ACTA SCIENTIARUM POLONORUM
Acta Sci. Pol. Hortorum Cultus, 18(3) 2019, 157–165
www.acta.media.pl ISSN 1644-0692 e-ISSN 2545-1405 DOI: 10.24326/asphc.2019.3.15
ORIGINAL PAPER
Accepted: 26.09.2018

EVALUATION OF GENETIC VARIABILITY WITHIN SWEET CHERRY (*Prunus avium* L.) GENETIC RESOURCES BY MOLECULAR SSR MARKERS

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ABSTRACT

Sweet cherry is a vegetatively propagated, perennial plant with high level of heterozygosity and ancient breeding history. Therefore, it is necessary to keep, conserve and evaluate known genetic resources for future breeding programs and fruit production stability. In present, the utilization of DNA molecular genetic analyses is the best suitable method for evaluation of individual accessions, thus we eliminated duplications and characterized the genetic relationships. In our work, we used PCR primer combinations for 19 SSR and 2 EST-SSR *loci* for analyses of 123 current, old and local sweet cherry cultivars from Czech genetic resources of Research and Breeding Institute of Pomology in Holovousy. In total, 115 polymorphic fragments were amplified, which we used for hierarchical cluster analysis of genetic variability. The result dendrograms were divided into three main clusters and ten subgroups. Clustering corresponded to genealogical and geobotanical characteristics of individual accessions as breeding history of several known accessions.

Key words: sweet cherry (*Prunus avium* L.), SSR and EST-SSR markers, genetic diversity, cultivars, hierarchical cluster analysis

INTRODUCTION

Sweet cherry (*Prunus avium* L.) is an out-breeding, self-incompatible diploid species in the *Rosaceae* family with a genome of $2n = 16$. The species is commonly grown in the temperate climatic zones with cooler temperatures to provide chilling requirement necessary for flower induction. It is believed that cherries originated in the area between the Black Sea and Caspian Sea in Asia Minor [Fernandez and Marti et al. 2012]. Breeding programs have developed new breeding lines based on improving traditional cultivars. But it seems that the genetic diversity in sweet cherry new varieties have been minimized due to repeated use of a few founding clones as parents [Lacis et al. 2009]. In the Czech Republic, the main sweet cherry breeding organization is Research and Breeding Institute of Pomology Holovousy Ltd., which is also the national center for maintaining and preserving the cherry genetic resources.

The importance of genetic diversity evaluation and accurate identification of plant material in fruit germplasm collections of cultivars and breeding material is obvious. In fruit crops, true identification is difficult since phenotypic characters are generally influenced by the environment and the growth stage of a plant [Struss et al. 2003]. The use of molecular techniques that detect variations at deoxyribonucleic acid (DNA) level is more objective. Effective genetic marker systems were developed during the past decades. Many

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marker systems have been created for various plant species since the first microsatellite marker identification in *Prunus* genus [Cipriani et al. 1999]. Microsatellites (Simple Sequence Repeat – SSR) have been widely used for genetic studies of cultivated and wild cherry, including genetic diversity analyses [Lacis et al. 2009, 2010], cultivar identification and fingerprinting [Struss et al. 2003], self-incompatibility and population genetic structure evaluation [Vaughan and Russell 2004, Mariette et al. 2010] and genetic mapping [Olmstead et al. 2008]. Nowadays, microsatellites markers have been found in gene sequences as Expressed Sequence Tag-Simple Sequence Repeat (EST-SSR) [Gasic et al. 2009]. A recent technical advance in next generation sequencing (NGS) opened a way to obtain sequence differences in genomes, useful as Single Nucleotide Polymorphism (SNP) markers [Fernandez and Marti et al. 2012, Ganopoulos et al. 2013].

In this paper, we present the use of SSR molecular markers for determination of genetic diversity and genetic relationships within the set of world current, old and local cherry cultivars from Czech genetic resources.

