Genetic Relationships among Slovenian Pears Assessed by Molecular Markers

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ABSTRACT. A sample of 94 pear (*Pyrus* L.) genotypes, traditionally present in the Slovenian landscape, was analyzed by amplified fragment length polymorphism (AFLP) and SSR (SSR), focusing on the assessment of genetic relationships. The analyzed samples involved germplasm of *Pyrus communis* L., *P. nivalis* Jacq., and *P. pyrauster* L. The AFLP technique, using five *Eco*RI and *Mse*I primer combinations, revealed molecular polymorphism at a level of 65.95%, representing 93 polymorphic bands among the total of 141 scored. With SSR analysis, 64 polymorphic alleles were found at seven microsatellite loci, with an average of 9.14 alleles per locus. Genetic distances among the individuals being studied were calculated using the Dice coefficient of similarity, and a dendrogram supported by AFLP and SSR data was constructed using the neighbor-joining method. The clustering method grouped the analyzed genotypes into three main clusters. The first cluster included the *P. communis* germplasm. The genotypes resembling *P. nivalis* were grouped in the second largest cluster, which could be divided into four subclusters. The germplasm of *P. pyrauster*, in this cluster, was found to be much less distinct than we had assumed. The most typical cultivar group in the third cluster was 'Vinska Mostnica'. The study indicated that *P. nivalis* germplasm is frequently present in Slovenia, but not as a pure species.

Pears belong to the family Rosaceae, subfamily Maloideae, and genus *Pyrus*. According to Challice and Westwood (1973), the most important pear species are distributed in five main groups: European species (*P. communis*, *P. cordata* Desv., and *P. nivalis*), West Asian species (*P. amygdaliformis* Vill., *P. elaeagnifolia* Pall., *P. syriaca* Boiss., *P. glabra* Boiss., *P. regelii* Rehd., and *P. salicifolia* Pall.), North African species (*P. longipes* Coss. et Dur., *P. mamorensis* Trab., and *P. gharbiana* Trab.), Asian pear pears (*P. betulifolia* Bunge, *P. calleryana* Decne., *P. dimorphophylla* Makino, *P. fauriei* Schneid., and *P. koehnei* Schneider), and medium-large fruited Asian pears (*P. hondoensis* Kik. et Nak., *P. pyrifolia* Nak., *P. ussuriensis* Maxim., and *P. pashia* D. Don.).

Perry pears or Mostnica genotypes are often present in Slovenia. The term "perry pear" is a technological name, closely associated with the production of alcoholic drinks. They are mostly grafted trees, but some of them originated directly from seed. At present, 50 to 60 cultivars of perry pears can be found, including local cultivars (limited to one or two villages), subregional cultivars (present in wider areas) and cultivars spread across borders (in other parts of central Europe). Today, these pears have a relatively low economic value, but owing to their perfect adaptation to existing ecological conditions and resistance/tolerance to some diseases and pests, they may represent an important resource for organic production of juice and other drinks such as perry. In the 18th and 19th centuries, fruit from perry pear trees played an important role in the

nutrition of the poor rural population. In the past, farmers made use of all fruit grown on their land. Fruit were fresh eaten, used as animal feed, dried, cooked, baked, stored for winter or used for production of juice, fruit wine, vinegar, or brandy. In the last decades, this traditional usage of fruit has been abandoned. Large fruit trees, such as perry pears, became remnants of traditional agriculture; in majority without their primary function (grown as fruit trees). In most cases, fruit were left to decompose and/or as food for wild animals (Siftar, 2008). Farmers rarely rejuvenate them and their number is decreasing rapidly. The most important species among pears in Slovenia is *P. communis*; others include *P. pyrauster*, *P. nivalis*, and *P. amygdaliformis*. According to some authors (Jogan et al., 2001), *P. nivalis* is the rarest pear species in Slovenia (Fig. 1B). As far as we know, the presence of *P. nivalis* as a "pure" species (described by Jacquin, 1774) has not yet been established. However, there are numerous cultivars that resemble it. The evolution of perry pears probably involved three species: *P. communis*, *P. pyrauster*, and *P. nivalis* (A. Siftar, personal communication). The fruit are distinguished by juicy fruit containing a high concentration of fruit acids and tannins that stabilize drinks and make them clean and transparent. In this way, perry remains drinkable for a longer period of time. The most typical characteristics of *P. communis*: a broad-pyramidal tree up to 15 m high, rarely to 20 m, sometimes spiny; young sprouts are glabrous or slightly pubescent, leaves are orbicular-ovate to elliptic, acute or short-acuminate, subcordate to broad-cuneate and 2 to 8 cm long; petioles slender, 1.5 to 5 cm long; inflorescences villous or nearly glabrous; flowers about 3 cm across, 6 to 9 flowers per inflorescence; pedicels 1.5 to 3 cm

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Fig. 1. Locations of *Pyrus* trees included in the study of genetic relationship (A); because of the high number of trees sampled, some of the locations on the map are overlapping. Localities of *Pyrus communis*, *P. nivalis*, and *P. pyraeaster* in Slovenia according to Jogan et al. (2001) (B). The maps were created with the help of the CKFF (Center za kartografijo favne in flore, Slovenia); I = Italy, A = Austria, H = Hungary, CRO = Croatia.

long; fruit turbinate or subglobose, on a slender stalk to 5 cm long; *P. nivalis*: a thornless tree, up to 16 m, young sprouts tomentose; leaves elliptic to obovate, acute, cuneate, 5 to 8 cm long and 2 to 4 cm broad, white tomentose when young, finally glabrous above; petioles 1 to 3 cm long; inflorescence white tomentose with an average of 10 flowers per inflorescence, flowers 3 to 4 cm across, fruit subglobose to 5 cm across yellowish green; a stalk as long or longer than fruit; *P. pyraeaster*

usually thorny, flowers 2 to 2.5 cm across, fruit 1.5 to 3 cm across (Rehder, 1940).

