Genetic diversity of Czech apple cultivars inferred from microsatellite markers analysis

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Abstract

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Genetic diversity and genetic relationships of Czech apple cultivars were evaluated. Trees of 33 Czech apple cultivars and 97 reference foreign cultivars were analysed using the set of 10 SSR (simple sequence repeat) primer pairs. The total of 89 polymorphic alleles were amplified, while the number of alleles per locus ranged from 4 to 14. The SSR dendrogram, based on the Jaccard's similarity coefficient, divided apple cultivars into three major groups: Cox's Orange Pippin, McIntosh and Golden Delicious ancestries. The clustering highly depended on pedigree and origin of apple cultivars. Spontaneous mutated cultivars were identical with their progenitors. We proved that microsatellite markers were useful for evaluation of genetic resources, collection management and cultivar identification.

Keywords: *Malus* × *domestica* Borkh.; current apple cultivars; genetic resources; SSR molecular markers

Apple (*Malus* × *domestica* Borkh.) belongs to the main fruit species and they are the most important fruit in Europe. Over 7,000 apple cultivars are known across the globe, whereas nowadays the world's production is based on a limited number of cultivars (Hokanson et al. 2001). Apple breeding programmes have developed new breeding lines based on improving traditional cultivars, mainly to introduce resistance to diseases from other *Malus* species into the better quality *Malus* × *domestica* Borkh. In the Czech Republic, there are several apple breeding organizations, developing new apple cultivars. One of them, Research and Breeding Institute of Pomology Holovousy is the national centre for maintaining and preservation of apple genetic resources (1,087 cultivars). Accurate and permanent identification of plant material within a germplasm collection is of utmost importance, especially for vegetatively propagated crops which are expensive to maintain, and should consist of a single genotype in the whole distribution area (Garkava-Gustavsson et al. 2008).

The development of DNA technology has provided a number of methods, which may be employed for characterisation of apple germplasm collections. During the past 10–15 years, microsatellites or simple sequence repeats have become the markers of choice for verification of cultivar identity and for diversity studies due to their abundance in plant genomes, large number of alleles per locus and high informativeness, codominant inheritance, and suitability for automatization (Garkava-Gustavsson et al. 2008). For apple, several hundreds of microsatellite markers were developed (Guilford et al. 1997; Hokanson et al. 1998; Liebhard et al. 2002; Silfverberg-Dilworth et al. 2006).

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SSR markers were used for assessment of genetic diversity in germplasm collections (Hokanson et al. 2001; Guarino et al. 2006; Pereira-Lorenzo et al. 2007; Garkava-Gustavsson et al. 2008; Gharghani et al. 2009; Patzak et al. 2009; Gasi et al. 2010), cultivar identification (Galli et al. 2005; van Treuren et al. 2010), construction of genetic linkage maps (Liebhard et al. 2003; Hemmat et al. 2003; Silfverberg-Dilworth et al. 2006) and for parent identification (Kitahara et al. 2005; Evans et al. 2011).

In this paper, we present the use of SSR molecular markers for determination of genetic diversity and genetic relationships within the set of Czech apple cultivars in comparison to selected current foreign apple cultivars.

MATERIAL AND METHODS

Plant material. In our experiment, we used in total 130 apple cultivars as was presented in Table 1. One tree of each of 33 Czech apple cultivars and 97 reference foreign cultivars were selected from apple genetic resources (1,087 cultivars) of Research and Breeding Institute of Pomology in Holovousy, Czech Republic. To monitor reproducibility and exactness between molecular analyses we included second tree of 10 apple cultivars: Ametyst, Goldstar, Šampion, Topaz, Golden Delicious, James Grieve, McIntosh, Resista, King of Pippins and Cox's Orange Pippin.

DNA isolation. Expanded green leaves were collected from June to August (2007–2010), immediately placed on ice and transported to the lab, where they were frozen by the liquid nitrogen and stored in –80°C until use. Leaves were powdered with liquid nitrogen in pre-cooled mortars. DNA was isolated from approximately 1 g of leaf powder using SDS isolation method according to Goulao et al. (2001).

