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PRIMER NOTE

CHARACTERIZATION AND TRANSFERABILITY OF MICROSATELLITE MARKERS DEVELOPED FOR *CARPINUS BETULUS* (BETULACEAE)[1]

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- *Premise of the study: Carpinus betulus* (Betulaceae) is an octoploid, ecologically important, common tree species in European woodlands. We established 11 nuclear microsatellite loci allowing for detailed analyses of genetic diversity and structure.
- *Methods and Results:* A microsatellite-enriched library was used to develop primers for 11 microsatellite loci that revealed high allele numbers and genetic diversity in a preliminary study.
- *Conclusions:* All of the loci developed here are informative for *C. betulus*. In addition, the loci are transferable to several species within the genus, and almost all loci cross-amplified in species of different genera of the Betulaceae.

Key words: Betulaceae; *Carpinus betulus*; cross-amplification; microsatellite loci; polyploidy.

The European hornbeam, *Carpinus betulus* L. (Betulaceae), is a common, late-successional, shade-tolerant tree often forming bushes and hedges. These edge communities between forest and pasture are highly valued for conservation due to their biodiversity. In addition, they provide refugia for plants and animals and connect biotopes. *Carpinus betulus* is also often used as an ornamental planting in gardens and nonforested landscapes.

Genetic analyses in *C. betulus* are scarce; they were based on universal chloroplast markers (Grivet and Petit, 2003) or anonymous amplified fragment length polymorphisms (AFLPs; Coart et al., 2005). Microsatellite markers were established for several species within the family (e.g., Barbará et al., 2007; Gürcan and Mehlenbacher, 2010), but not for *C. betulus*.

The species is octoploid and thus complex fragment patterns are expected using codominant microsatellite markers. Recent advances in statistical methods and new software allow for analysis of genetic diversity even in polyploid species (e.g., Wiehle et al., 2014).

METHODS AND RESULTS

Genomic DNA was extracted from young leaves of an adult tree of *C. betulus* growing in Göttingen, Germany (Appendix 1), using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). A microsatellite-enriched library was generated based on the protocol of Fischer and Bachmann (1998) with some modifications (Prinz et al., 2009). We used biotinylated oligonucleotides with the motif of $(GA)_{10}$ for hybridization at 60°C. All steps of the enrichment procedure were repeated once. Final PCR products were purified and ligated into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, California, USA). The vectors were transformed chemically to One Shot TOP10 Competent cells (Invitrogen). Ninety-six positive clones were sequenced forward and reverse in an ABI Prism 3100 automatic sequencer (Applied Biosystems, Foster City, California, USA), and 44 sequences were suitable for primer design. The remaining fragments showed low quality, short sizes of the flanking regions, or were identified as duplicates. A total of 35 primers were designed applying Primer3 version 2.2.3 (Rozen and Skaletsky, 1999) and tested for amplification. PCR assays were conducted in a final volume of 15 µL containing approximately 10 ng of genomic DNA, 1× Hot Start Buffer (0.8 M Tris-HCl [pH 9.0], 0.2 M $(NH_4)_2SO_4$, 0.2% w/v Tween-20; Solis BioDyne, Tartu, Estonia), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 unit Hot Start DNA Polymerase (5 U/µL HOT FIREPol; Solis BioDyne), and 0.3 pmol of each primer. Forward primers of each pair were labeled with a fluorescent tag. PCR was performed applying a touchdown program adapted to the annealing temperatures (T_a) of each primer provided by Primer3 version 2.2.3 (Rozen and Skaletsky, 1999) and producers. The general protocol contained cycles of 1 min at 94°C, 1 min at T_a + 3–5°C to T_a − 3–5°C reducing the temperature at 1°C in each cycle, 1 min at 72°C, followed by 25 cycles at the final annealing temperature without further touchdown. PCR products were checked for quality and approximate lengths of the fragments. Nineteen primer pairs revealed unambiguously observable fragments in an expected size range. A test for variability was performed in 25 individuals of *C. betulus* sampled in Germany and Romania as well as in 13 individuals of several species of the Betulaceae (Appendix 1). After amplification, fragments were separated in an ABI Prism 3100 automatic sequencer (Applied Biosystems), and fragment sizes were scored using GeneScan 3.7 analysis software based on the internal standard GeneScan 500 ROX (Applied Biosystems).