Perry pears are genetically and morphologically very heterogeneous. Besides the many characteristics of cultivated genotypes of *P. communis*, they have several characteristics of wild species; the fruit are generally smaller, sour or bitter, and generally contain more seeds. They can be classified in various ways, the most appropriate of which appears to be according to the dominant germplasm of *P. communis*, *P. nivalis*, or *P. pyraeaster*. In the evolution of perry pears, *P. nivalis* probably played a much more important role than *P. pyraeaster*. According to our experience, *P. nivalis* trees are much more allogamous and, therefore, participated in more genetic recombination than *P. pyraeaster*. This may also be one of the reasons that pure *P. nivalis* germplasm is hard to find.

Perry pears are probably much younger than traditional cultivars of *P. communis* and *Vitis vinifera* L. The first selection was most likely aimed at creating genotypes that could be used as food. As they are extensively cultivated at higher elevations where *V. vinifera* cannot grow, we can assume that they were purposely selected for the production of alcoholic drinks.

Early efforts to identify cultivars by means of phenotypic data (Westwood, 1982) proved to be reliable only for a limited number of cultivars under certain conditions. The phenotypic variability seen among accessions grown in areas with slightly different environments and production practices demonstrates a number of problems associated with this approach (Hokanson et al., 1998). Owing to the high variability of morphological, anatomical, and/or physiological characteristics, conclusions about relatedness between genotypes based on phenotypic markers are not always reliable.

More appropriate are molecular markers, which appear to be more stable.

In the genus *Pyrus*, several studies have been carried out using molecular markers to characterize different genotypes and to establish the genetic relationships among species and/or genotypes. The earliest studies associated with the identification of *Pyrus* cultivars involved isozymes (Cerezo and Socias, 1989; Santamour and Demuth, 1980). Later studies involved

Table 1. Identification and phenotype of the 94 sampled pears included in the study of genetic relationships.

D ^z	<i>Pyrus</i> species	Accession name ^y	Pubescence intensity of young shoots (1–3 scale) ^x	Position of fruit maximum diam (1–3 scale) ^w	Size of fruit (1–5 scale) ^v	Fruit profile of sides (1–9 scale) ^u	Time of maturity for consumption/ processing (1–5 scale) ^t
80	<i>P. communis</i>	PP	1	3	2	7	4
48	<i>P. communis</i>	PP	1	1	1	2	2
41	<i>P. communis</i>	‘Gelbmöstler’ (‘Rumenka’), PP	1	2	3	1	2
60	<i>P. communis</i>	‘Gelbmöstler’ (‘Rumenka’), PP	1	2	3	1	2
97	<i>P. communis</i>	‘Doyene de Julliet’ (Julijska Lepotica)	1	2	1	2	1
11	<i>P. communis</i>	‘Kleine Landbirne’ (Dunjacka), PP	1	1	2	1–2	4
14	<i>P. communis</i>	‘Kleine Landbirne’ (Dunjacka), PP	1	1	2	1–2	4
23	<i>P. communis</i>	‘Kleine Landbirne’ (Dunjacka), PP	1	1	2	1–2	4
28	<i>P. communis</i>	‘Kleine Landbirne’ (Dunjacka), PP	1	1	2	1–2	4
40	<i>P. communis</i>	‘Kleine Landbirne’ (Dunjacka), PP	1	1	2	1–2	4
83	<i>P. communis</i>	‘Lapsica’ – LN, PP	1	1	3	2	4
89	<i>P. communis</i>	‘Lapsica’ – LN, PP	1	1	3	2	4
90	<i>P. communis</i>	‘Lapsica’ – LN, PP	1	1	3	2	4
96	<i>P. communis</i>	‘Packham’s Triumph’	1	1	5	1	4
10	<i>P. communis</i>	‘Speckbirne’ (‘Vinska Mostnica’), PP	1	2	4	1	4
15	<i>P. communis</i>	‘Speckbirne’ (‘Vinska Mostnica’), PP	1	2	4	1	4
27	<i>P. communis</i>	‘Speckbirne’ (‘Vinska Mostnica’), PP	1	2	4	1	4
29	<i>P. communis</i>	‘Speckbirne’ (‘Vinska Mostnica’), PP	1	2	4	1	4
30	<i>P. communis</i>	‘Speckbirne’ (‘Vinska Mostnica’), PP	1	2	4	1	4
34	<i>P. communis</i>	‘Speckbirne’ (‘Vinska Mostnica’), PP	1	2	4	1	4
35	<i>P. communis</i>	‘Speckbirne’ (‘Vinska Mostnica’), PP	1	2	4	1	4
42	<i>P. communis</i>	‘Speckbirne’ (‘Vinska Mostnica’), PP	1	2	4	1	4
84	<i>P. communis</i>	‘Speckbirne’ (‘Vinska Mostnica’), PP	1	2	4	1	4
16	<i>P. communis</i>	‘Unterlaibacher’	1	2	3	6	4
		(‘Ljubljanska Mostnica’), PP					
99	<i>P. communis</i>	‘Williams Bon Chrétien’	1	2	3–4	3	3
		(‘Viljamovka’)					
98	<i>P. communis</i>	‘Zgodnja Dekanka’	1	2–3	2	8	1
49	<i>P. communis</i>	‘Zieregger Mostbirne’	1	1	2	5	4
		(‘Cirinska moštnica’), PP					
7	<i>P. communis</i>	PP	1	1	3	4	4
13	<i>P. communis</i>	PP	1	1	3	1	4
18	<i>P. communis</i>	PP	1	1	3	6	3
19	<i>P. communis</i>	PP	1	1	3	6	3
20	<i>P. communis</i>	PP	1	1	3	6	3
21	<i>P. communis</i>	PP	1	1	3	6	3
22	<i>P. communis</i>	PP	1	1	1	2	3
25	<i>P. communis</i>	PP	1	1	3	6	3
31	<i>P. communis</i>	PP	1	1	3	6	3
36	<i>P. communis</i>	PP	1	1	3	6	3
52	<i>P. communis</i>	PP	1	3	3	6	3
53	<i>P. communis</i>	PP	1	1	3	2–5	2
63	<i>P. communis</i>	PP	1	1	3	1	2
64	<i>P. communis</i>	PP	1	1	2	6	1
68	<i>P. communis</i>	PP	1	1	2	7	1
69	<i>P. communis</i>	PP	1	1	2	1	1
72	<i>P. communis</i>	PP	1	1	3	1	3
73	<i>P. communis</i>	PP	1	1	2	3	2
85	<i>P. communis</i>	PP	1	1	1	2	2
45	<i>P. communis</i>	Tree with <i>Viscum album</i> , PP	1	2	1	1–2	3
1	<i>P. communis</i>	‘Speckbirne’, (‘Vinska Mostnica’), PP	1	2	4	3–6	4
62	<i>P. communis</i>	× <i>P. nivalis</i> developed from seed-seedling, PP	2	1	1	1–2	4

continued next page

Table 1. Continued.

