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Development of microsatellites from *Cornus mas* L. (Cornaceae) and characterization of genetic diversity of cornelian cherries from China, central Europe, and the United States

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ABSTRACT

Cornelian cherry (*Cornus mas* L.) is indigenous to central and southeastern Europe and is an ecologically and economically important shrub or small tree. The aim of this study was to develop molecular tools for assessing genetic diversity and provide unique molecular identification of *C. mas* samples from central Europe and United States. A microsatellite-enriched library was used to develop nine polymorphic microsatellite loci. The loci amplified perfect and imperfect repeats with 2 to 11 alleles detected per locus. Observed heterozygosity ranged from 0.00 to 0.71 and expected heterozygosity ranged from 0.00 to 0.82. Additionally, cross species transfer to *Cornus eydeana* was observed. The multilocus allelic data was used to cluster 37 *C. mas* samples and 1 *C. eydeana* sample based on the allele sharing distance matrix. The similarity coefficient ranged from 0.05 to 0.73 among all genotypes. All *C. mas* individuals clustered into two main clades, with the single *C. eydeana* sample used to root the dendrogram. All samples in group I belong to the botanical form Macrocarpa and originated from Austria, Poland, or Ukraine, whereas group II included samples that originated from Poland, Romania, and the United States. Five loci (CM007, CM010, CM031, CM037, and CM043) were used to develop a molecular identification key that successfully delineated all samples. The loci described in this study will facilitate further investigations of genetic diversity, gene flow, and genetic structure among populations of *C. mas*.

1. Introduction

Cornelian cherry (*Cornus mas* L.) is a shrub or small tree native to central and southeastern Europe and produces one of the most valuable fruits within the *Cornaceae* (Eyde, 1988). Cornelian cherry is both a slow-growing and a long-lived plant up to 300 years (Piórecki, 2007). The shiny fruits are either yellow or deep-red (dark cherry to almost black), and are spherical or oval- shaped stone fruits.

Cornelian cherry was, and still is, known for its flavor, nutritional and medicinal benefits. Biologically active compounds, such as vitamin C, organic acids (mainly malic acid), pectins (Seeram et al., 2002; Kucharska et al., 2011; Kucharska, 2012), phenolic acids (gallic and ellagic, and derivatives of hydroxycinnamic acids) (Pantelidis et al., 2007 Kucharska, 2012), flavonoids (anthocyanins, flavonols) (Tural and Koca, 2008; Pawlowska et al., 2010), triterpenoid (ursolic acid) (Yayaprakasam et al., 2006), and – recently identified – iridoids (loganic acid, cornuside, loganin, sweroside) (West et al., 2012; Deng et al., 2013) have been reported in fruits of cornelian cherry. These compounds are purported to be beneficial for the prevention of heart disease and diabetes.

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Antibacterial, anti-inflammatory and antioxidant properties are also often ascribed to the fruits (Seeram et al., 2002; West et al., 2012). Cornelian cherry fruits also were used in folk medicine for the treatment of various fever-related diseases (flu, sore throat, and malaria) and gastrointestinal disorders.

Seed propagation and long term human selection have given rise to a great diversity of trees. Today, selections of large-fruited cultivars are characterized by the following: different fruit shapes, smaller stones, desirable chemical composition, and different maturity dates on the same plant. Only in recent decades breeding programs aimed at the development of large fruit and high-yielding trees have been launched in several countries. Most of these programs have been successful and new cultivars of cornelian cherry were registered in Ukraine [Elegantny, Koralovy, Nezhny, Yantarny, Svetlyachok, Exotichesky, Evgenia, Semen, Elena, Radost, Nikolka (Klimenko, 2004, 2007)], in Bulgaria (Kazanlytsky, Pancharevsky), in Slovakia (Dvin, Titus), in Austria (Jolico), in Russia (Ispolinskij, Karazogal, Kyrymzy-zogal, Gjul-zogal), in Azerbaijan (Armudi-Zogal, Ag-Zogal). Efficient selection and breeding programs have recently been established in Yugoslavia, Georgia, Czech Republic, Serbia, France, Turkey, Germany as well as in Poland.

In Poland, cornelian cherry has been cultivated for about 400 years (Piórecki, 2007). Until World War II, it was a well-known plant, often planted in parks neighboring Polish palaces and manors. Starting in the eighteenth-century, trees have been preserved in gardens and parks of many Polish towns (Piórecki, 2012). In the dendrological collection of Stanislaw Wodzicki in Niedzwiedz (near Cracow) during the first half of the nineteenth century, the following five cultivars of cornelian cherry were grown: Alba (1833), Flava (1817), Macrocarpa (1833), Variegata (1817) and Violacea (1833) (Dolatowski, 2013). Fruits are eaten both as fresh and processed, and in the form of jams, jellies, wines liqueurs, compotes, and pickled in Poland, (Burgsdorf, 1809; Wodzicki, 1818; Gerald-Wyżycki, 1845; Seneta, 1994).

