1990; Wagner, 1995; Navasaitis et al., 2003; Borzan, 2004). The yellowish fruits of wild non-crabs are globose and may have yellow sides. We hypothesize that the absence of anthocyanin production in the fruit of wild apple without over colour appears to arise as a direct result of mutations in the genes of anthocyanin biosynthesis pathway. Purplish-red primary veins of leaves, purplish-red petioles, purplish-red over colour of ripening fruit, and red-brown shoots are the evidence of *Malus sieversii* (Ledeb.) M. Roem. characters in the phenotype of crab apple tree No. 22. According to Forte et al. (2001), *Malus sieversii* is the primary progenitor of *Malus domestica*.

In spite of the fact that the relationships between peroxidase patterns and morphological traits are not clear, the results indicate that the progenies of crab apple trees No. 23 and No. 22 have the higher proportion of identical siblings in comparison to the progenies of non-crab apple trees No. 4 and No. 13 (Table 4). Thereby, sexual and asexual reproduction might occur simultaneously in wild apple if considering the resemblance of some siblings in the progenies revealed by the simultaneous comparison of their peroxidase isozyme patterns. It's worth noting that DNA markers would be better suited to investigating a system as precise as apomixis, as they are more sensitive and not affected by environmental factors.

In the zone with Rf values 0.25−0.90 peroxidase enzyme system of wild apple shows polymorphic pattern (Table 4): this surpasses the findings on apple cultivars with the polymorphic pattern of peroxidase isozymes within Rf values 0.46−0.80 (Gelvonauskis & Šikšnianienė, 2001). Failure to show peroxidase band at Rf = 0.43 demonstrates the heterogeneity of wild apple siblings. Peroxidase isozymes at Rf = 0.67 (exact value of Rf within the progeny No. 4 is 0.66: Fig. 1) are obligatory for the identification of *Malus sylvestris* Mill. The phenotypic characterization of rapidly growing reproductive structures (e.g. fruit characters) may be useful for the identification of *Malus sylvestris* Mill. independent of the remaining characters of wild apple trees.

CONCLUSIONS

This study demonstrates some shortcomings of the method of isozyme analysis. However, autogamy or even apomixis might be the factor in wild apple if considering the resemblance of some siblings in the progenies revealed by the simultaneous comparison of their peroxidase isozyme patterns. In the zone with Rf values 0.25−0.90 peroxidase enzyme system of wild apple shows a polymorphic pattern, therefore wild apple progenies could be identified by the simultaneous comparison of the isozyme patterns. Species-specific peroxidase markers of *Malus sylvestris* Mill.: present band at Rf = 0.67 and absent band at Rf = 0.43.

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