D ^z	<i>Pyrus</i> species	Accession name ^y	Pubescence intensity of young shoots (1–3 scale) ^x	Position of fruit maximum diam (1–3 scale) ^w	Size of fruit (1–5 scale) ^v	Fruit profile of sides (1–9 scale) ^u	Time of maturity for consumption/ processing (1–5 scale) ^t
3	<i>P. nivalis</i>	‘Welsche Bratbirne’ (Koroška Mostnica), PP	3	1	1	5	4
44	<i>P. nivalis</i>	‘Hirschbirne’ (Bela tepka), PP	3	2	2	7	4
55	<i>P. nivalis</i>	‘Steierische Scheibelbirne’ (Dolicanka), PP	3	1	1	2	4
32	<i>P. nivalis</i>	‘Welsche Bratbirne’ (Koroska Mostnica), PP	3	1	1	5	4
43	<i>P. nivalis</i>	‘Welsche Bratbirne’ (Koroska Mostnica), PP	3	1	1	5	4
65	<i>P. nivalis</i>	‘Welsche Bratbirne’ (Koroska Mostnica), PP	3	1	1	5	4
81	<i>P. nivalis</i>	‘Welsche Bratbirne’ (Koroska Mostnica), PP	3	1	1	5	4
46	<i>P. nivalis</i>	‘Sózókörte’ (Šoza), PP	3	1	1	5	4
50	<i>P. nivalis</i>	seedling	3	1	1	6	4
8	<i>P. nivalis</i>	PP	3	1	2	5–7	4
2	<i>P. nivalis</i>	PP	3	1	1–2	5	4
12	<i>P. nivalis</i>	‘Steierische Scheibelbirne’ (Dolicanka), PP	3	1	1	2–5	4
17	<i>P. nivalis</i>	‘Steierische Scheibelbirne’ (Dolicanka), PP	3	1	1	2–5	4
24	<i>P. nivalis</i>	‘Crnivka’, PP	3	1	1	7	4
26	<i>P. nivalis</i>	‘Crnivka’, PP	3	1	1	2–5	4
33	<i>P. nivalis</i>	PP	3	1	1	2–5	4
37	<i>P. nivalis</i>	PP	3	2	1	1	4
38	<i>P. nivalis</i>	‘Welsche Bratbirne’ (Koroska Mostnica), PP	3	2	1	5	4
54	<i>P. nivalis</i>	PP	3	1	1	1–5	4
56	<i>P. nivalis</i>	PP	3	2	1–2	1–5	5
58	<i>P. nivalis</i>	PP	3	2	1–2	1–5	5
75	<i>P. nivalis</i>	PP	3	2	1	7	4
76	<i>P. nivalis</i>	PP	3	1	1	2	4
77	<i>P. nivalis</i>	PP	3	1	1	7	4
78	<i>P. nivalis</i>	PP	3	1	1	7	4
5	<i>P. nivalis</i>	PP	3	1	1	2	4
70	<i>P. nivalis</i>	‘Betzelsbirne’, PP	2	2	3	1	5
71	<i>P. nivalis</i>	‘Betzelsbirne’, PP	2	2	3	1	5
59	<i>P. nivalis</i>	‘Tepka’, PP	3	1	2	1–2	5
88	<i>P. nivalis</i>	‘Tepka’, PP	3	1	2	1–2	5
91	<i>P. nivalis</i>	‘Tepka’, PP	3	1	2	1–2	5
92	<i>P. nivalis</i>	<i>f. austriaca</i>	2	1	1	5	4
4	<i>P. pyraeaster</i>	PP	1	1	1	2	3
9	<i>P. pyraeaster</i>	PP	1	1	1	2	3
47	<i>P. pyraeaster</i>	PP	1	1	1	2	3
57	<i>P. pyraeaster</i>	‘Snopsarca’ – LN, PP	1	1	1–2	1–2	3
61	<i>P. pyraeaster</i>	PP	1	1	1	5	3
66	<i>P. pyraeaster</i>	PP	1	1	1	2	3
67	<i>P. pyraeaster</i>	PP	1	1	1	2	3
74	<i>P. pyraeaster</i>	PP	1	1	1	2	3
79	<i>P. pyraeaster</i>	PP	1	1	1	7	3–4
82	<i>P. pyraeaster</i>	PP	1	1	1	2	3
87	<i>P. pyraeaster</i>	PP	1	1	1	2	3
39	<i>P. pyraeaster</i>	PP	1	1	1	2	3
6	<i>P. salicifolia</i>		3	1	2	8	4

^zNumbers refer to the labels of Fig. 5.^yPP = perry pear, LN = local name.^x1 = weak, 2 = medium, 3 = strong [No. 14; International Union for the Protection of new Varieties of Plants (UPOV), 2000].^w1 = in middle, 2 = slightly toward calyx, 3 = clearly toward calyx (No. 40; UPOV, 2000).^vLength × width: 1 = very small, 2 = small, 3 = medium, 4 = large, 5 = very large (No. 41; UPOV, 2000); this parameter was adjusted according to Schmidthaler (2001).^u1 = blunt-whirling, 2 = round, 3 = bell shaped, 4 = barrel shaped, 5 = round-flat, 6 = whirling, 7 = ovoid, 8 = bottle shaped, 9 = drop shaped (No. 43; UPOV, 2000); this parameter was adjusted according to Schmidthaler (2001).^t1 = very early ‘Doyenné de Juillet’, 2 = early ‘Précoce de Trevoux’, 3 = medium ‘Coscia’, 4 = late ‘Beurré Hardy’, ‘Doyenné du Comice’, ‘Jeanne d’Arc’, 5 = very late ‘Doyenné d’Hiver’, ‘Nordhäuser, Winterforelle’, ‘Président Drouard’ (No. 65; UPOV, 2000).