The fruits that were used for food and medicine before World War II generally were small, with an average weight of 1.6–2.6 g, and had large stones. Today, selections of large-fruited cultivars are characterized by the following: different fruit shapes, smaller stones, desirable chemical composition, and different maturity dates on the same plant. Such fruits are desired by both producers and consumers. Therefore, breeding programs have concentrated on the selection of the finest cultivars of cornelian cherry. At the end of the last century, new cultivars of cornelian cherry were also registered in Azerbaijan (1990), Austria (1991), Bulgaria (1985), Slovakia (1989), as well as in Georgia and Czech Republic. However, most cultivars were registered in Ukraine in the years 1987–1999 and were the result of research done at the National Botanic Garden of the Ukrainian Academy of Sciences in Kiev (Klimenko, 2004, 2007).

Valuable ecotypes and forms from southeastern Poland and Ukraine were described in 1928 (Wierdak, 1928). The first modern Polish arboretum collections of cornelian cherry were founded in Bolestraszyce near Przemysl, in the late 1970s and early 1980s (Piórecki, 2007). The starting material was collected primarily from the nineteenth-century bushes in Zwierzyniec near Zamosc, in Bolestraszyce and in Pralkowce near Przemysl. During this time, 240 trees were planted at the Arboretum Bolestraszyce. From those collections 12 cultivars of cornelian cherry were selected and 10 were registered in Poland in the first decade of the twenty-first century. The fruits of selected Polish cultivars differ in both the time of ripening and harvesting as well as in antioxidant content and activity. Differences in the morphology and physico-chemical composition between Polish and European cultivars were also observed (Klimenko, 2004; Kucharska et al., 2007; Kucharska, 2011, 2012). However information from these traits is not sufficient to unambiguously identify cornelian cherry genotypes; the differences between them are often subtle or misleading. Thus, it would be useful to investigate DNA markers as tools for cultivar identification. The accessibly of reliable genetic markers is essential for variety identification and distinction, for development breeding method to create new cultivars, and to guarantee their proprietary protection. Various types of DNA markers such as DNA amplification fingerprinting (DAF) (Caetano-Anollés et al., 1999; Culpepper et al., 1991), microsatellites (Cabe and Liles, 2002; Hadziabdic et al., 2010, 2012; Wadl et al., 2008a, 2008b, 2010, 2012, 2013; Wang et al., 2008) and amplified fragment length polymorphism (AFLPs) (Mmbaga and Sauve, 2007; Smith et al., 2007) have been used to determine genetic diversity in *Cornus* species.

The accessibly of reliable genetic markers is essential for variety identification and distinction, for development breeding method to create new cultivars, and to guarantee their proprietary protection. Microsatellite, or simple sequence repeats (SSRs), markers have proven to be highly informative and useful for developing a molecular identification key for cultivars and lines of *Cornus florida* and *C. kousa* (Wadl et al., 2008a). Therefore, the aims of this study were to clarify the origin of Polish cultivars of cornelian cherry, which are part of the collection at the Arboretum in Bolestraszyce and to estimate genetic variation in European and US cultivars. Microsatellites can be used for cultivar differentiation in cornelian cherry because all individuals of a cultivar originate from the same progenitor by vegetative propagation (clonal).

The objectives of this study were the following: (1) to develop microsatellite loci for *C. mas*; (2) to characterize the genetic diversity and relationships and establish a molecular identification key of samples of cornelian cherry from China, central Europe (Austria, Poland, Romania, and Ukraine), and the United States; (3) to clarify the origin of Polish cultivars of cornelian cherry, which are part of the collection at the Arboretum in Bolestraszyce.

2. Materials and methods

2.1. Plant materials and DNA extraction

A single tree of *C. mas* 'Golden Glory' ($2n = 2x = 22$) was used to develop a small insert genomic library enriched for microsatellites. A total of 38 *Cornus* samples (Table 1), composed of 37 samples of *C. mas* and a sample of *Cornus eydeana* ($2n = 2x = 22$) were used to characterize microsatellite loci. Genomic DNA was extracted from leaves or unopened flower buds using the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). DNA was quantified with the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and quality was assessed by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized using the 2000 Gel Documentation System (Bio-Rad Laboratories, Hercules, CA, USA).