Molecular SSR analysis. Ten simple sequence repeat (SSR) primer pairs were used for molecular analyses. Eight primer pairs were obtained from Liebhard et al. (2002): CH01c06, CH01d09, CH01f12, CH02b10, CH02c02a, CH03d01, CH03g07 and CH05g08, and two primer pairs were obtained from Hokanson et al. (1998): GD 100 and GD 162. In a typical PCR reaction (Taq PCR master mix kit, Qiagen, Hilden, Germany), we used the following amplification conditions: 2 min at 94°C, 35 cycles (30 s at 94°C; 60 s at 54°C, 90 s at 72°C); 10 min at 72°C, in Genius thermocycler (Techne, Cambridge, UK) or TGradient thermocycler (Biometra, Goettingen, FRG). Amplification products were resolved via 5% denaturing (8M urea) polyacrylamide gel vertical electrophoresis and visualized by silver-staining (Patzak 2001). Stained and dried gels were duplicated to opaque daylight film (Promega, Madison, USA). The products were scored for the presence or absence in each sample, based on size measured with 20 bp DNA Marker (Bio-Rad, Hercules, USA). To monitor reproducibility between runs, we included the duplicated 10 apple cultivars, mentioned above, in every run.

Data analysis. Observed DNA fragments were recorded by the number of base pairs for each microsatellite locus in order to convert the recorded values to discrete alleles. Microsatellite data were transformed into binary scores in order to calculate similarity values. Genetic similarity was estimated using Jaccard's similarity coefficient (Jaccard 1908), which ranges from 0 (all products between evaluated cultivars were different) to 1 (all products between evaluated cultivars were identical). The genetic diversity analysis was evaluated by cluster analysis, which was revealed by NTSYS-pc v. 2.11V for Windows (Exeter Software, New York, USA). The dendrogram was generated using the unweighted pair group method with arithmetic mean (UPGMA) clustering procedure.

RESULTS AND DISCUSSION

Characterization and evaluation of plant genetic resources is one of the purposes of "National program for conservation and utilization of genetic resources of plants, animals and microorganisms" of Ministry of Agriculture of the Czech Republic. In this study, we presented the results of utilization of ten microsatellite SSR loci to characterize 33 Czech cultivars and 97 reference foreign cultivars obtained from the national apple genetic resources collection in the Research and Breeding Institute of Pomology in Holovousy. All ten SSR primer pairs amplified clearly distinguishable and highly polymorphic PCR products. These microsatellite loci yielded a total of 89 polymorphic alleles in the set of 130 apple cultivars. The number of alleles per locus ranged from four (GD 100) to fourteen (CH02c02a). There were the specific allelic compositions for each analysed apple cultivar. In our previous work, we used only six SSR primer

Table 1. List of 130 apple cultivars, which were used for molecular simple sequence repeat (SSR) analyses

Cultivar	Pedigree	Origin	Year
Akane	Jonathan × Worcester Pearmain	JP	1939
Alkmene	Oldenburg × Cox's Orange Pippin	DE	1930
Ametyst	Nela (Prima × Krasava) × Vista Bella	CZ	2005
Angold	A 28/39 (Antonovka o.p.) × Golden Delicious	CZ	1995
Antonovka	chance seedling	RU	1826
Apollo	Cox's Orange Pippin × Oldenburg	DE	1976
Arlet	Golden Delicious × Idared	CH	1958
Bancroft	Forest × McIntosh	CA	1930
Baumanns Renette	chance seedling	BE	1811
Belréne	King of the Pippins mutation	FR	1975
Biogolden	ÚEB 1200/1 × Golden Delicious	CZ	2001
Blaník	Florina × Šampion	CZ	2003
Blenheim Orange	chance seedling	UK	1740
Bohemia	Rubín mutation	CZ	1994
Boskoop	chance seedling	NL	1856
Braeburn	Lady Hamilton o.p.	NZ	1952
Breuhahn	seedling of Halberstädter Jungfernapfel	DE	1895
Britemac	Melba × Kildare	US	1934
Cox's Orange Pippin	Ribston Pippin × Blenheim Orange	UK	1825
Delicious Richared	Red Delicious mutation	US	1915
Denár	Golden Delicious × Cox's Orange Pippin	CZ	1989
Desert	Golden Delicious × Cox's Orange Pippin	CZ	1993
Diadém	James Grieve × Jonathan	CZ	1992
Discovery	Worcester Pearmain × Beauty of Bath	UK	1949
Dublet	Golden Delicious × Jonathan	CZ	1991
Dukát	Golden Delicious × Cox's Orange Pippin	CZ	1965
Ecolette	Elstar (Golden Delicious × Ingrid Marie) × Prima	NL	1995
Empire	McIntosh × Red Delicious	US	1945
Empire Red	Empire mutation	US	1966
Fantasia	McIntosh × Linda	PL	1944
Florina	Golden Delicious × (Rome Beauty × *Malus floribunda* 82) × Starking Simpson's Giant Limb × Jonathan	FR	1980
Fuji	Ralls Janet × Red Delicious	JP	1939
Gala	Kidd's Orange Red × Golden Delicious	NZ	1934
Gavin	Worcester Pearmain × DG 20-9	UK	1965
Glencross	seedling of McIntosh	CA	1991
Glockenapfel	chance seedling	CH	1865
Gloster	Glockenapfel × Richared Delicious	DE	1969
Golden Delicious	seedling of Grimes Golden	US	1890
Golden Spur	Golden Delicious mutation	US	1960
Goldstar	Rubín × Vanda (Jolana × Lord Lambourne)	CZ	1998
Granny Smith	chance seedling	AT	1868
Granny Smith Spur	Granny Smith mutation	AT	1981
Greensleeves	James Grieve × Golden Delicious	UK	1966
Hana	Prima × Krasava	CZ	1999