RAPD markers, which were often used for diversity analyses in pears (Botta et al., 1998; Lee et al., 2004; Oliveira et al., 1999; Teng et al., 2002). The majority of the most recent studies are based on AFLP markers (Monte-Corvo et al., 2000, 2002; Shengua et al., 2002) for the identification and estimation of the genetic similarity among pear species and cultivars. Regarding SSR, Yamamoto et al. (2001), screened apple (*Malus* L.)-derived microsatellite markers in 36 pear accessions belonging to five *Pyrus* species (one of which was *P. communis*) to evaluate their usefulness for pear characterization. Later, other authors screened more apple SSR markers for polymorphism in pears (Hemmat et al., 2003; Pierantoni et al., 2004). Yamamoto et al. (2002a and 2002b) were the first to develop SSR markers from *P. pyrifolia*, also known as asian pear. SSR markers are now frequently used for the assessment of genetic diversity in pears: Ghosh et al. (2006) screened 28 pear accessions (economically important cultivars and selections from breeding programs), mostly belonging to the species *P. communis*, with 18 SSR primer pairs; Volk et al. (2006) used 13 microsatellite loci for determination of relationships among 145 wild and cultivated individuals of *P. communis*; Wünsch and Hormaza (2007) identified 63 European pear cultivars with seven microsatellite loci. According to our knowledge, ≈ 90 SSR markers are available now for genetic diversity studies within the genus *Pyrus*.

The main objectives of this study were to evaluate the genetic diversity and relationships among pears and to explain the taxonomic position of perry pears, which are the trees most typical of the Slovenian landscape, comparing them with the most common species such as *P. communis*, *P. nivalis*, and *P. pyraster*. To our knowledge, such research has not yet been conducted.

Materials and Methods

PLANT MATERIAL. A total of 94 *Pyrus* genotypes traditionally present in the Slovenian landscape were included in the study. The analyzed samples involved the germplasm of *Pyrus communis*, *P. nivalis*, and *P. pyraster*, which were collected in different parts of Slovenia (Fig. 1A). The exception is sample number 92 (*P. nivalis*), which was collected in the Botanical Garden of the University of Vienna (Vienna, Austria). Most of the 94 samples collected included perry pears. As a reference, four commercial cultivars of *P. communis* (sample numbers 96–99), *P. nivalis* from the Botanical Garden of the University of Vienna, and *P. pyraster* from the Ljubljana Botanical Garden (Ljubljana, Slovenia) were included. A majority of the investigated accessions were determined according to the morphological descriptors published by Grill and Keppel, 2005; Handlechner and Schmidhalter, 2007; Hartmann, 2003; Kessler, 1948; Löschig et al., 1912; Lucke et al., 1992; Petzold, 1982; Schmidhalter, 2001, and Terpó and Amaral Franco, 1968.

The studied genotypes are listed in Table 1. The grouping of perry pears is based on similarity to three crucial species: *P. communis*, *P. nivalis*, and *P. pyraster* (e.g., an accession was considered as a member of the *P. nivalis* perry pear group when characteristics of *P. nivalis* predominated). Accessions were grouped according to the visual survey of morphological traits (e.g., intensity of pubescence, fruit size and shape, and time of maturity for consumption). The fruit of these three common types of perry pears are shown in Fig. 2.

Fig. 2. Groups of perry pear fruit based on similarity to the three crucial species: *Pyrus communis*, *P. nivalis*, and *P. pyraster*.

DNA ISOLATION. DNA was extracted from fresh, young leaves using the cetyl trimethylammonium bromide (CTAB) protocol. To ≈ 2 to 3 cm² of fresh leaf tissue, 1 mL of preheated (68 °C) CTAB extraction buffer (Doyle and Doyle, 1987) was added and well homogenized with a mortar and pestle, and transferred to a 1.5-mL tube. Samples were incubated for 1.5 h at 68 °C in a water bath. After incubation, 600 μ L of chloroform:isoamyl alcohol in a 24:1 proportion was added, and the samples were thoroughly mixed. The mixtures were centrifuged at 14,200 g_n for 10 to 15 min. After centrifugation, the supernatant was transferred to a fresh tube and the DNA was precipitated by the addition of 1 volume of 3 M sodium acetate and 10 volumes of ice-cold isopropanol and kept at –20 °C for

20 to 30 min. Samples were again centrifuged at 14,200 g_n for 10 to 15 min. The pellet was washed in 70% ethanol for 20 min, air dried, and rehydrated in 100 μ L of TE buffer. The DNA concentration was estimated using a DNA fluorometer TKO 100 (Hoefer, Holliston, MA). Two separate extractions per tree were performed.

MOLECULAR ANALYSIS. To obtain more reliable information, two types of molecular markers (AFLP and SSR) were used. We assumed that the use of different molecular marker systems could reveal more information about the existing polymorphism and therefore enable more reliable results.