2.2. Microsatellite development

For isolation of microsatellites, protocols previously described by Wang et al. (2007) and Wadl et al. (2011) were followed. Genomic DNA (2.5 μg) was digested with *Alu*I, *Hae*III, and *Rsa*I (New England BioLabs, Beverly, MA, USA) and ligated to SNX linker adaptors (Hamilton et al., 1999). The SNX-ligated fragments were hybridized to $(GT)_{12}$ biotinylated oligonucleotides to enrich for sequences containing microsatellites and these fragments were ligated to the pBluescript SK II (+) vector (Fermentas, Glen Burnie, MD, USA) and transformed into TOP-10 *Escherichia coli* cells (Invitrogen, Carlsbad, CA, USA). To identify clones that were positive for microsatellite containing sequences, PCR was performed using the following 10 μL reaction: 1× GeneAmp PCR Buffer (Applied Biosystems, Carlsbad, CA, USA), 2.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.25 μM T3 primer, 0.25 μM T7 primer, 0.25 μM $(GT)_{12}$ primer,

Table 1
Samples of *Cornus eydeana* and *C. mas* used in microsatellite analysis.

Sample	Collection source	Sample	Collection source
C. eydeana	China	*C. mas* 'Macrocarpa'	Arboretum Bolestraszyce, Poland, Ukraine
C. mas 'Alosza'	Arboretum Bolestraszyce, Poland Ukraine	*C. mas* 'Nikolka'	Arboretum Bolestraszyce, Poland, Ukraine
C. mas 'Bilda'	Arboretum Bolestraszyce, Poland Ukraine	*C. mas* 'P5′	Arboretum Bolestraszyce, Poland
C. mas 'Bolestraszycki'	Arboretum Bolestraszyce, Poland Collected in Florianka, Poland	*C. mas* 'Paczoski'	Arboretum Bolestraszyce, Poland Poland
C. mas 'Dublany'	Arboretum Bolestraszyce, Poland Collected in Florianka, Poland	*C. mas* 'Podolski'	Arboretum Bolestraszyce, Poland Collected in Bolestraszyce, Poland
C. mas 'Elegantnyj'	Arboretum Bolestraszyce, Poland Ukraine	*C. mas* 'Raciborski'	Arboretum Bolestraszyce, Poland Poland
C. mas 'Elena'	Arboretum Bolestraszyce, Poland Ukraine	*C. mas* 'Radist'	Arboretum Bolestraszyce, Poland Ukraine
C. mas 'Flava'	Arboretum Bolestraszyce, Poland Bulgaria	*C. mas* 'Schönbrunner Gourmet-Dirndl'	Arboretum Bolestraszyce, Poland Austria
C. mas 'Florianka'	Arboretum Bolestraszyce, Poland Collected in Florianka, Poland	*C. mas* 'Semen'	Arboretum Bolestraszyce, Poland Ukraine
C. mas 'Golden Glory'	United States	*C. mas* 'Słowianin'	Arboretum Bolestraszyce, Poland Collected in Ukraine
C. mas 'Grovmas'	United States	*C. mas* 'Starokijevskij'	Arboretum Bolestraszyce, Poland Ukraine
C. mas 'Jantarnyj'	Arboretum Bolestraszyce, Poland Crimea, Ukraine	*C. mas* 'Szafer'	Arboretum Bolestraszyce, Poland Collected in Florianka, Poland
C. mas 'Jewgienia'	Arboretum Bolestraszyce, Poland Ukraine	*C. mas* 'Tricolor'	Arboretum Bolestraszyce, Poland Poland
C. mas 'Jolico'	Arboretum Bolestraszyce, Poland Austria	*C. mas* 'Variegata'	Arboretum Bolestraszyce, Poland
C. mas 'Juliusz'	Arboretum Bolestraszyce, Poland Collected in Pralkowce, Poland	*C. mas* 005351	Arboretum Bolestraszyce, Poland Ukraine
C. mas 'Koralovyj'	Arboretum Bolestraszyce, Poland Ukraine	*C. mas* 5-2	Romania
C. mas 'Kostia'	Arboretum Bolestraszyce, Poland Ukraine	*C. mas* 5-3	Romania
C. mas 'Kotula'	Arboretum Bolestraszyce, Poland Poland	*C. mas* 16	Romania
C. mas 'Lukjanovskij'	Arboretum Bolestraszyce, Poland Ukraine	*C. mas* 25	Romania

