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PRIMER NOTE

ISOLATION AND CHARACTERIZATION OF MICROSATELLITE LOCI FOR *CORNUS SANGUINEA* (CORNACEAE)[1]

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- *Premise of the study:* To facilitate genetic and conservation research of *Cornus sanguinea*, microsatellite loci were isolated and 29 individuals from 11 German populations were genotyped.
- *Methods and Results:* Sixteen microsatellite loci were characterized from an enriched small insert genomic library. The number of alleles detected ranged from five to 11 per locus, observed heterozygosity ranged from 0.00 to 1.00, expected heterozygosity ranged from 0.65 to 0.90, and polymorphic information content ranged from 0.59 to 0.88.
- *Conclusions:* The markers described in the study will allow further investigation of population dynamics and the degree of clonal reproduction within populations of *C. sanguinea*.

Key words: bloodtwig dogwood; Cornaceae; *Cornus sanguinea*; population genetics.

Cornus sanguinea L. (Cornaceae) is a 4–5 m tall shrub (Liesebach and Götz, 2008) and is distributed over almost the entire European continent and in Asia in the Caucasus Mountains, northern Iran, Turkey, Syria, and Lebanon (see distribution map in Liesebach and Götz, 2008). *Cornus sanguinea* grows from lowlands to 1500 m in the Alps (Schütt et al., 1994). It can form hedges and grows along forest edges, riversides, and in floodplain forests and is often planted as an ornamental in cities and along roads. Genetic analysis of the species, based on isozymes (Leinemann et al., 2002) and chloroplast markers (Liesebach and Götz, 2008), was conducted to assess genetic variation in natural populations and to address conservation issues. Isozyme analyses were conducted on a small scale and revealed that most of the specimens within one natural shrub community were the result of vegetative reproduction (Leinemann et al., 2002). Previous studies have analyzed chloroplast markers for a panel of range-wide samples detecting lower genetic variation in *C. sanguinea* compared to other European tree species (e.g., *Fraxinus excelsior* L. [Heuertz et al., 2004]; *Corylus avellana* L. [Palmé and Vendramin, 2002]), with the latter study focusing mainly on large-scale differentiation in Europe. Small-scale genetic differentiation should be determined by analyzing nuclear microsatellites.

Genetic markers have become an important prerequisite for selection and management of genetic resources (Geburek and Turok, 2005). To select high-quality seeds from populations of *C. sanguinea*, knowledge of the genetic composition of natural stands is required. For the establishment of seed orchards, population genetic research is essential to increase the productivity of the selected clones. Understanding the genetic composition of the selected clones is necessary, especially for clonally reproducing shrub and tree species such as *C. sanguinea*. Polymorphic nuclear microsatellites can establish and determine the degree of clonal reproduction in natural populations.

METHODS AND RESULTS

Samples of *C. sanguinea* were collected from 11 distinct locations in Germany, and representative voucher specimens were deposited at the Botanische Staatssammlung München (Appendix 1), Munich, Germany. For isolation of microsatellites, we followed protocols previously described by Wang et al. (2007) and Wadl et al. (2011). Briefly, genomic DNA (2.5 µg) was digested with the restriction enzymes *Alu*I, *Hae*III, and *Rsa*I (New England BioLabs, Beverly, Massachusetts, USA) and ligated to SNX linker adapters (Hamilton et al., 1999). To enrich for sequences containing microsatellites, the SNX-ligated fragments were hybridized to $(GT)_{12}$ biotinylated oligonucleotides. Microsatellite-enriched fragments were ligated to the pBluescript II SK (+) vector (Fermentas, Glen Burnie, Maryland, USA) and transformed into *Escherichia coli* TOP10 cells (Invitrogen, Carlsbad, California, USA). PCR screening to select positive clones was performed using the following reaction: 1× GeneAmp PCR Buffer (Applied Biosystems, Carlsbad, California, 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, 0.3 U AmpliTaq Gold DNA Polymerase (Applied Biosystems), and sterile water. The reaction mixtures were PCR amplified using the following conditions: one 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 one cycle at 72°C for 1 min. Clones that exhibited a smear when separated on 2% agarose gels were considered as positive for a microsatellite, and positive colonies (n = 192) were sequenced using universal T3 and T7 primers (Wang et al., 2007). Of the 192 clones sequenced, 148 (77.1%) contained microsatellites when searched using the default settings of the program Imperfect SSR Finder (Stieneke and Eujayl, 2007). Sequences were randomly