MATERIAL AND METHODS

In our experiment, we totally used 123 cherry accessions of current, old and local cultivars (Tab. 1) from cherry genetic resources collections of Research and Breeding Institute of Pomology in Holovousy (50°22'31.19"N, 15°34'39.04"E, CR). One g of young green leaves was collected, powdered in liquid nitrogen and used for DNA isolation by SDS isolation method according to Goulão et al. [2001]. Isolated DNAs were afterwards cleaned by ChargeSwitch® gDNA Plant Kit (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA). Twenty SSR primer pairs: UDP96001, UDP96005, UDP98021, UDP98022, UDP98412 [Testolin et al. 2000], UCDCH12, UCDCH14, UCDCH17, UCDCH21, UCDCH31 [Struss et al. 2003], EMPA004, EMPA005, EMPA018 [Clarke and Tobutt 2003], EMPaS001, EMPaS006, EMPaS012 [Vaughan and Russell 2004], BPPCT002, BPPCT005, BPPCT026 and BPPCT034 [Dirlewanger et al. 2002] and five EST-SSR primer pairs: CN911135, CN896269, CO414802, CN907352 and CO753161 [Gasic et al. 2009] were used for molecular analyses. In a PCR reaction (*Taq* PCR master mix kit, Qiagen, Hilden, FRG), 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 TGradient thermocycler (Biometra, Goettingen, FRG). Amplification products were resolved *via* 5% denaturing (8 M urea) polyacrylamide gel vertical electrophoresis and visualized by silver-staining [Patzak 2001]. Stained and dried gels were duplicated to opaque daylight film (Promega, Madison, WI, USA). The products were scored for the presence or absence of fragments in each sample, based on the size measured with 20 bp DNA Marker (Bio-Rad, Hercules, CA, USA). Fragments were recorded by the number of base pairs for each microsatellite *locus* in order to convert the recorded values to discrete alleles. Expected and observed heterozygosities, polymorphic information content, Hardy-Weinberg equilibrium and Weir and Cockerham F-statistics within population were calculated using GENEPOP version 3.4 [Raymond and Rousset 1995]. Hierarchical cluster analysis was used for evaluation of cherry genotypes genetic relationships. They were based on Jaccard's similarity coefficient and unweighted Neighbor-Joining (NJ) clustering in DARwin v. 5.0.155 (Dissimilarity Analysis and Representation for Windows, http://darwin.cirad.fr/darwin). The dendrogram was visualized by Geneious Pro 4.8.2 (Biomatters Ltd., Auckland, New Zealand).

RESULTS

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 twenty microsatellite SSR and five EST-SSR *loci* to characterize 123 world current, old and local cultivars obtained from the national cherry genetic resources collection in Research and Breeding Institute of Pomology in Holovousy. All primer pairs amplified clearly distinguishable and highly polymorphic PCR products. UDP96005 *locus* was multi-allelic and there were found null alleles in five SSR *loci* (UCDCH14, UCDCH31, EMPA004, EMPA005 and EMPaS006). The number of alleles per *locus* ranged from three (UCDCH14)

Table 1. List of 123 current, old and local sweet cherry cultivars with their origins

Cultivar	Origin	Pedigree
1	2	3
'Raná Černá Edra'	BGR	unknown
'Raná Laskovská'	BGR	unknown
'Sam'	CAN	'V160140' ('Windsor' × OP) × OP
'Star'	CAN	'Deacon' × OP
'Stella'	CAN	'Lambert' × 'J12420'
'Stella Compact'	CAN	'Lambert' × 'JI 2420'
'Sue'	CAN	'Bing' × 'Schmidt'
'Summit'	CAN	'Van' × 'Sam'
'Sunburst'	CAN	'Van' × 'Stella'
'Sweetheart'	CAN	'Van' × 'Newstar' ('Van' × 'Stella')
'Van'	CAN	'Empress Eugenie' × OP
'Van Compact'	CAN	'Empress Eugenie' × OP
'Vega'	CAN	'Bing' × 'Victor' ('Windsor' × OP)
'Velvet'	CAN	'Windsor' × OP
'Venus'	CAN	'Hedelfinger' × 'Windsor'
'Vic'	CAN	'Bing' × 'Schmidt'
'Vineland'	CAN	unknown
'Viva'	CAN	'Hedelfingen' × 'Windsor'
'Vogue'	CAN	'Hedelfinger' × 'Windsor'
'Buketova'	CZE	unknown
'Černá špička'	CZE	unknown
'Černá z Hořan'	CZE	unknown
'Děkanka'	CZE	unknown
'H 21/40 Černá'	CZE	unknown
'Holovouská chrupka'	CZE	unknown, seedling of 'Hedelfinger'
'Chlumecká Černá'	CZE	unknown
'Karešova'	CZE	unknown
'Kordia'	CZE	unknown
'Ladeho pozdní'	CZE	seedling of 'Ed Lade', syn. 'Hildesheim'
'Libějovická raná'	CZE	unknown
'Moravská rychlice'	CZE	unknown
'Mramorovaná chrupka'	CZE	unknown
'Pivka'	CZE	unknown
'Pivovka'	CZE	unknown
'Plavečský granát'	CZE	unknown
'Samofertilní'	CZE	unknown
'Semenáč č.13'	CZE	unknown
'Srdcovka přeúrodná'	CZE	unknown
'Šakvická'	CZE	unknown
'Těchlovan'	CZE	'Van' × 'Kordia'