0.3 U AmpliTaq Gold® DNA Polymerase (Applied Biosystems) and sterile water. DNA was supplied as 1 μL of clonal bacterial cells. The reaction mixtures were PCR amplified using the following conditions: 1 cycle at 95 °C for 3 min; 35 cycles at 95 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, and 1 cycle at 72 °C for 1 min. Amplified DNA from clones ($n = 192$) that exhibited a smear when separated on 2% agarose gels were considered as positive for a microsatellite containing insert (Wang et al., 2007). Plasmid DNA was sequenced with Big-Dye 3.1 terminators (Applied Biosystems) on an ABI 3730XL capillary electrophoresis DNA analyzer (Applied Biosystems) with a 50 cm array employing universal T3 and T7 primers that flank the cloned insert. The vector and adapter sequences were removed from the sequenced DNA fragments using the program Sequencer 4.8. Microsatellites motifs were identified and primers designed using BatchPrimer3 v1.0 (You et al., 2008) using a minimum of six repeats for di-nucleotide, four repeats for tri-nucleotide and three repeats for tetra- to hexa-nucleotide motifs. Primer pairs, 18–23 base pairs (bp) long, were designed with 50% GC content and a minimum annealing temperature of 55 °C.

2.3. Microsatellite amplification

The PCR conditions described by Wadl et al. (2011) were used to screen and characterize microsatellite loci for *C. mas* with the exception that 8 ng of genomic DNA was used instead of 4 ng in the PCR reactions. Each 10 μL PCR reaction consisted of the following: 8 ng genomic DNA, 2.5 mM $MgCl_2$, 1× GeneAmp PCR Buffer II (Applied Biosystems), 0.2 mM dNTPs, 0.25 μM primer (forward and reverse), 5% dimethyl sulfoxide [(DMSO) Fisher Scientific, Pittsburgh, PA, USA], 0.4 U AmpliTaq Gold® DNA polymerase (Applied Biosystems) and sterile water. PCR amplifications for all loci used the following thermalcycling conditions: 95 °C for 5 min; 35 cycles of 94 °C for 40 s, 52 °C for 40 s and 72 °C for 30 s, and a final extension ending at 72 °C for 4 min. The QIAxcel Capillary Electrophoresis System (Qiagen) using an internal 25–300-bp size standard was used to separate the PCR products.

2.4. Data analysis

Raw allele length data for each sample were binned into allelic classes using the program FLEXIBIN (Amos et al., 2007). We used a conservative ±2–3 bp allelic class size range because of the 2–5 bp resolution of the QIAxcel Capillary Electrophoresis System. One of the limitations of the QIAxcel system compared to other systems such as the ABI is the potential of not detecting heterozygotes of a 1 bp difference. When two flowering dogwood (*C. florida*) individuals were compared at 19 loci using the ABI or the QIAxcel system there was only a single individual with a 1 bp resolution difference on the ABI, thus confirming the utility of the QIAxcel system (Hadziabdic et al., 2012). The multilocus genotypic data for each individual was used to calculate the number of alleles per locus (A), number of effective alleles (N_e), expected (H_E) and observed heterozygosity (H_O) using GenAlEx 6.5 (Peakall and Smouse, 2006, 2012) for 9 *C. mas* and 2 *C. florida* microsatellite loci (Wang et al., 2007, 2008; Wadl et al., 2010). Data from the microsatellite loci were analyzed with POPULATIONS version 1.2.32 (Langella, 2002) to estimate the shared allele distance (Jin and Chakraborty, 1994) and to create a pairwise matrix of genetic similarities. A

Table 2
Characteristics of two *Cornus florida* microsatellite loci and nine *C. mas* microsatellite loci.