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TABLE 1. Characteristics of 16 microsatellite loci isolated from *Cornus sanguinea*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Allelic class size range (bp)	T_a (°C)	GenBank accession no.
CS4	F: AAAGGAATGTGCTTGGTTGAAT	$(AC)_{10}$	200–221	52	KC175472
	R: ACCTAGCAATGATTGGGACTTG				
CS5	F: AGCAATATGCAGCAGACTCAAA	$(TA)_5(TG)_8$	160–179	52	KC175473
	R: GGTCCACATTGATGAACAGAGA				
CS6	F: TCTGCTTGCTTGATTGAAATGT	$(AC)_6AT(AC)_{12}$	217–230	52	KC175474
	R: GGTGAGTGTTGACGTCATGTTT				
CS9	F: CAATGCTCTCTATCCTCGGTGT	$(TG)_{14}$	234–249	52	KC175475
	R: GGATTCGTTAAACGGCAAATAA				
CS15	F: AATCCAATTTGAACCCAGTACC	$(CA)_{14}$	149–163	52	KC175476
	R: TGATCATCAGTAAGGGAAAGTGAA				
CS16	F: GATGTTGACATATGCCAGTGGT	$(TG)_{13}$	156–180	52	KC175477
	R: GCTAAATAAAGGTTTAAACAGATTGG				
CS17	F: TTCGGTGAACTCGACTTCCTAT	$(TG)_{11}TT(TG)_6$	211–257	52	KC175478
	R: AAAACGAGCATGGAAAGGTTTA				
CS19	F: CAAACATATCTACGTCAATGCAAA	$(TG)_{18}$	155–182	52	KC175479
	R: GCTGTAGGGATTCACTGCTCTT				
CS21	F: GCAAGAAGTGCCTGTGTGTTTA	$(TC)_5TGT(ATAC)_6$	182–217	52	KC175480
	R: CATTTGGTGCTAAAGGTGATGA				
CS22	F: CAGAAGCCTTGAGAACATGGTTA	$(TG)_{12}$	156–170	52	KC175481
	R: TGTGGAATTGAAGAGAGCACAT				
CS24	F: TGATTTCTCATTTCCCCTCTCT	$(GT)_3G(GT)_5$	181–211	52	KC175482
	R: CTCGAATGAAGGCGCAGT				
CS25	F: TGATTTCTCATTTCCCCTCTCT	$(TG)_{18}$	196–235	52	KC175483
	R: CCAACAAGTGCAACTAAATCACA				
CS26	F: GGTTTGGAGAAGGTTAGCATGT	$(TG)_{12}$	138–176	52	KC175484
	R: TTGTACGCATAGCACATCTTCA				
CS27	F: GTCAACTTTCAAGTCGTCACCA	$(AC)_9$	160–187	52	KC175485
	R: CACACCAACTTTTGAAAACCAA				
CS29	F: GGTCCATTCATAATTGCGATCT	$(TG)_{13}$	159–180	52	KC175486
	R: CGTGACATTTAGTCCTGCAATC				
CS30	F: ATTTGGAGTAGCCCAAACTCAA	$(CA)_{13}$	151–177	52	KC175487
	R: TGATGGGTAAACATGGTGGTAA				

Note: T_a = annealing temperature.
[a]All values are based on 29 individuals from 11 populations in Germany (see Appendix 1).

selected and primers were designed for 30 microsatellite loci using Primer3 (Rozen and Skaletsky, 2000).