Table 1 cont.

1	2	3
'Těchlovická'	CZE	unknown
'Vanda'	CZE	'Van' × 'Kordia'
'Vlachova'	CZE	unknown
'Vosenka'	CZE	unknown
'Žalanka'	CZE	unknown
'Alma'	DEU	'Rube' × 'Allers Späte Knorpel'
'Badeborner'	DEU	unknown
'Büttners späte Knorpelkirsche'	DEU	unknown ('Napoleon')
'Dönissens Gelbe'	DEU	unknown
'Drogans Gelbe'	DEU	unknown
'Emperor Francis'	DEU	unknown
'Erika'	DEU	'Rube' × 'Stechmanns Bunte'
'Frühe von Boppard'	DEU	unknown
'Germersdorfer'	DEU	unknown
'Grolls Schwarze Knorpelkirsche'	DEU	unknown
'Hedelfinger'	DEU	unknown
'Hildesheim'	DEU	unknown
'Kassins Frühe'	DEU	unknown
'Knauffs Schwarze'	DEU	unknown
'Leopoldskirsche'	DEU	unknown
'Meckenheimer Frühe'	DEU	unknown
'Müncheberger'	DEU	'Flamentiner' × 'Früheste de Mark'
'Napoleon'	DEU	unknown
'Napoleon Compact'	DEU	unknown
'Německá rychlice'	DEU	unknown
'Oktavia'	DEU	'Schneiders Späte Knorpel' × 'Rube'
'Querfurter Königskirsche'	DEU	unknown ('Napoleon')
'Rebekka'	DEU	'Rube' × 'Schubacks Frühe Schwarze'
'Regina'	DEU	'Schneiders' × 'Rube'
'Simonis'	DEU	unknown
'Spitze Braune'	DEU	unknown
'Thurn Taxis'	DEU	'Schneiders'
'Troprichters Schwarze Knorpelkirsche'	DEU	unknown
'Valeska'	DEU	'Rube' × 'Stechmanns Bunte'
'Velká Černá Chrupka'	DEU	'Grosse Schwarze Knorpelkirsche'
'Viola'	DEU	'Schneiders Späte Knorpel' × 'Rube'
'Winkler's Frühe'	DEU	unknown
'Zeisberger'	DEU	unknown
'Bigarreau Charmes'	FRA	unknown
'Burlat'	FRA	unknown
'Burlat C1'	FRA	unknown

Table 1 cont.