Eleven out of 19 loci revealed unambiguously scorable patterns that were polymorphic among samples of *C. betulus* (Table 1). Eight loci revealed ambiguous and nonvaluable patterns (Appendix 2). The 11 informative loci were resequenced for some samples to verify the specific amplification products. In total, 252 alleles were detected ranging from 15 to 30 per locus (Table 2). Three to six alleles per locus were most frequently observed in each individual polyploid plant. Lower average numbers of alleles for individual plants were observed only at locus Cb_33, but they were not fixed. Thus, genetic diversity is high, ranging from 0.199 to 0.320, calculated from a converted binary data matrix in which present alleles are represented by "1" and absent alleles by "0" (e.g., Sampson and Byrne, 2012).

Cross-amplification was successful for almost all loci (Table 3). Thus, six loci amplified in all *Carpinus* species, and one additional locus was successfully applied in seven out of eight *Carpinus* samples. The reduced number of transferred loci is likely caused by species-specific taxonomic relationships to the species of origin. Successful cross-amplification among species of different Betulaceae genera was observed for three loci amplified in all individuals and two additional loci amplified in four out of five species. Most alleles were shared with *C. betulus*, whereas two loci showed more than 50% additional alleles (Table 3). Reduced genetic diversity of transferred loci can be explained

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TABLE 1. Characterization of microsatellite loci developed for *Carpinus betulus*.

Locus	Primer sequences (5′–3′)	Repeat motif[a]	Allele size range (bp)	T_a (°C)[b]	GenBank accession no.
Cb_12b	F: CATAATTAGCATCTCCCCACCT R: AATGCGGCGAAGACACAT	$(CT)_x$	97–137	TD 68–58	KP844902
Cb_15b	F: CCTCCATTCACGAACCAATC R: GCCTCTGCATGTTGTGTGAG	$(CT)_x$	63–115	TD 68–58	KP844903
Cb_17	F: GCAGGCGGATATGTTTGTG R: CGGCGAAGACACATTGAG	$(GA)_x$	56–100	61	KP844904
Cb_27	F: CTTCACGGCCTCACTGAAAC R: CATCGATCATCCCAGTCCTT	$(GA)_x$	77–150	64	KP844905
Cb_29	F: CTTCGACACAACCTCCCAAC R: ATTGCCAATGGACCTTTCTC	$(GA)_x$	55–95	61	KP844906
Cb_33	F: GACAGTCTAGAGGCTGTACAAGAA R: TGGAACAAAATTATGAGAAATTGA	$(GA)_xN_x(GA)_x$	128–172	TD 66–54	KP844907
Cb_35	F: TGCGTGTTGGTTTTGTCC R: TGCAATTAAGGTATGATTGATCG	$(GA)_x$	77–144	TD 68–60	KP844908
Cb_37a	F: GAAGGTTGTAGCCAGCCTAA R: ATCTTAAGAGAAAGCGAAACCCTA	$(GA)_x$	70–136	TD 68–58	KP844909
Cb_43	F: ACATTGAGTGATCCATACGAGA R: TCCATTTGCATATGTGTGCTC	$(GA)_x$	81–138	TD 66–54	KP844910
Cb_48a	F: CAAGAATAAGCTAGAAAGAGAGAAGC R: TGAAGGTAGACTTTGATGGAACA	$(GA)_x$	130–188	TD 66–57	KP844911
Cb_49a	F: AATCAGCGATTCTGCCAAAG R: CGTCGTCCTCAGCTGCAC	$(GA)_xN_x(GAG)_x$	143–182	TD 68–58	KP844912

Note: T_a = annealing temperature.
[a]N_x signifies a microsatellite motif interrupted by an ongoing DNA sequence of different lengths.
[b]A touchdown (TD) protocol was applied. Annealing starts at the highest temperature and decreases at 1°C in each PCR cycle.

by low sample size, the general observation of reduced amplification success and genetic diversity after cross-amplification (e.g., Selkoe and Toonen, 2006; Barbará et al., 2007), and finally by differing ploidy levels, which are not known for all species.