Table 1. to be continued

Cultivar	Pedigree	Origin	Year
Holiday	Macoun × Jonathan	US	1940
Idared	Jonathan × Wagener	US	1935
Imperial Red	McIntosh mutation	UK	1932
Ingrid Marie	seedling of Cox's Orange Pippin	DK	1910
Jamba 69	Melba × James Grieve	DE	1955
James Grieve	seedling of Pott's Seedling	UK	1893
James Grieve Red	James Grieve mutation	UK	1922
Jerseymac	Melba × (Wealthy × Starr) × (Red Rome × Melba) × Julyred	US	1956
Jester	Worcester × Starkspur Golden Delicious	UK	1981
Jonafree	855-102 (Jonathan, Golden Delicious, Rome Beauty) × New Jersey 31(Galia Beauty × Red Spy)	US	1972
Jonagold	Golden Delicious × Jonathan	US	1943
Jonalord	Jonathan × Lord Lambourne	CZ	1993
Jonamac	McIntosh × Jonathan	US	1944
Jonared	Jonathan mutation	US	1962
Julia	Discovery × Quinte	CZ	1991
Julyred	(Petrel × Early McIntosh) × [Melba × (Williams × Starr)]	US	1955
Karin Schneider	Ingrid Marie mutation	DK	1953
Karmen	Lord Lambourne × Linda	CZ	1966
Karmína	Karmen × 1725 × 6	CZ	1995
Katja	James Grieve × Worcester Pearmain	SE	1947
King Jonagold	Jonagold mutation	BE	1986
King of the Pippins	chance seedling	UK	1800
Krasava	Otcovo × Wagener	CZ	1965
Liberty	Macoun × Purdue 54-12	US	1955
Ligol	Linda × Golden Delicious	PL	1972
Lobo	seedling of McIntosh	CA	1898
Lord Lambourne	James Grieve × Worcester Pearmain	UK	1907
Macoun	McIntosh × Jersey Black	US	1909
Mantet	seedling of Tetofsky	CA	1928
McFree	McIntosh × PRI 48-177	CA	1975
McIntosh	seedling of Fameuse	CA	1796
McIntosh Double Red	McIntosh mutation	US	1930
McIntosh Red	McIntosh mutation	CA	1811
McIntosh Spur	McIntosh mutation	CA	1978
Melba	seedling of McIntosh	CA	1898
Melba redRed	Melba mutation	CA	1936
Melodie	Šampion × DIR-38-T-16	CZ	1989
Melrose	Jonathan × Red Delicious	US	1937
Mio	Worcester Pearmain × Oranie	SE	1932
Mollie's Delicious	(Golden Delicious × Edgewood) × NJ 4(Gravensteiner Red × Close)	US	1948
Oldenburg	Hammerstein × Baumanns Renette	DE	1897
Oldenburg Red	Oldenburg mutation	CZ	1983
Ontario	Wagener × Northern Spy	CA	1820