AFLP MARKERS. The DNA was restricted using two restriction enzymes (*Eco*RI and *Mse*I) and was ligated to *Eco*RI and *Mse*I adapters. The total genomic DNA (500 ng) was incubated for 2 h at 37 °C together with 2.5 U of *Eco*RI and 2.5 U of *Mse*I restriction enzymes (New England Biolabs, Ipswich, MA) and buffer solution (50 mM NaCl, 100 mM Tris-HCl, 10 mM MgCl₂, and 0.025% Triton X-100, pH 5.59) in final volume of 40 μ L. During the incubation, from time to time, samples were gently stirred. On the next day, 10 μ L of ligation solution [5 pmol- μ L⁻¹ *Eco*RI-adapter, 50 pmol- μ L⁻¹ *Mse*I-adapter; 1 $\times$ T4 DNA ligase buffer (50 mM Tris-HCl, 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP, and 25 μ g- μ L⁻¹ BSA, pH 7.5, New England Biolabs, and 1 U of Weiss T4-DNA ligase] was added and kept incubated at the same temperature for another 2 to 3 h. Pre-amplifications were performed in 50- μ L volumes of 1 $\times$ PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 75 ng of *Eco*RI and *Mse*I primers, 1.5 U of Taq polymerase, and 5 μ L of ligated DNA, using 72 °C for 120 s, followed by 20 cycles of 94 °C for 30 s, 56 °C for 60 s, and 72 °C for 105 s. Two-microliter aliquots of the diluted (1:10) pre-amplification product were used as templates for the selective amplifications with five primer combinations: E-AGC + M-CTG, E-AGC + M-CAT, E-AGC + M-CTC, E-AGC + M-CTT, and E-AGC + M-CTA. These five combinations were chosen among 10 primer pairs previously tested on four samples. The five most polymorphic primers were used in the experiment. *Eco*RI primers were labeled with Cy5 at the 5' end for selective amplification, thus permitting fluorescence detection. The selective amplification was carried out in a 10- μ L volume using the following temperature profile: 94 °C for 150 s followed by 13 cycles of 94 °C for 30 s, 65 °C for 30 s, with a decrease of annealing temperature of 0.7 °C per cycle, and 72 °C for 60 s, followed by 23 cycles at the annealing temperature of 56 °C. To the PCR products, equal volumes of formamide loading dye were added and denatured at 94 °C for 4 min. The samples were separated on a 7.5% polyacrylamide denaturing gel in an automated ALFexpressII sequencer

(Amersham Biosciences, Piscataway, NJ). A fluorescent-labeled size marker (Cy5 Sizer 50-500; Amersham Biosciences) was used as a molecular weight reference.

SSR MARKERS. Seventeen SSR loci developed earlier (Gianfranceschi et al., 1998; Guilford et al., 1997; Hokanson et al., 1998; Yamamoto et al., 2001, 2002b) were tested: KA4b, KA14, KA16, KB16, KU10, BGA35, BGT23b, GD15, CH01H10, CH02B10, CHO2D11, CH01F02, CH02B03b, CH01E12, O2B1, 05g8, and 28f4. Because some of the loci resulted in unspecific amplification and amplified more than one locus or because of the presence of a monomorphic allele, only seven of them were selected and used for further experimentation (Table 2).

Ten microliters of PCR mixture contained 20 ng of DNA, 0.25 U of Taq DNA polymerase (Promega, Madison, WI), reaction buffer (50 mM KCl, 1.5 mM MgCl₂, and 10 mM Tris-HCl; Promega), 0.5 μ L of each primer, and 200 μ M of each dNTP. PCR conditions consisted of an initial denaturation at 94 °C for 5 min, followed by 5 cycles of 94 °C for 45 s, 60 °C for 30 s, with a decrease of annealing temperature of 1 °C per cycle, and 72 °C for 1 min, followed by 25 cycles at the annealing temperature of 55 °C and extension at 72 °C for 90 s, and a final step of 8 min at 72 °C. For the locus KB16, 32 cycles were used, and for locus 28f4 and CH01H10, only 25 cycles were used. The annealing temperatures were different for the locus KU10 (58.6 °C), BGA 35 (56.7 °C), CH01F02 (58.6 °C), and 28f4 (55.8 °C). The PCR was performed using a Gene AMP 9700 thermocycler (Applied Biosystems, Foster City, CA). PCR products were separated and detected on a 7.5% polyacrylamide denaturing gel in an automated ALFexpressII sequencer (Amersham Biosciences). A fluorescent-labeled size marker (Cy5 Sizer 50-500; Amersham Biosciences) was used as a molecular weight reference.

DATA ANALYSIS. AFLP and SSR data were analyzed using the software ALFwinTM Fragment Analyser 1.01 (Amersham Biosciences), which forms part of the computer equipment of the ALFexpres II sequencer. All unambiguous fragments were scored for the presence (1) or absence (0) of each band. Only clear and reproducible fragments were taken for data analysis. The binary data matrix was used to calculate Dice's similarity coefficients (Dice, 1945); $D_{(i,j)} = (b + c) / [(2a + (b + c))]$, where $D_{(i,j)}$ is the similarity between two individuals i and j ; a is the number of bands present in both i and j ; b is the number of bands present in i and absent in j ; and c is the number of bands present in j and absent in i . The values of Dice's coefficients are between 0 (there is no common band) and 1 (two genotypes have identical markers, thus they are identical). Dice similarity

Table 2. SSR loci analyzed and parameters of genetic variability calculated for different microsatellite loci of the 94 pear genotypes: number of alleles (n), effective number of alleles (n_e), observed (H_o) and expected (H_e) heterozygosity, polymorphic information content (PIC), and probability of identity (PI).

Locus	Reference	n	n_e	H_o	H_e	PIC	PI
KB16	Yamamoto et al. (2002a)	4	2.845	0.968	0.646	0.582	0.329
KU10	Yamamoto et al. (2002a)	15	5.791	0.303	0.824	0.809	0.091
BGA35	Yamamoto et al. (2002a)	4	1.776	0.538	0.430	0.389	0.460
CH01H10	Gianfranceschi et al. (1998)	12	4.108	0.235	0.748	0.729	0.161
CH01F02	Gianfranceschi et al. (1998)	10	5.428	1.000	0.815	0.797	0.100
02B1	Guilford et al. (1997)	11	6.070	0.479	0.814	0.489	0.087
28f4	Guilford et al. (1997)	8	3.841	0.793	0.738	0.711	0.157
Avg		9.14	4.266	0.617	0.716	0.691	—

coefficients were calculated using the DARWIN computer package (Perrier and Jacquemoud-Collet, 2005).