CONCLUSIONS

In this study, we developed microsatellite markers for *C. betulus* despite complex fragment patterns resulting from the octoploid nature of the species. We also tested their transferability to other species within *Carpinus* and other genera of the Betulaceae with ploidy levels that differ and are not known for all species. Highly polymorphic and codominant microsatellite markers allow for detailed analyses of genetic diversity and structure, i.e., gene flow within and among species.

TABLE 2. Species-specific genetic diversity of microsatellite loci among 25 *Carpinus betulus* individuals represented by number of alleles, number of rare alleles (<10%), and unbiased genetic diversity (GenAlEx version 6.4; Peakall and Smouse, 2006).

Locus	*A*	No. rare alleles	Genetic diversity[a]
Cb_12b	26	16	0.224
Cb_15b	20	8	0.271
Cb_17	15	5	0.283
Cb_27	30	11	0.278
Cb_29	20	11	0.226
Cb_33	20	13	0.199
Cb_35	29	17	0.200
Cb_37a	27	11	0.263
Cb_43	26	13	0.248
Cb_48a	18	9	0.248
Cb_49a	21	6	0.320

Note: *A* = number of alleles.
[a]The parameter replaces the expected heterozygosity in the polyploid species.

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TABLE 3. Number of alleles per microsatellite locus resulting from cross-amplification of loci developed for *Carpinus betulus* and observed in single plants of related species.

Species	Cb_12b	Cb_15b	Cb_17	Cb_27	Cb_29	Cb_33	Cb_35	Cb_37a	Cb_43	Cb_48a	Cb_49a
Carpinus caroliniana	4	2	—	1	—	2	1	2	2	2	2
C. caucasica	3	5	3	5	3	2	4	7	8	1	5
C. koreana	3	1	—	—	—	2	—	1	3	2	2
C. orientalis	2	1	—	1	1	1	—	1	2	1	2
*C. turczaninovii*_1	3	1	—	—	3	3	—	2	2	2	2
*C. turczaninovii*_2	4	1	2	—	1	7	—	4	3	—	4
*C. turczaninovii*_3	2	1	—	—	—	2	—	1	2	2	2
C. viminea	5	1	2	2	1	2	1	1	2	2	2
Alnus glutinosa	5	—	—	3	—	1	—	—	—	1	—
Betula pendula	4	—	—	3	—	1	—	—	4	—	—
Corylus avellana	2	4	—	7	—	1	—	—	2	1	—
Ostrya carpinifolia	5	2	3	2	1	2	—	2	2	3	—
Ostrya virginiana	3	—	—	5	3	2	—	2	2	1	2
Private for *C. betulus*	15	13	9	17	13	12	23	16	13	13	11
Absence in *C. betulus*	3	5	3	—	—	9	1	3	4	8	4

Note: — = no cross-amplification.

Appendix 1. Origin and voucher information for all samples included in the establishment of newly developed microsatellite markers for *Carpinus betulus*.