Table 1. to be continued

Cultivar	Pedigree	Origin	Year
Orangenburg	Cox's Orange Pippin × Oldenburg	DE	1973
Otava	Šampion × Jolana	CZ	1997
Paulared	chance seedling	US	1960
Pilot	Clivia (Oldenburg × Cox's Orange Pippin) × Undine	DE	1962
Pinova	Clivia × Golden Delicious	DE	1965
Pott's Seedling	chance seedling	UK	1844
Priam	PRI 14-126 × Jonathan	US	1974
Prima	PRI 14-510 × NJ 123249	US	1970
Primula	(Golden Delicious, Melba, Red Rome, Rome Beauty, *M. floribunda* 821)	PL	1967
Priscilla	Starking × PRI 610-2	US	1961
Produkta	Antonovka × Goldspur	CZ	1998
Quinte	Crimson Beauty × Red Melba	CA	1964
Rajka	Šampion × ÚEB 1200/1(Katka)	CZ	1999
Red Free	Raritan × PRI 1018-101	US	1982
Red Mantet	Mantet mutation	CZ	1993
Red Spur Delicious	Starking Delicious mutation	US	1954
Red Wealthy	Wealthy mutation	US	1933
Relinda	Undine × B5(44,14)	DE	1989
Resista	Prima × NJ 56 (Golden Delicious, Rome, Jonathan, Yellow Newtown, Cox's Orange Pippin)	CZ	1997
Rewena	(Cox's Orange Pippin × Oldenburg) × BX 44,14	DE	1989
Rode Boskoop	Boskoop mutation	NL	1923
Rogo	King of the Pippins mutation	YU	1972
Romus 1	Camuzat × PCF9-100 (F4)	RO	1986
Rosana	Jolana × Lord Lambourne	CZ	1994
Royal Gala	Gala mutation	NL	1977
Rozela	Vanda × Bohemia	CZ	2007
Rubín	Lord Lambourne × Golden Delicious	CZ	1983
Rubinola	Prima × Rubín	CZ	1997
Šampion	Golden Delicious × Cox's Orange Pippin	CZ	1977
Šampion red	Šampion mutation	CZ	2003
Schweizer Orangenapfel	Ontario × Cox's Orange Pippin	CH	1935
Selena	Britemac × Prima	CZ	1994
Spartan	McIntosh × Newton Pippin	CA	1926
Spartan Compact	Spartan mutation	CA	1965
Stark Earliest	chance seedling	US	1938
Starkrimson Delicious	Starking Delicious mutation	US	1952
Topaz	Rubín × Vanda	CZ	1997
Vista Bella	Julyred × Starr × Williams × Melba × Sonar	US	1956
Wagener	chance seedling	US	1796
Wealthy	seedling of Cherry Crab	US	1869
Witos	Fantazja × Primula	PL	1995
Worcester Pearmain	seedling of Devonshire Quarrenden	UK	1874
Zlatava	Lord Lambourne × Bláhova Reneta (Cox's Orange Pippin × Wagener)	CZ	1991

Information about apple cultivars is from the official database of national apple repository centre in RBIP Holovousy

pairs for successful genetic diversity analysis of 102 old and local Czech apple genotypes. The use of ten SSR loci was better for sufficient evaluation of genetic biodiversity. Hokanson et al. (2001) used eight SSR primer pairs, Guarino et al. (2006) and Gharghani et al. (2009) used nine SSR primer pairs, Pereira-Lorenzo et al. (2007), Garkava-Gustavsson et al. (2008) and Gasi et al. (2010) used ten SSR primer pairs for assessment of genetic diversity in germplasm collections, respectively. Silfverberg-Dilworth et al. (2006) reported that 86 reliable, highly polymorphic, and overall the apple genome well-scattered SSRs cover about 85% of apple genome. Patocchi et al. (2009) included these SSRs into 21 different multiplex PCR and multiplex Hi12a of six SSRs was enabled to differ 125 used apple genotypes (Evans et al. 2011).