For each microsatellite locus, the number of alleles per locus (n), allele frequencies, observed heterozygosity (H_O), and expected heterozygosity (H_E) were calculated using the 'IDEN-TITY 1.0' computer program (Wagner and Sefc, 1999). Also, the number of effective alleles (n_e) (Morgante et al., 1994) was estimated using the following equation:

$$n_e = \left(\sum_i^n p_i^2 \right)^{-1}$$

where p is the frequency of the i -th allele and the polymorphism information content (PIC) (Botstein et al., 1980), a measure of the allelic diversity at a locus, was calculated using the program Cervus 2.0 (Marshall et al., 1998).

The average distance between pairs of accessions was obtained by taking into account microsatellite and AFLP data, and a neighbor-joining tree was constructed using the DARWIN computer package (Perrier and Jacquemoud-Collet, 2005).

A matrix of Dice similarity coefficients was used for assessing relationships among 94 genotypes using the neighbor-joining algorithm developed by Saitou and Nei (1987).

Results

The five AFLP primer combinations (Table 3) amplified a total of 141 fragments in the 94 analyzed genotypes in sizes from 40 to 388 bp. Ninety-three bands were polymorphic, representing a 65.95% rate of polymorphism.

With the SSR analysis, 64 polymorphic alleles were found at seven microsatellite loci. The number of alleles (Table 2) detected per locus ranged from 4 (KB16, BGA35) to 15, (KU10), with an average of 9.14 alleles per locus and 0.691 PIC value per locus. The effective number of alleles was calculated at 4.26 alleles. The observed heterozygosity ranged between 0.235 (locus CH01H10) and 1.00 (locus CH01F02), with an average of 0.617. The expected heterozygosity ranged between 0.430 (locus BGA35) and 0.824 (locus KU10), with an average of 0.716. The differences between the observed and expected heterozygosity were observed on all loci. The largest difference was observed on locus CH01H10 (0.513) and the lowest on locus BGA35 (0.055). The average of observed (0.617) and expected (0.716) heterozygosity was very similar. The reliability of microsatellite markers in cultivar genotyping was determined on the basis of the following criteria: the complexity of the banding patterns, the amplification of quality PCR products, the stability of the microsatellite repeated structure, and the polymorphic information content of markers. On the basis of PIC values, all the microsatellite loci except BGA35 were classified as very informative loci ($PIC > 0.5$), and five loci proved suitable for mapping ($PIC > 0.7$).

The allele sizes, frequencies, and variability parameters calculated for each locus are shown in Tables 2 and 4. Figure 3 and 4 show examples of the obtained results for AFLP and SSR markers.

Both marker systems demonstrated a high level of genetic variability in these pear genotypes and proved their reliability in assessing genetic relationships.

The dendrogram (Fig. 5) based on joined AFLP and SSR data arranged the 94 samples under investigation in three main

Table 3. The total number of AFLP markers, number of polymorphic markers, and percentage of polymorphisms amplified in pear genotypes using five AFLP primer combinations.

Primer combination	Amplified fragments (no.)	Polymorphic fragments (no.)	Polymorphic fragments (%)
E-AGC + M-CTG	30	24	80.00
E-AGC + M-CAT	44	30	68.18
E-AGC + M-CTC	21	11	52.38
E-AGC + M-CTT	25	17	68.00
E-AGC + M-CTA	21	11	52.38
Σ	141	93	65.95

clusters. When looking at the length of the lines, we observed a larger distance between accessions than between clusters. The within-cluster heterogeneity was much more expressed than the one between clusters.

The cluster I included *P. communis* germplasm. In this cluster, we found all four commercial cultivars of *P. communis* included in our research: Packham's Triumph, Doyene de Juliet, Zgodnja Dekanka, and Williams Bon Chretien, as well as an old cultivar group, Laprsica.

The subcluster contained the cultivar group Dunjacka, with sample numbers 23, 11, 14, and 28. This group of samples included sample 31, which was considered as an undefined cultivar of *P. communis*. Our molecular results suggest that this sample probably belongs to the cultivar group Dunjacka.

The genotypes resembling *P. nivalis* were grouped in the second cluster, which could be divided in to three sub-clusters. The sub-cluster 1 contained an unidentified group of Mostnica pears, characterized by the dominant *P. nivalis* germplasm. In this sub-cluster we also found some cultivars known as Betzelbirne, represented by samples 70 and 71. In the same sub-cluster, there was also an old cultivar called Zimska Strdenka (numbers 46 and 50). Owing to their close positions in the cluster, we can assume that they belong to the same cultivar. The differences may be the consequence of an accumulation of mutations over a long time period.

In the subcluster 1, we found three undefined genotypes of *P. nivalis* (samples 54, 56, and 58). Samples 52 and 53 were morphologically similar to *P. communis*. However, the molecular analyses showed that they occupied positions close to *P. nivalis*. The subcluster 3 was very small and contained only three samples of *P. pyraeaster*; the first was sample 9 from the Ljubljana Botanical Garden; the second was 39 (used as rootstock in the recently created pear tree lane in Kuzma, Slovenia), and the third was sample 57 from Ratkovci (north-eastern Slovenia).

The most typical cultivar group in the cluster III is 'Vinska Mostnica'. This includes samples 34, 27, 30, 29, 35, 10, 1, 15, and 42.

When comparing the data obtained by molecular markers with the morphological characteristics listed in Table 1, one can observe that, in the cluster I, weakly expressed young shoot pubescence predominated (17 accessions of 23) and the position of the maximum fruit diameter was in the middle, indicating that fruit were round or nearly round (involving 18 accessions of this cluster). In this cluster, all fruit sizes can be found, from very small to very large. All accessions in the subcluster 'Dunjacka' exhibited weak young shoot pubescence, and the position of the maximum fruit diameter was in the

Table 4. Allele size (bp) and allele frequencies (in parenthesis) of the 94 pear genotypes at seven microsatellite loci.