1	2	3
‘Burlat Spur’	FRA	unknown
‘Ramon Oliva’	FRA	unknown
‘Early Rivers’	GBR	‘Early Purple’ × OP
‘Merchant’	GBR	‘Merton Glory’ × OP
‘Merla’	GBR	‘Merton Late’ (‘Bella Agatha’ × ‘Napoleon’) × OP
‘Mermat’	GBR	‘Merton Glory’ × OP
‘Merton Favourite’	GBR	unknown
‘Merton Glory’	GBR	‘Ursula Rivers’ × ‘Noble’
‘Merton Premier’	GBR	‘Emperor Francis’ × ‘Bedford Prolific’
‘Baltavarská’	HUN	unknown
‘Alfa’	CHE	‘Basler Adler’ × ‘Erstfrühe’
‘Basler Adlerkirche’	CHE	unknown
‘Basler Langstieler’	CHE	unknown
‘Beta’	CHE	‘Zweitfrühe’ × ‘Basler Adlerkirsche’
‘Beta VF’	CHE	‘Zweitfrühe’ × ‘Basler Adlerkirsche’
‘Delta’	CHE	‘Basler Adlerkirsche’ × ‘Zweitfrühe’
‘Gamma’	CHE	‘Mischler’ × ‘Zweitfrühe’
‘Schöne von Marien’	CHE	unknown
‘Zweitfrühe’	CHE	unknown
‘Durone Nero 1’	ITA	unknown
‘Nero 1’	ITA	unknown
‘Nero 2’	ITA	unknown
‘Kišiněvskaja’	MDA	unknown
‘Skierniewice 1’	POL	unknown
‘Skierniewice 3’	POL	unknown
‘Skorospielka’	RUS	unknown
‘Asenova raná’	SRB	unknown
‘Ladzanská 1’	SVK	‘Grosse Schwarze Knorpelkirsche’
‘Medňanská’	SVK	unknown
‘Huldra’	SWE	‘Eriane’ × ‘Allmän Gulröd’
‘Rivan’	SWE	‘Early Rivers’ × ‘Van’
‘Szwecija’	SWE	unknown
‘Valerij Tschkalov’	UKR	‘Rozornaja’ (‘Cherry Rose’) × OP
‘Bing’	USA	‘Black Republican’ × OP
‘Gil Peck’	USA	‘Napoleon’ × ‘Giant’
‘Hudson’	USA	‘Oswego’ × ‘Giant’
‘Kristin’	USA	‘Emperor Francis’ × ‘Gil Peck’
‘Lambert’	USA	‘Napoleon’ × ‘Blackheart’
‘Lapins’	USA	‘Van’ × ‘Stella’
‘Mona Cherry’	USA	unknown
‘Seneca’	USA	‘Early Purple Guigne’ × OP
‘Starking Hardy Giant’	USA	unknown

OP – open pollinated

Table 2. Allelic diversity, genetic and statistic characteristics of the microsatellite *loci*

SSR *locus*	Size range (bp)	N_A	H_E	H_O	*PIC*	*P-value*	F_{is}
UDP96001	108–126	4	0.652	0.488	0.650	0.000	0.253
UDP98021	100–112	6	0.588	0.626	0.586	0.076	–0.064
UDP98022	92–114	7	0.724	0.707	0.228	0.207	0.023
UDP98412	114–140	7	0.789	0.723	0.715	0.000	0.083
UCDCH12	173–200	6	0.680	0.585	0.712	0.000	0.139
UCDCH14	139–147	3	0.645	0.545	0.653	0.095	0.156
UCDCH17	186–190	8	0.826	0.724	0.823	0.000	0.125
UCDCH21	114–122	4	0.679	0.374	0.676	0.000	0.451
UCDCH31	111–148	6	0.614	0.528	0.648	0.002	0.140
EMPA004	177–195	5	0.645	0.707	0.647	0.015	–0.098
EMPA005	230–262	5	0.659	0.707	0.655	0.078	–0.074
EMPA018	92–110	6	0.545	0.488	0.480	0.019	0.106
EMPaS001	225–254	4	0.590	0.545	0.588	0.069	0.077
EMPaS006	200–230	9	0.810	0.683	0.813	0.001	0.157
EMPaS012	121–152	5	0.782	0.805	0.812	0.043	–0.030
BPPCT002	180–200	6	0.699	0.618	0.696	0.012	0.116
BPPCT005	154–204	7	0.742	0.813	0.478	0.113	–0.097
BPPCT026	162–182	8	0.767	0.813	0.560	0.094	–0.060
BPPCT034	212–234	4	0.598	0.675	0.596	0.029	–0.129
CN911135	178–180	2	0.143	0.154	0.146	1.000	–0.081
CN896269	294–300	3	0.465	0.447	0.466	0.433	0.044

N_A – number of alleles, H_E – expected heterozygosity, H_O – observed heterozygosity, *PIC* – Polymorphic Information Content, *P-value* – Hardy-Weinberg equilibrium, F_{is} – Weir and Cockerham F-statistics within population