Thus in total, 89 polymorphic SSR alleles were sufficient and useful for the cluster analysis of 130 apple cultivars by NTSYS-pc v. 2.11V (Exeter Software, New York, USA), with the aim to evaluate genetic diversity and relationships. The resulting dendrogram showed that apple cultivars were divided into three major groups (Fig. 1). The dendrogram showed that there was a genotype dependence on pedigree and origin of apple cultivars (Table 1). Therefore SSR markers were useful for parentage study (Kitahara et al. 2005; Evans et al. 2011) and cultivar identification (Galli et al. 2005; van Treuren et al. 2010) of apple genotypes. The first group (I.) was divided into three subgroups, surrounded around cvs James Grieve (I.a), Cox's Orange Pippin (I.b) and Jonathan ancestry (I.c). The second group (II.) included apple cultivars with cv. McIntosh ancestry and it was divided into three subgroups depending on genetic diversity. The third group (III.) was also divided into three subgroups: with cv. Golden Delicious ancestry (III.a, b), Czech breeding program origin (III.b) and undefined, more diverse, origin (III.c). One-half of Czech apple cultivars was grouped in the third group, mainly in subgroup III.b (Fig. 1). This group was influenced by cv. Golden Delicious ancestry and Czech bred cultivars and breeding clones. Czech parents cvs. Karmen and Krasava of cultivars Karmína and Hana, respectively, were grouped, due to their ancestry (Table 1), in subgroup I.a (Fig. 1). But cultivars Karmína and Hana were grouped with *Vf* scab (*Venturia inaequalis* CKE) resistant cultivars Goldstar, Topaz, Melodie, Rajka, Biogolden, Angold, Otava and Rosana from Czech resistance breeding program (Zoufalá et al. 2009). Next *Vf* scab resistant Czech cultivars Selena, Rubinola and Ametyst were grouped with their parent resistant cultivar Prima in subgroup II.a (Table 1, Fig. 1). Genome of another cv. Prima derived cultivar Resista was influenced by other ancestors and cv. Resista was included in subgroup II.b (cv. McIntosh ancestry group). *Vf* scab resistant cultivar Blaník was also included in this subgroup, while its parental genotypes were grouped in subgroups I.c (cv. Florina) and III.b (cv. Šampion). Last *Vf* scab resistant cultivar Dukát was grouped in subgroup I.b, with parent cultivar Cox's Orange Pippin (Fig. 1). Evans et al. (2011) reported that cultivar Dukát could not be derived from cv. Golden Delicious based on SSR analysis. Hence, cultivars Desert and Denár, from similar breeding program (Table 1), were grouped in subgroups I.a and I.b, respectively (Fig. 1). The additional Czech apple cultivars were also clustered due to their pedigree (Table 1, Fig. 1). Cultivar Julia was grouped in subgroup II.a, with parent cultivar Quinte. Cultivars Jonalord and Dublet were grouped in cv. Jonathan ancestry subgroup I.c. Cultivar Zlatava was grouped in cv. Cox's Orange Pippin ancestry subgroup I.b. Cultivar Diadém was grouped in cv. James Grieve ancestry subgroup I.a.

It is a known fact that genetic diversity analyses are always influenced by the range of evaluated molecular data and genotypes. Although, several apple genetic diversity studies based on SSR markers were done (Hokanson et al. 2001; Guarino et al. 2006; Pereira-Lorenzo et al. 2007; Garkava-Gustavsson et al. 2008; Gharghani et al. 2009; Patzak et al. 2009; Gasi et al. 2010), very different germplasm collections were used for them. Spontaneous mutations always had identical allele composition (Hokanson et al. 2001; Pereira-Lorenzo et al. 2007; Garkava-Gustavsson et al. 2008). Therefore, we also did not find microsatellite differences between original and mutated (spur, red, etc.) apple cultivars James Grieve, McIntosh, Oldenburg, Melba, Mantet, Golden Delicious, Gala, Jonagold, Empire, Spartan, Šampion, Wealthy, Boskoop and Granny Smith (Fig. 1). Only retrotransposon markers were possible to distinguish spontaneous mutations (Venturi et al. 2006; Zhao et al. 2010). We also found no differences between cvs Belrené and King of Pippins, Mantet and Glencross, Red Spur Delicious, Starkrimson Delicious and Delicious Richared, respectively (Fig. 1). Pereira-Lorenzo et al. (2007) also found that cultivars Red Spur, Top Red, Erovan and Oregon were indistinguishable.