GenBank accession no.	Locus	Primer sequence (5′-3′)	Repeat motif	Expected size (bp)	No. of alleles (*A*)	No. of effective alleles (N_e)	Observed heterozygosity (H_o)	Expected heterozygosity (H_e)
ED651732	CF48	L: gctttgacatcctctttgcttctc R: aagaggcttcacaagacaatcagc	$(TG)_9$	144	1	1.00	0.00	0.00
ED651738	CF55	L: tggagtagggcaaaagatcaagag R: tccagggaatgttcggtagattag	$(GT)_7T(TG)_{10}$	155	2	1.70	0.58	0.41
KF550288	CM007	L: gtttaggtgtgagtgcagatgg R: caatgctaacaagcaccattcc	$(GT)_{24}$	189	10	4.00	0.69	0.75
KF550289	CM008	L: tcgttaatgtgaaattggaacg R: caccgtacacgcaaagtcc	$(GT)_{11}$	156	6	2.78	0.01	0.64
KF550290	CM010	L: gctagcagaagcacagttagcc R: tccaacatgtaaaacctagatgc	$(CA)_{12}$	230	9	4.20	0.20	0.76
KF550291	CM020	L: tggcagactaggttcttgttagc R: ctccactgtctggcttacttgg	$(TG)_{10}$	194	2	1.06	0.00	0.05
KF550292	CM026	L: gaattcatgtaatgttgttgtctgc R: cctgcatataattcaggtaaagagc	$(CA)_{14}$	192	7	2.92	0.14	0.66
KF550293	CM031	L: taccctctcttgctctttgtcc R: aaacaatcaaacccaaacaacc	$(AG)_{26}(TG)_{13}$	208	11	5.36	0.31	0.81
KF550294	CM037	L: aacacagagaaacacgtgcaa R: tggagatctttgaagaacagga	$(TG)_{20}$	184	10	5.53	0.66	0.82
KF550295	CM039	L: gggtattgtaatcaatgtaaaaccaa R: tcacaccaccagcaatcact	$(GT)_{18}$	242	8	3.53	0.71	0.72
KF550296	CM043	L: gtccacacctgttgttcagc R: ggttgcaatgctttcttggt	$(TG)_{16}(TA)_5$	220	8	4.16	0.54	0.76
Mean					6.73	3.29	0.36	0.58

dendrogram was generated from the similarity matrix by the unweighted pair group method using arithmetic averages (UPGMA) with the SAHN function with numerical taxonomy and the multivariate analysis system (NTSYSpc Version 2.20q) (Rohlf, 2005). To measure the goodness of fit between the similarity and cophenetic matrices, the cophenetic correlation coefficient (r) was calculated. Five polymorphic microsatellite loci (CM007, CM010, CM031, CM037, and CM043) were used to develop a molecular key to unambiguously identify *C. mas* and *C. eydeana* samples. In this molecular key, locus CM037 was used first to separate the samples into groups and the other loci were used to further delineate the groups until each sample was uniquely identified.

3. Results

3.1. Microsatellite development

The $(GT)_{12}$ enriched small insert genomic library was constructed from *C. mas* 'Golden Glory'. A total of 480 colonies of *E. coli* were screened via PCR to select putative inserts containing a microsatellite locus in which the three primer reaction revealed that 232 (48.3%) colonies putatively harbored sequences with the $(GT)_n$ motif. We sequenced 192 of these colonies and 152 (79.2%) were of suitable quality to analyze further for microsatellites. After searching the sequences for di- to hexa-nucleotide repeats, 149 of the sequences contained inserts with various microsatellite motifs. The majority (87.3%) of the microsatellite motifs were di-nucleotide repeats, with 4, 6, 2, and 0.7% of the motifs being tri-, tetra-, penta-, or hexa-nucleotide repeats respectively. Among the dinucleotide repeats, the GT/TG (61.5%) motifs were most common, followed by 23% AC/CA repeats. Repeat lengths ranged from 6 to 33 for dinucleotide, 4–6 for trinucleotide, 3 for tetranucleotide, 3 for pentanucleotide, and 3 for hexanucleotide motifs. Primer pairs for the 149 were designed for all of the sequences containing microsatellites.

3.2. Characterization of microsatellite loci

From the 149 microsatellite loci, 50 were randomly selected and tested for amplification against a panel of four *C. mas* cultivars. Nine markers were suitable for further characterization, whereas 41 markers did not support amplification, produced a smear pattern, produced more than 2 amplicons/locus, or were monomorphic. The nine markers from *C. mas* and two microsatellites from *C. florida* were used to assess genetic diversity in 37 *C. mas* samples and 1 *C. eydeana* sample (Table 2). The nine loci from *C. mas* and one locus (CF55) from *C. florida* were polymorphic and 73 alleles were detected in all samples. Both loci from *C. florida* and five loci (CM007, CM026, CM031, CM037, and CM043) from *C. mas* amplified DNA from the *C. eydeana* accession and all loci were homozygous. Number of alleles per locus ranged from 1 to 11 and averaged 6.73, whereas the effective number of alleles per locus ranged from 1.00 to 5.53 with an average of 3.29. Observed heterozygosity of the 38 accessions was 0.00–0.71, whereas expected heterozygosity was 0.00–0.82. Tests of Hardy-Weinberg equilibrium and linkage disequilibrium were not conducted due to clonal reproduction that occurs with selection of plants for specific characteristics in cultivar development, which would result in patterns of nonrandom mating.

