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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,