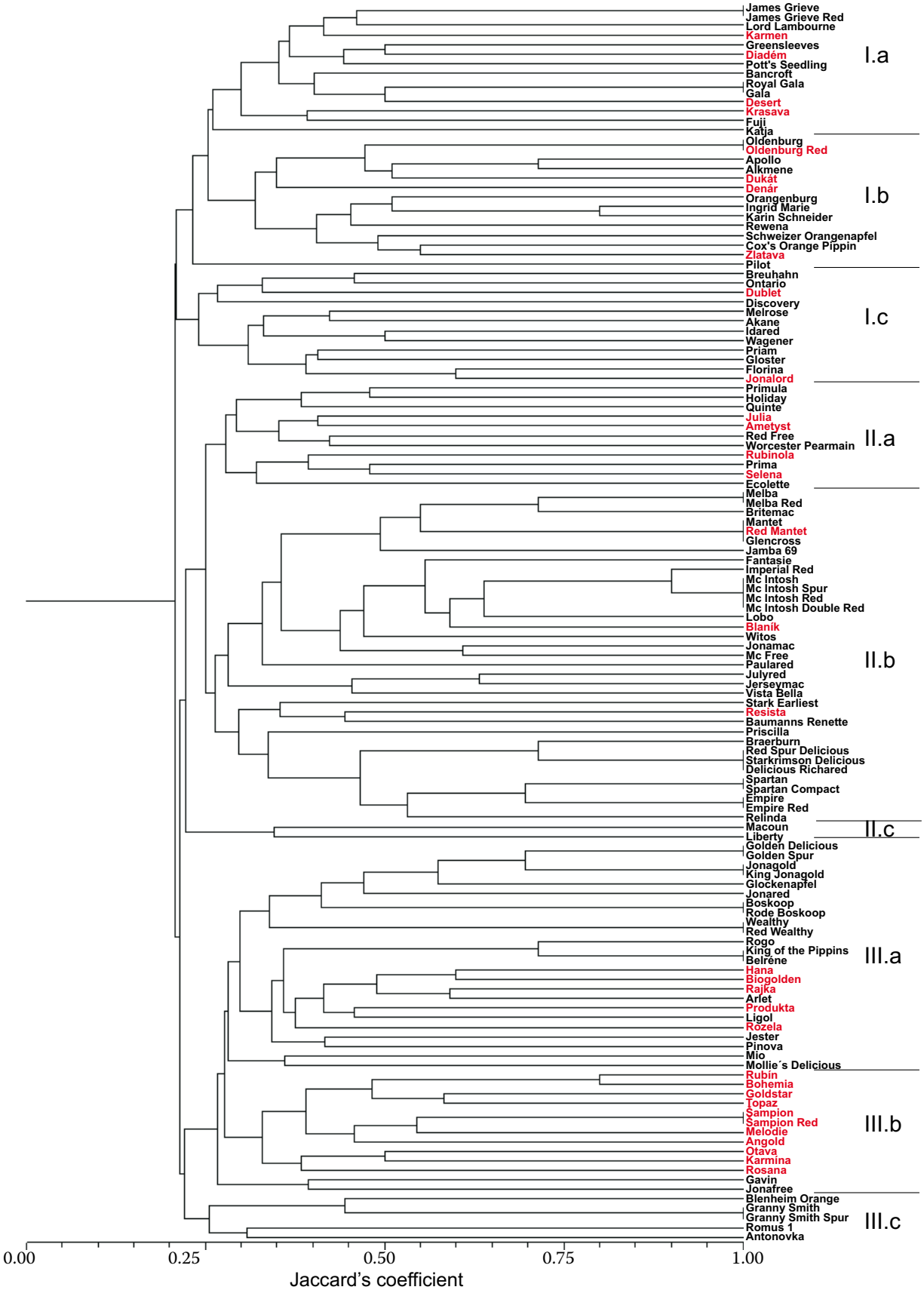

Fig. 1. Dendrogram of 130 apple cultivars revealed by software NTSYS-pc v. 2.11V cluster analysis determined using 89 SSR markers of ten microsatellite loci

Cv. Belrené is a mutation of cv. King of Pippins (Table 1). Identical microsatellite profiles of cvs Mantet and Glencross could be due to mistake in the reported pedigree or potential documentation error. Similar results were published for known pedigrees (Evans et al. 2011) or incorrect documented cultivar names (van Treuren et al. 2010) in apple collections using SSR markers. Therefore for apple, microsatellite genotyping appeared a very efficient tool for enhancing collection management of genetic resources and for cultivar identification.

CONCLUSIONS

We proved that microsatellite markers were useful for evaluation of genetic diversity of Czech apple cultivars and determination of genetic relationships with current foreign apple cultivars. The most of Czech cultivars were clustered due to their pedigree with parent cultivars and molecular marker analyses confirmed their origin. However, SSR analyses were able to find the additional breeding background, incorrections in reported pedigrees and other documentation errors.

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