to nine (EMPaS006). The level of observed and expected heterozygosities ranged from 0.374 to 0.813 and from 0.545 to 0.826 within SSR *loci*, respectively (Tab. 2). Polymorphic Information Content (PIC) ranged from 0.228 to 0.813 within SSR *loci* (Tab. 2). Seven microsatellite *loci* showed significant deviation from Hardy-Weinberg equilibrium ($p < 0.01$) and calculated positive F_{IS} values indicated heterozygosity deficiency within twelve microsatellite *loci* (Tab. 2). The level of polymorphism within EST-SSR *loci* was very low that CO414802, CN907352 and CO753161 were monomorphic within studied cherry genotypes. There were found null alleles for CN911135. Statistic characteristics of EST-SSR *loci* were summarized in Tab. 2. Twenty-one microsatellite *loci* yielded a total of 115 polymorphic amplified fragments in cherry cultivars. Each cherry cultivar was characterized by individual allelic profile and results were useful for the hierarchical cluster analysis. Jaccard's similarity coefficient and unweighted Neighbor-Joining clustering (DARwin v. 5.0.155) were the best choice for an evaluation of genetic diversity and relationships of cherry accessions. The resulting dendrogram showed that cherry cultivars were divided into three main clusters and ten subgroups (Fig. 1). No molecular differences were found between 'Napoleon', 'Stella' and 'Van' cultivars and their compact variants, 'Burlat' cultivar and their spur and compact variants. Virus free plants of 'Beta' cultivar was also the same as original cultivar. We also did not find any duplications of accessions in genetic resources collection. Cluster one included early dark red cherry cultivars, mainly originated from Switzerland in (Ia) and other old European in (Ib). Bigarreau cultivars originated from

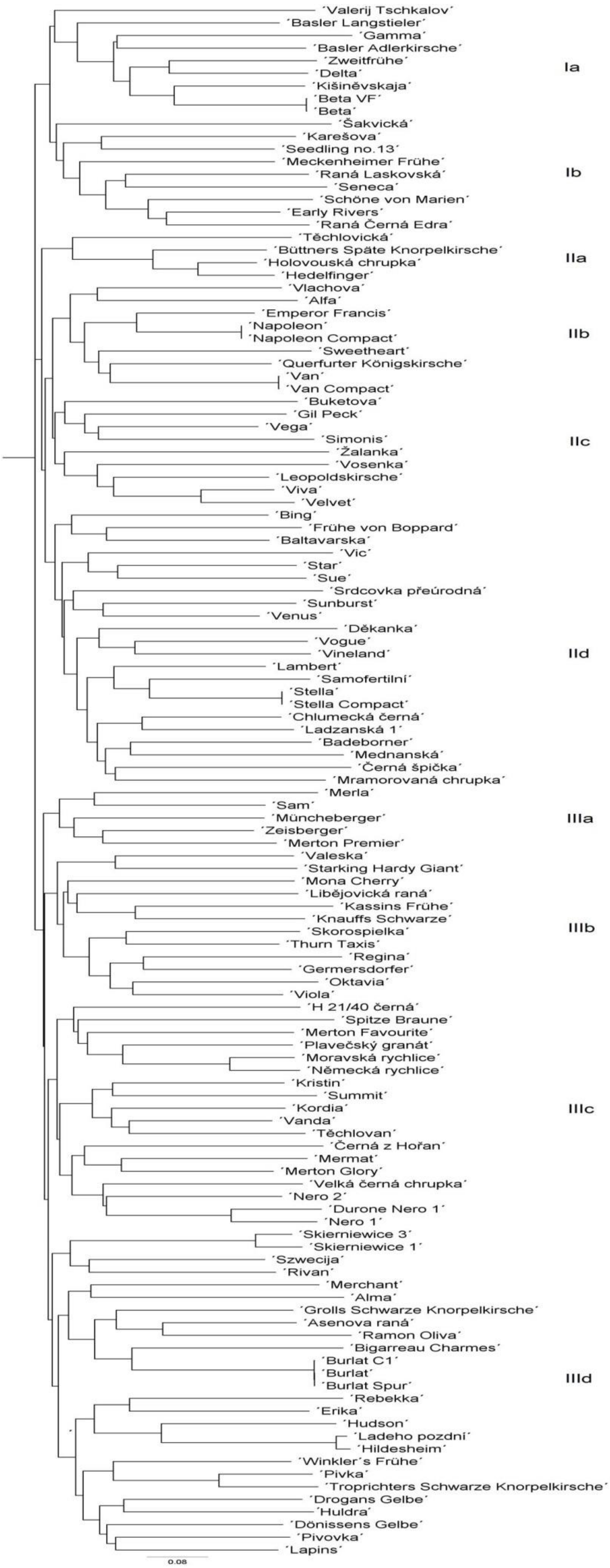

Fig. 1. Dendrogram of genetic distances of 123 current, old and local cherry cultivars revealed by unweighted Neighbor-Joining (NJ) clustering based on Jaccard’s similarity coefficient determined by 115 polymorphic molecular markers