Species	Collection locality	Collector	Voucher/Plant ID	Herbarium[a]
Carpinus betulus L.[b]	Göttingen-Geismar, Germany	Prinz, Müller	KP_Cb0002	GOET
Carpinus betulus	Göttingen, Germany	Prinz, Müller, Dolynska	KP_Cb0001, 0003, 0004, 0020, 0022	n.a.
Carpinus betulus	Eschwege, Germany	Dolynska	KP_Cb0021	n.a.
Carpinus betulus	Kleve, Germany	Prinz	KP_Cb0023–0025	n.a.
Carpinus betulus	Brasov, Romania	Finkeldey	KP_Cb0005–0014	n.a.
Carpinus betulus	Valley of the beeches, Romania	Finkeldey	KP_Cb0015–0019	n.a.
Carpinus caroliniana Walter	Forest Botanical Garden, Göttingen, Germany	Prinz, Müller	KP_Cb0040	GOET
Carpinus caucasica Grossh.	Forest Botanical Garden, Göttingen, Germany	Prinz, Müller	KP_Cb0029	GOET
Carpinus koreana Nakai	Experimental Botanical Garden, Göttingen, Germany	Prinz, Müller	KP_Cb0041	GOET
Carpinus orientalis Mill.	Greece	Vidalis	KP_Cb0039	n.a.
Carpinus turczaninovii Hance	Experimental Botanical Garden, Göttingen, Germany[c]	Prinz, Müller	KP_Cb0043–0045	GOET
Carpinus viminea Lindl.	Experimental Botanical Garden, Göttingen, Germany	Prinz, Müller	KP_Cb0042	GOET
Alnus glutinosa (L.) Gaertn.	Forest Botanical Garden, Göttingen, Germany	Prinz, Müller	KP_Cb0032	GOET
Betula pendula Roth	Forest Botanical Garden, Göttingen, Germany	Prinz, Müller	KP_Cb0036	GOET
Corylus avellana L.	Forest Botanical Garden, Göttingen, Germany	Prinz, Müller	KP_Cb0030	GOET
Ostrya carpinifolia Scop.	Forest Botanical Garden, Göttingen, Germany[c]	Prinz, Müller	KP_Cb0037	n.a.
Ostrya virginiana (Mill.) K. Koch	Forest Botanical Garden, Göttingen, Germany[c]	Prinz, Müller	KP_Cb0031	n.a.

[a] Frozen leaves from all samples are stored in the Section Forest Genetics and Forest Tree Breeding, Georg-August-Universität Göttingen, Germany. Voucher herbarium specimens from almost all species are deposited in the Herbarium Göttingen (GOET), Georg-August-Universität Göttingen, Germany. German samples from *Carpinus betulus* are expected to be closely related. Greek and Romanian samples from *C. betulus* are genetically different from German samples due to geographical distances rather than phylogenetic differences.

[b] DNA of the tree was used to generate a microsatellite-enriched library for marker development (51°30.555′N, 09°57.773′E).

[c] The two *Ostrya* species and one individual of *Carpinus turczaninovii* were recently removed from their respective botanical gardens.

Appendix 2. Details for additional microsatellite loci developed for *Carpinus betulus* that revealed ambiguous and nonvaluable patterns.

Locus	Primer sequences (5′–3′)	Repeat motif[a]	Allele size (bp)	T_a (°C)[b]
Cb_15	F: GCCAACATGATTTTTGATTTAGA R: GCTAGGAAAGTGAAAGAGCTTAAGTG	$(GA)_x$	102	TD 68–58
Cb_16.1	F: GGACCATGAAGCAAGTGGAG R: ATTGTTGTTGGCTTCGCTG	$(GA)_x$	133	TD 66–54
Cb_16.2	F: GGGTGGCTGAAAATGGAT R: GAGACCCAAGGAGTAGTAGAACCA	$(GA)_x$	88	TD 66–57
Cb_29a	F: CCCACCTCTTCTCAGTTCTCC R: GTGAGCTTAGCAATGGCGAG	$(GA_x)_x$	141	61
Cb_33a	F: AGTTGCACCCTGCAATATCT R: TCAGGCGATTCATCGTTATG	$(CT)_x$	88	TD 66–57
Cb_37	F: AACACAAGAAAACTGGAGAGAGA R: GTTGCTTATTGCGTCTCATG	$(GA)_x$	93	60
Cb_39a	F: CGAGAATATGGGGCAATGAA R: TGCTCATTCTAATCTTATCTGGACT	$[(GA)_x(TG_x)(GA)_x]_5$	180	58
Cb_46	F: CATTTCTAGAAGTTATTTTAC R: GTTGATTAATCATTATCTTGG	$(GA)_x$	94	53

Note: T_a = annealing temperature.

[a] N_x signifies a microsatellite motif interrupted by an ongoing DNA sequence of different lengths.

[b] A touchdown (TD) protocol was applied. Annealing starts at the highest temperature and decreases at 1°C in each PCR cycle.