3.3. Genetic relationships and molecular identification key

The multilocus allelic data was used to create the allele sharing distance matrix for the *C. mas* and *C. eydeana* samples and then a UPGMA tree was generated to cluster the genotypes (Fig. 1). The similarity coefficient ranged from 0.05 to 0.73 among all genotypes. All *C. mas* samples clustered into two main groups, with the single *C. eydeana* sample clustered separately. Group I included all samples that belong to the Macrocarpa form (botanic variety). All of these samples are derived from a single population, but were selected and registered in countries in Central Europe (Austria, Poland, and Ukraine). Group II included samples that constitute another botanical form ('Flava', 'Tricolor', and 'Variegata'). All of the samples in group II were selections from Poland or the United States. Also included in this group were wild samples collected from Romania and grown at the University of Tennessee Forest Resources AgResearch and Education Center (Oak Ridge, TN, USA). The cophenetic correlation coefficient value, $r = 0.83$ suggested a good fit between the dendrogram cluster and the similarity matrix from which it

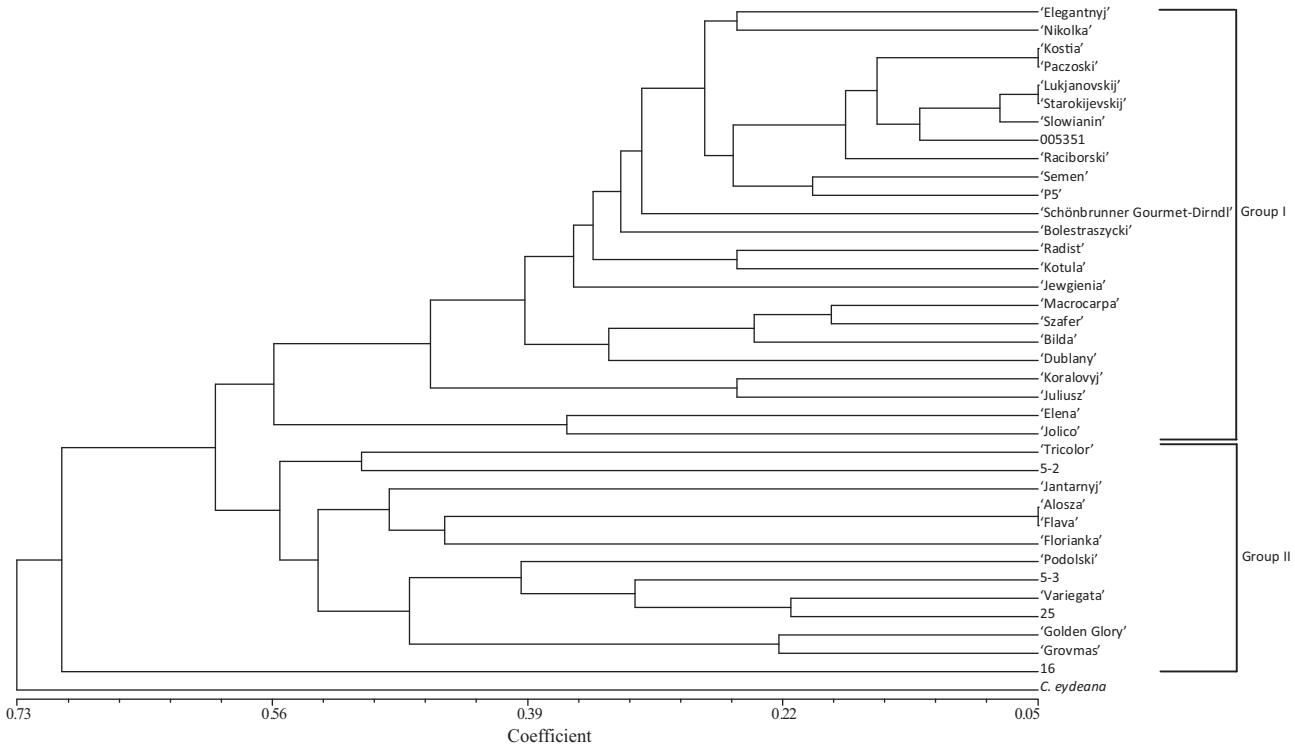

Fig. 1. Dendrogram of 37 *C. mas* samples and 1 sample of *C. eydeana* generated by unweighted pair group method with arithmetic mean (UPGMA) cluster analysis based on the shared allele distance (Jin and Chakraborty, 1994) matrix. Smaller coefficient values indicate greater genetic similarity and larger values indicate greater genetic diversity between taxa.

was derived (Sneath and Sokal, 1973). Five loci (CM007, CM010, CM031, CM037, and CM043) were used to develop a molecular key that successfully delineated all individuals (Fig. 2).