'Napoleon', 'Winsdor', 'Van' and 'Bing' were grouped in cluster two. Cultivars originated from Germany were included in (IIa) and (IIb), and cultivars originated from Canada and USA in (IIc) and (IId). Czech old cultivars were spread through whole cluster, depending on their genetic origin. Cluster (IIIa) grouped cultivars genetically close with cluster two in origin. The rest of cluster three included black or dark red old sweet cherry cultivars from Europe. Cultivars mainly from Germany, originated from 'Schneiders', were in cluster (IIIb), cultivars from United Kingdom were spread through the whole cluster three, mainly in cluster (IIIc). Cultivars from Italy were in cluster (IIIc). Cultivars from France, Germany, Sweden and Poland were grouped in cluster (IIId). There were also American cultivars: 'Sam', 'Starking Hardy Giant', 'Mona Cherry', 'Kristin', 'Summit', 'Hudson' and 'Lapins', in cluster (III).

These obtained results were in agreement with breeding history of several accessions with known pedigree and origin, and with genealogical and geobotanical characteristics of individual accessions.

DISCUSSION

It is a known fact that genetic diversity analyses are always influenced by used type and range of molecular markers and by number and population of evaluated genotypes. For our used SSR markers, the number of alleles per *locus* was similar to previously published results [Dirlewanger et al. 2002, Vaughan and Russell 2004, Gasic et al. 2009], ranged from one to six. Higher number of alleles, up to 31, were found when different *Prunus* species [Wünsch 2009] or wild cherries cultivars [Turkoglu et al. 2012, De Rogatis et al. 2013] were studied. Turkoglu et al. [2012] also found null alleles within 37 studied cherry genotypes. Mariette et al. [2010] reported that average number of alleles was 7.6 for wild cherries and 8.8 for all sweet cherries, but only 4.3 for modern cherry cultivars. This loss of diversity within modern cultivars was due to breeding *vice versa* wild and landrace genotypes. Observed and expected heterozygosities, PIC and F_{is} corresponded to previous results in the range and average according to Lacis et al. [2009, 2010], Turkoglu et al. [2012] and De Rogatis et al. [2013]. The level of polymorphism and other statistical characteristic for EST-SSR *loci* were very low and their utilization was limited. These markers were derived from *Malus* genome by Gasic et al. [2009] and their transferability was only 25% to *Prunus* species. Previous sweet cherry genetic diversity analyses based on SSR markers, have been aimed mainly at evaluating the wild germplasm collections [Lacis et al. 2009, Turkoglu et al. 2012, Ganopoulos et al. 2013, De Rogatis et al. 2013], therefore we could not exactly compare obtained results of genetic diversity analyses. Either methodology or accession germplasm could influence the genetic diversity analysis. Fernandez and Marti et al. [2012] reported comparison of dendrograms from SSR and SNP markers. There was 75% similarity when inconsistency in relationships with presumed pedigree of accessions was shown. The result clustering for SSR markers was similar to our results. 114 analyzed cherry cultivars included only cultivars from our cluster two. The absence of cultivars from our other clusters could be due to the group of cultivars 'Sam', 'Kristin' and 'Lapins' inside three groups found. But depending on pedigree, these three cultivars could be also in our cluster two and not in cluster three. Nevertheless, we found very good agreement with suggested origins of old unknown cherry cultivars: 'Holovouská chrupka' from 'Hedelfinger', 'Pivka' from 'Troprichters Schwarze Knorpelkirsche', 'Ladeho pozdní' from 'Hildesheim' [Paprštein and Kloutvor 2015]. Therefore, SSR molecular markers can be successfully used for origin identification of old and local cultivars.

CONCLUSIONS

We proved that microsatellite SSR and EST-SSR molecular markers can be utilized for determination of genetic diversity and genetic relationships within 123 current, old and local cherry cultivars. The result dendrogram corresponded with genealogical and geobotanical characteristics of individual accessions. Microsatellite molecular markers can be useful in a management of genetic resources collections to eliminate duplications and mislabelling and to create core collections with maintenance of wide genetic diversity.

ACKNOWLEDGMENTS

This work was supported by the National Agency for Agricultural Research of the Ministry of Agriculture of CR in project QJ1510001.

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