Alleles	Locus						
	KB16	KU10	BGA35	CH01H10	CH01F02	02B1	28f4
A	166 (0.086)	110 (0.011)	140 (0.005)	108 (0.006)	176 (0.054)	268 (0.181)	114 (0.011)
B	168 (0.398)	118 (0.045)	142 (0.198)	120 (0.154)	180 (0.125)	270 (0.096)	116 (0.098)
C	170 (0.091)	120 (0.006)	144 (0.725)	122 (0.056)	182 (0.231)	272 (0.282)	118 (0.161)
D	172 (0.425)	122 (0.090)	146 (0.071)	124 (0.395)	184 (0.119)	274 (0.202)	120 (0.172)
E	—	124 (0.185)	—	126 (0.247)	186 (0.038)	276 (0.149)	122 (0.437)
F	—	126 (0.219)	—	128 (0.012)	188 (0.081)	280 (0.027)	124 (0.069)
G	—	128 (0.275)	—	130 (0.080)	192 (0.300)	282 (0.021)	126 (0.034)
H	—	130 (0.067)	—	132 (0.019)	194 (0.025)	284 (0.016)	130 (0.017)
I	—	132 (0.006)	—	134 (0.006)	196 (0.019)	286 (0.005)	—
J	—	134 (0.051)	—	138 (0.006)	202 (0.006)	292 (0.005)	—
K	—	138 (0.017)	—	142 (0.006)	—	296 (0.016)	—
L	—	144 (0.006)	—	146 (0.012)	—	—	—
M	—	148 (0.006)	—	—	—	—	—
N	—	150 (0.011)	—	—	—	—	—
O	—	158 (0.006)	—	—	—	—	—
Σ	4	15	4	12	10	11	8

Fig. 3. An example of AFLP results for the primer combination E-AGC/M-CTG, using curve option. Above each detected peak, its size in bps is shown. Each row represents one sample of pear genotype (10 samples of 94 are presented).

Fig. 4. Microsatellite alleles of 38 pear genotypes on locus 28f4. The first and the last column represent size marker standards (50–500 bp); columns 2 to 39 represent analyzed samples.

middle. Accessions in this subcluster were characterized by small fruit; the exception was the accession 31, which had medium fruit size. In the cluster II, subcluster 1, strong intensity of young shoot pubescence predominated (20 accessions of 25) and the position of the maximum fruit diameter was in the middle in 17 accessions in this subcluster. Fruit were mostly very small (17 accessions) and many different fruit profiles could be found. In the subcluster 2 (cluster II), weakly expressed young shoot pubescence predominated (15 accessions of 18) with the position of the maximum fruit diameter in the middle (15 accessions among 18). Sizes of fruit varied from very small to medium. Accessions in the subcluster 3 were morphologically very similar to those characterized by weakly expressed young shoot pubescence, the position of the maximum fruit diameter was in the middle, and fruit were very small and round in profile. In the subcluster 'Vinska Mostnica' (cluster III), all accessions exhibited weak young shoot pubescence, with the position of the maximum fruit diameter in the middle or slightly closer to calyx residual, with medium or large fruit and different side profiles. The last subcluster of the cluster III involved morphologically quite different accessions.

The accessions closely linked in the dendrogram are, in most cases, morphologically similar. For instance, accessions 23 and 11 that lie together in the dendrogram are identical in all studied morphological traits presented in Table 1. A similar conclusion can be made for several other accessions such as 70 and 71, 39 and 9, 34 and 27, and 21 and 19. Accessions 17 and 24, 1 and 10, 2 and 3, 37 and 38, and 54 and 55 differ only in one trait: the fruit profile of sides.

The studied accessions were collected in different parts of Slovenia (Fig. 1A). The correspondence between the genetic structure and the geographical distribution indicates that some

accessions from different geographical locations probably have a very similar genetic background because of their position in the same cluster or subcluster. As an example can be the accessions collected from Dovje (northwestern Slovenia), the distance of ≈ 240 km, to Kuzma (northeastern Slovenia), which are placed in the same subcluster, named 'Vinska Mostnica'.

Within the cluster I, accessions 99, 97, 98, 89, 84, 80, 96, 23, 11, 14, 31, and 28 were classified as *P. communis*; accessions 82 and 79 as *P. pyraeaster*; and 88, 77, 91, 78, 92, and 8 as *P. nivalis*. In the subcluster 1 (cluster II), most accessions belonged to the *P. nivalis* group, with two exceptions: number 66, which belonged to *P. pyraeaster* and numbers 52 and 53, which belonged to *P. communis*. In the subcluster 2 (cluster II), accessions classified as *P. communis* predominated, with the exceptions 75 and 76, which were classified as *P. nivalis*, and 74, 87, 61, 47, and 67, which were classified as *P. pyraeaster*. In the subcluster 3 (cluster II), all samples belong to *P. pyraeaster*. The subcluster 'Vinska Mostnica' (cluster III), was composed of *P. communis* genotypes. The most heterogeneous was the subcluster 2 (cluster III), with four *P. communis*, two *P. nivalis*, one *P. pyraeaster*, and one *P. salicifolia* tree. Here, we have to mention that in several cases, it was very difficult to position the perry pear accessions to appropriate species groups when the criteria were based solely on morphological markers.