4. Discussion

Cornus has been divided into four major subgroups (big-bracted, dwarf, and blue-fruited dogwoods and cornelian cherries) and the phylogenetic relationships of these four groups are well resolved by molecular and morphological data (Fan and Xiang, 2001, 2003; Xiang et al., 2006, 2008). The genus is ecologically and economically important worldwide. Microsatellites are important markers for plant breeders and conservationists investigating gene flow. Microsatellites are tandemly repeated mono-, di-, tri, tetra-, or penta-nucleotide units that are co-dominantly inherited and occur in abundance within the genomes of most eukaryotes (Powell et al., 1996). Small insert genomic libraries enriched for microsatellites have been developed for *C. florida* [flowering dogwood (Cabe and Liles, 2002; Wang et al., 2007, 2008)], *C. kousa* [kousa dogwood (Wadl et al., 2008b)], and *C. sanguinea* [blood twig dogwood (Wadl et al., 2013)]. In our study, the microsatellite enrichment rate for *C. mas* (79.2%) is similar to *C. kousa* [72.2% (Wadl et al., 2008b)] and *C. sanguinea* [77.1% (Wadl et al., 2013)] and is lower than *C. florida* [88.2% (Cabe and Liles, 2002), 95.8–99.7% (Wang et al., 2007), and 96.5–99.4% (Wang et al., 2008)].

This is the first study to develop and use microsatellites for genetic fingerprinting and determining relationships among the cornelian cherries. Accurate comparisons of genetic diversity between studies are best accomplished when estimates of expected heterozygosity (H_e) are used (Allendorf et al., 2013). The H_e values ranged from 0.00 to 0.82 and the mean H_e was 0.58 in our analysis. An ISSR analysis of *C. mas* accessions from Iran reported H_e values that ranged from 0.43 to 0.49 and a mean H_e of 0.42 (Hassanpour et al., 2013). The higher H_e value using microsatellites indicates that these loci are potentially more useful in studies of genetic structure in *C. mas*. These markers can be used to determine parentage within germplasm collections and to distinguish genotypes where morphological characters make identity difficult.

We studied a genetic pool of *C. mas* from Austria, Poland, Romania, Ukraine, and United States that originated from wild and cultivated sources. There were registered cultivars as well as hybrids developed from *C. mas* breeding and selection program at Arboretum Bolestraszyce. Improvement and selection of cornelian cherry is recent therefore many indigenous varieties, unfortunately have been lost in central Europe. Efforts have been initiated in Europe and America on cornelian cherry cultivation and on developing new varieties (Dudukal and Rudnko, 1984; Pirc, 1990; Reich, 1996).

Extensive investigations of the existing gene pool as well as the breeding of new cultivars of *C. mas* are ongoing at Arboretum Bolestraszyce. From the collection, ten cultivars have been selected: Bolestraszycki, Dublany, Florianka, Juliusz, Kresowiak, Paczoski, Podolski, Raciborski, Slowianin, and Szafer. Among these cultivars there is large variation among fruits, including physicochemical properties and also in terms of maturation and harvesting. The fruit colors are dark red, cherry red, pink or yellow, while the fruit can be oval, pear, or bottle shaped. Yellow fruited cultivars are either rare or no longer occur in the wild (Klimenko, 2004). We found that cultivars with yellow fruits (Alosza, Florianka, and Jantarnyj) clustered together in our study and are valuable components of the germplasm collection at Arboretum Bolestraszyce. The last yellow fruited variety discovered was found growing in a Crimean forest and is thought to be the progenitor of the existing varieties (Simirenko, 1973).

Identification of the most promising candidates for improvement of desired trait(s) is the first step in a successful breeding program. The next step is development of hybrids with specific