To screen and characterize the microsatellite loci for *C. sanguinea*, the PCR conditions described by Wadl et al. (2011) were used with the exception that 8 ng of DNA was used instead of 4 ng of DNA in the PCR reaction mixtures. 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, Pennsylvania, USA), 0.4 U AmpliTaq Gold DNA polymerase (Applied Biosystems), and sterile water. PCR amplifications for all loci used the following thermocycling 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, Hilden, Germany) using an internal 25–300-bp size standard was used to separate the PCR products. After separation, the raw allele length data 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 (e.g., ABI 3130xl Genetic Analyzer; Applied Biosystems) is the potential of not detecting heterozygotes with a 1-bp difference. When two flowering dogwood (*Cornus florida* L.) individuals were compared at 19 loci using both the ABI and the QIAxcel systems, only a single individual with a 1-bp resolution difference was detected on the ABI system, thus confirming the accuracy of the QIAxcel system (Hadziabdic et al., 2012). The multiallelic genotypic data for each individual was used to calculate the number of alleles per locus (A), expected (H_e) and observed heterozygosities (H_o), and polymorphic information content (PIC) using CERVUS version 3.0 (Kalinowski et al., 2007).

Of the 30 loci screened, 16 (53.3%) amplified DNA from the individual that was used in the microsatellite library constructions. Twenty-nine *C. sanguinea* individuals collected from 11 populations in Germany (Appendix 1) were then genotyped using the 16 polymorphic microsatellite loci (Table 1). The number of alleles detected ranged from five to 11 per locus, H_o ranged from 0.00 to 1.00, H_e ranged from 0.65 to 0.90, and PIC ranged from 0.59 to 0.88 (Table 2). The sampled populations of *C. sanguinea* exhibited considerable adventitious shoots originating from the roots, which indicates clonal reproduction; as this violates one of the tenets of the Hardy–Weinberg principle, we did not perform tests for Hardy–Weinberg equilibrium or linkage disequilibrium.

TABLE 2. Microsatellite characterization of 16 loci in 29 individuals of *Cornus sanguinea*.

Locus	A	H_o	H_e	PIC
CS4	6	1.00	0.78	0.72
CS5	7	0.39	0.68	0.63
CS6	5	0.08	0.78	0.70
CS9	5	0.00	0.75	0.69
CS15	7	0.79	0.80	0.76
CS16	8	1.00	0.76	0.71
CS17	9	0.32	0.79	0.75
CS19	8	0.76	0.73	0.67
CS21	11	0.86	0.86	0.83
CS22	6	0.45	0.65	0.59
CS24	12	0.54	0.78	0.74
CS25	16	0.59	0.90	0.88
CS26	9	1.00	0.79	0.75
CS27	11	0.76	0.86	0.83
CS29	8	0.83	0.76	0.71
CS30	10	1.00	0.75	0.71

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; PIC= polymorphic information content.

CONCLUSIONS

Understanding the scales over which dispersal, genetic drift, and selection operate requires knowledge of population structure. There is a lack of knowledge of the genetic structure of *C. sanguinea*, in particular of clonal reproduction, which may exacerbate the effects of low gene flow by seed between populations. We expect that the microsatellites described in this study will be highly useful for population genetic studies and for assessing the degree of clonal reproduction in *C. sanguinea* in both natural populations and seed orchards.

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Appendix 1. *Cornus sanguinea* voucher specimens used in this study. All specimens are deposited at the Botanische Staatssammlung München (M), Munich, Germany.

Collection no. (no. of individuals genotyped)	Locality (GPS coordinates)
BERT 1 (3)	Bertholdsheim, Germany (48°43.984′N, 11°01.550′E)
BUCH 1 (3)	Buch, Germany (48°38.317′N, 11°03.784′E)
DET 1 (3)	Dettenheim, Germany (49°09.250′N, 8°25.417′E)
DOR 1 (3)	Dorsbrunn, Germany (49°05.750′N, 10°54.167′E)
DOR 2 (3)	Dorsbrunn, Germany (59°05.683′N, 10°55.000′E)
GEI 1 (3)	Geichet, Germany (48°20.267′N, 11°34.000′E)
09 1 (1)	Holstein, Germany (53°55.984′N, 10°03.416′E)
07 1 (1)	Holstein, Germany (53°52.834′N, 9°46.117′E)
MAU 1 (3)	Mauern, Germany (48°26.533′N, 11°05.100′E)
SCH 1 (3)	Schnödhof, Germany (48°42.334′N, 11°04.350′E)
TRU 1 (3)	Trugenhofen, Germany (48°46.084′N, 11°00.050′E)