Discussion

Efficient molecular research requires the selection of highly informative markers from the available molecular marker systems. Before the development of microsatellite markers in apples and pears, RAPD markers were extensively used for pear genome research. In our research, AFLP and SSR markers were

Fig. 5. Neighbor-joining dendrogram describing genetic relationships among 94 pear genotypes based on a combination of AFLP and SSR markers. Each genotype is represented by a number indicated in Table 1.

applied to 94 pear genotypes. Both molecular marker techniques proved their reliability in assessing genetic relationships among the pear genotypes being studied. All seven SSR primers produced PCR amplifications that were polymorphic and easily scorable in all accessions included in the study. From this point of view, the SSR markers used appeared to be very informative markers. The average number of alleles detected per locus in our study ranged from 4 to 15, with a mean value of 9.14 per locus. This value is very similar to the results obtained by Yamamoto et al. (2002b), which got with seven SSR primers an average nine alleles per locus, and Gianfranceschi et al. (1998), where the number of alleles per SSR ranged from 5 to 12 with an average of 8.2. It is known from the literature that

with self-pollinating and/or annual crops, the average number of alleles per locus is lower and for plant species such as long lived woody perennials, which are usually out-crossed, these values are higher (Hokanson et al., 1998), as confirmed by our results. According to Powell et al. (1996), expected heterozygosity is a good measure of information content and corresponds to the probability that two alleles taken at random from a population can be distinguished using the marker in question. In our study, the average expected heterozygosity (Table 2) for all loci was calculated at 0.716, and this value falls within the range of values being reported for plant SSR studies and is quite similar to the 0.66 value reported by Guilford et al. (1997) in their study of 21 apple cultivars, and the 0.693 value in the study of 66 *Malus × domestica* Borkh. accessions reported by Hokanson et al. (1998).

Pears represent an extremely heterogeneous group of species and genotypes, suggesting that their evolution was complex. The evolution of the pears included in our investigation probably involved all types of genetic recombination (self-fertilization, and intra- and interspecific hybridization).

Breeding experience with other genera shows that interspecific hybrids, from the molecular point of view, usually lie somewhere between the parental species, but after several generations of segregation, they usually drift closer to one of them (Simonovik et al., 2007). In the case of pears, if interspecific recombination had occurred between cultivated and wild genotypes, the progeny probably drifted closer to the wild type because wild

cultivars usually have a better chance of surviving.

It is very difficult to explain the genetic origin of perry pears. Based on our results (Fig. 5), it is possible to assume that they do not represent an independent species. Their evolution may include five main possibilities:

(a) Cultivated or semicultivated genotypes of *P. communis* (they probably do not exist any more) genetically recombined with *P. nivalis* [cluster II: subclusters 1 and 2 (e.g., accessions 5, 12, and 17)] or *P. pyraeaster* [cluster II: subcluster 3 (e.g., 3, 39, and 57)]. Wild *P. communis* should probably be excluded because of the specific nature of perry pears (e.g., the extremely large fruit of some genotypes).

(b) Backcrosses involving one of the parental species; interspecific hybrids between wild *P. communis* and *P. nivalis* or *P. pyraeaster* were probably backcrossed to cultivated *P. communis* (e.g., accession *P. nivalis* from cluster II and subcluster 1 was crossed with one of the cultivated or semi-cultivated *P. communis* genotypes, probably several centuries ago, and one of the vital hybrids was backcrossed to another cultivated or semicultivated genotype of *P. communis*). Cultivated genotypes of *P. communis* included in the investigation are relatively young and therefore we could not assume that they participated in the formation of perry pears.

(c) Involvement of a third species, probably after many generations of self-fertilization, sib-recombination, and/or crossing among individuals belonging to the same interspecific combination; e.g., *P. nivalis* (cluster II: subcluster 1; accession 71 was crossed with a cultivated genotype of *P. communis*, and after several generations of seed and vegetative propagation associated with artificial selection, one of the plants with several improved characteristics was crossed with *P. pyraeaster* from cluster II: subcluster 3; accession 9).

(d) Genetic recombination involving two different interspecific hybrids such as (*P. communis* × *P. pyraeaster*) × (*P. communis* × *P. nivalis*); e.g., a cultivar of cultivated or semicultivated *P. communis* was crossed with *P. pyraeaster* (accession 57) from cluster II: subcluster 3; in the same region, another or the same genotype of a cultivated or semicultivated *P. communis* was crossed with *P. nivalis* (accession 60) from cluster II: subcluster 2; two vital hybrid plants intercrossed and one of the most desired and productive individuals was multiplied as a perry pear cultivar.

(e) Backcrosses of tri-species hybrids with cultivated or semicultivated *P. communis*. A plant obtained from possibility “c” was grown close to cultivated or semicultivated genotypes of *P. communis*, enabling the genetic recombination (backcross). Some of the offspring individuals attracted local growers because of bigger fruit or other desired agronomical traits, and were multiplied vegetative as new cultivars.

P. communis, *P. nivalis*, and *P. pyraeaster* are allogamous and predominately entomophilous species. During evolution, crosses among them probably occurred and one of the results is the perry pear group. To obtain additional information regarding the role of each of the wild species (i.e., *P. nivalis* and *P. pyraeaster*) in the evolution of perry pears, it would be necessary to include in investigation more genotypes, especially of the wild species. It would also be good to define the reference genotype of *P. nivalis* and to involve more genetically inherited morphological traits (e.g., fruit height-fruit stalk ratio, seed length-width ratio, shape of cotyledons after germination, and shape of the first true leaves). Another question that has to be answered is the level of cross-(in)compatibility among the involved pears species and various groups of perry pears. The best way to find the exact answer is to conduct a semidiallel cross, which should involve as many genotypes as possible, and to count the number of viable seeds obtained.

This study indicates that the germplasm of *P. nivalis* is often present in Slovenia, but not as a botanically “pure” species. *P. nivalis* is associated with several taxonomic problems. When searching for “reference” trees, we faced serious difficulties. For the determination, we used several keys; the most useful was that published by Jacquin (1774). However, it was not possible to find a tree that could be considered a typical *P.*

nivalis according to this author’s botanical descriptors. Even the tree in the Botanical Garden of the University of Vienna, which was included in our study, differed in several traits. In the dendrogram, it was placed in the cluster I characterized by dominant *P. communis* germplasm.

The germplasm of *P. pyraeaster* was found to be much less distinctive than we had assumed. There may be several reasons for this. The investigation included a limited number of sampled trees, the sampling did not cover all regions, and only a few trees represented typical wild *P. pyraeaster* genotypes. Another reason could be the unclear taxonomic distinction between *P. pyraeaster*, *P. nivalis*, and wild *P. communis*.

The molecular analyses applied enabled us to place some of the unidentified genotypes in known cultivar groups. In some cases, the genetic differences were so small that it was possible to assume that the samples belonged to the same cultivar and could be considered as subclones.

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