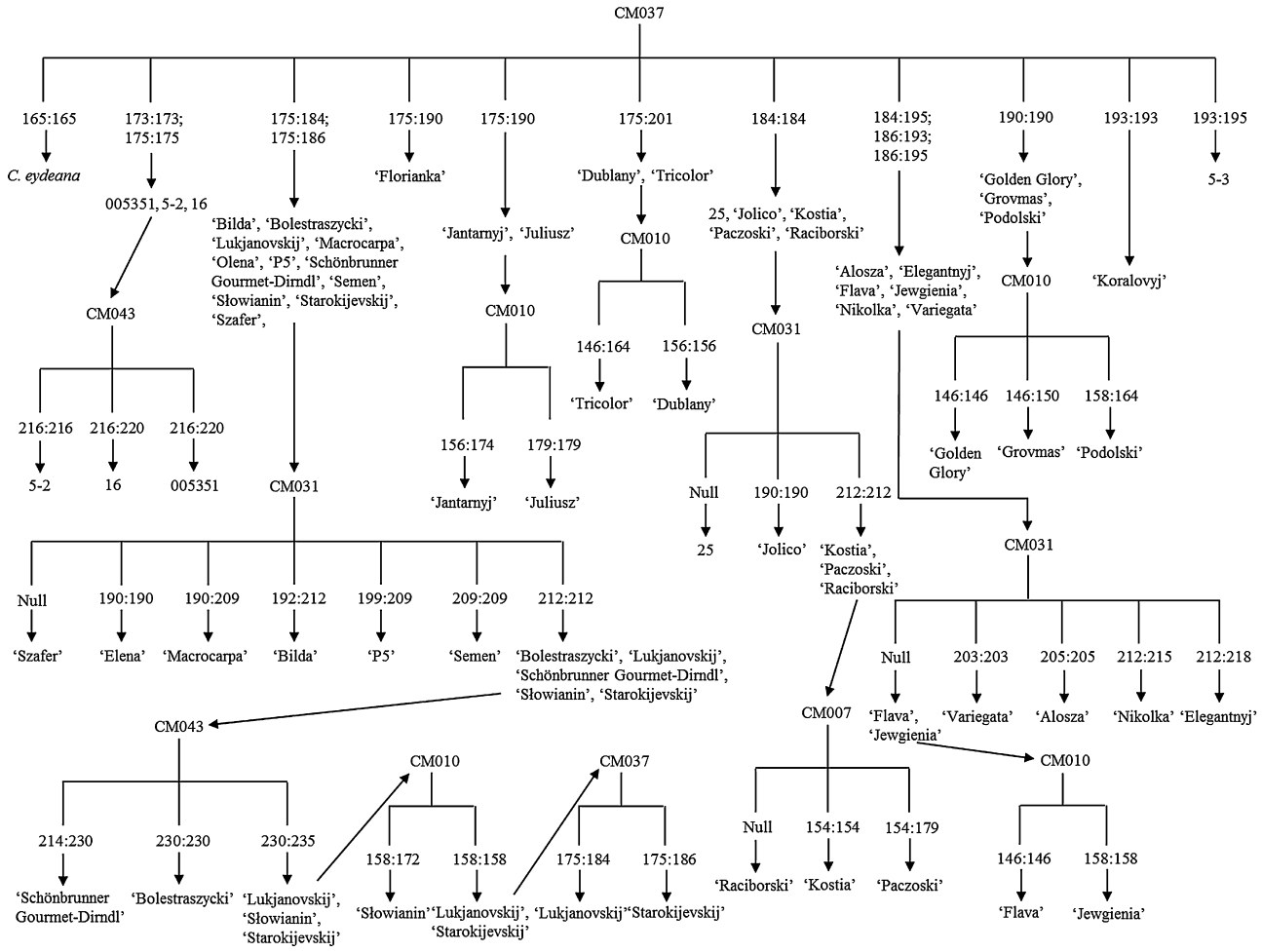

Fig. 2. Molecular identification key for 37 *Cornus mas* samples and 1 sample of *C. eydeana* based on microsatellite loci CM007, CM010, CM031, CM037, and CM043.

properties and features. Preservation of the gene pool is essential to maintain a stable level of traits within improved lines. The microsatellites described in this study will be useful in determining the level of genetic diversity in subsequent generations and particularly valuable for the identification of varieties that are "in fact derived", that is, those that are consistent with the initial varieties in terms of essential characteristics. Even very phenotypically similar varieties are expected to have a numerous differences contained in their genetic information. The development of specific markers for *C. mas* provides a tool to study DNA polymorphism and has application in the plant breeding program at Arboretum Bolestraszyce.

5. Conclusions

In this study, nine polymorphic microsatellite loci were developed and used to determine the genetic relationships between accessions of cornelian cherries (*C. eydeana* and *C. mas*) and five loci were used to develop a molecular identification key to delimit each sample. We demonstrated cross species transfer of *C. florida* loci, which is in a different subgroup (clade) than *C. eydeana* and *C. mas* (Xiang et al., 2008) using the same amplification conditions. Our results indicated the potential for these markers in studying genetic diversity, gene flow, and genetic structure among populations of *C. mas* and the potential in populations of *C. eydeana*. The results confirmed our suspicions about the distinct varieties of 'Podolski' and 'Florianka' as Polish origin.

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