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Development and characterization of polymorphic microsatellite markers for *Conopholis americana* (Orobanchaceae)[1]

Anuar G. Rodrigues[2,4], Alison E. L. Colwell[3], and Saša Stefanović[2]

[2] Department of Biology, University of Toronto Mississauga, Mississauga, Ontario L5L 1C6, Canada; and [3]Division of Biological Resources, Yosemite National Park, El Portal, California 95318 USA

- *Premise of the study: Conopholis americana* is an obligate root parasite with highly reduced morphology. To investigate the population structure, genetic diversity, and mating system of this predominantly eastern North American species, we developed polymorphic microsatellite markers for *C. americana*.
- *Methods and Results:* Using an enrichment cloning protocol, we report the isolation and characterization of 11 microsatellite markers. Product size varied from 198–370 bp. These loci show moderate levels of allelic variation (averaging 4.182 alleles per locus) and very low levels of heterozygosity (average observed heterozygosity = 0.054).
- *Conclusions:* These microsatellite markers will be useful in obtaining estimates of population-level genetic diversity and in phylogeographic studies of *C. americana*.

Key words: *Conopholis americana*; microsatellite; Orobanchaceae; parasitic plants.

Members of the genus *Conopholis* Wallr. are perennial achlorophyllous obligate root parasites that form haustorial connections to the vascular system of oaks (Kuijt, 1969). *Conopholis americana* (L.) Wallr. specifically parasitizes red oaks (*Quercus* L. section *Lobatae* Loudon) in moist, deciduous, or mixed forests and is found across eastern North America, from Florida north to Nova Scotia west to Wisconsin and south to Alabama (Haynes, 1971). To date, very little is known of the relationships among populations and of the species's postglacial history. The populations are best described as locally abundant but rare and isolated, often separated by kilometers of forest. These plants do not possess floral nectaries and are not known to produce a fragrance that would attract insect pollinators. Bagging experiments to specifically investigate pollination by wind or insects suggest a predominant selfing mode of pollination (Baird and Riopel, 1986). In addition, studies of flowers postanthesis have found the anthers to be in physical contact with the stigma. Dispersal occurs either by the washing away of seeds after periods of rain or through the consumption of the inflorescence by mammals such as deer (Baird and Riopel, 1986).

At the height of the last glacial maximum, ranges of many species in eastern North America, particularly those that currently occupy temperate habitats, were restricted south of the Laurentian ice sheets that dominated the northern part of the continent (see McLachlan et al., 2005 and references therein). With the retreat of the ice, populations of *C. americana* are likely to have migrated northward, together with its host, to their present-day ranges. The "southern refugia hypothesis" postulates higher diversity in the southern nonglaciated regions and loss of this diversity in populations moving northward. As part of our broader research on systematics and evolutionary history of *Conopholis* (Rodrigues et al., 2011), in this study we isolate and characterize fast-evolving codominant microsatellite markers to gain a better understanding of the mating system, relationships among populations, and the amount and geographic distribution of genetic diversity for *C. americana*.

METHODS AND RESULTS

Total genomic DNA was extracted from fresh or silica-dried material using a modified hexadecyltrimethylammonium bromide (CTAB) technique from Doyle and Doyle (1987) containing polyvinylpyrrolidone (PVP) in concentrations of 0.3–4%, used to bind and remove tannins and other secondary plant compounds (Palmer, 1986). All DNA extracted from multiple individuals per population was purified using Wizard minicolumns (Promega, Madison, Wisconsin, USA). A microsatellite-enriched *C. americana* genomic library was constructed according to the Fast Isolation by AFLP of Sequences Containing Repeats (FIASCO) protocol by Zane et al. (2002). Briefly, DNA of *C. americana* was simultaneously digested with *Mse*I and ligated to an *Mse*I AFLP adapter (5′-GACGATGAGTCCTGAG-3′). The digestion-ligation mixture was then diluted (1:10) and directly amplified with AFLP adapter-specific primers. The products were denatured, hybridized to a biotinylated probe, $(AC)_{17}$, and fragments containing microsatellite sequences were captured by streptavidin-coated magnetic beads (Promega). Nonspecific DNA was removed by three nonstringency washes and three stringency washes. DNA fragments were separated from the bead-probe complex by two denaturation steps (Elution 1 and Elution 2). The last nonstringency wash, the last stringency wash, and the two elution steps should harbor increasing proportions of repeat-enriched DNA fragments carrying the *Mse*I-N primer target site at each end. Each of the four recovered fractions was amplified by PCR using the *Mse*I-N primer. PCR products

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[4] Author for correspondence: anuar.rodrigues@utoronto.ca

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TABLE 1. Characteristics of 11 microsatellite loci developed for *Conopholis americana* (n = 72 individuals).

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	T_a (°C)	GenBank Accession No.
SSR6	F: TGAAACATCGAACATGTGTGT	$(GT)_{13}(GA)_{20}$	245	59	JN050982
	R: CCTCAAGCGACACATAGAGC**				
SSR10	F: TTCGCACCATAGATCTTGACC*	$(GA)_{10}C(AG)_{15}$	257	59	JN050983
	R: TCCCCTTATGATTTAGATTGAATTG				
SSR27	F: CCAAATTCGACAATCTAAAACA	$(CA)_{13}$	249	61	JN050984
	R: AGCCTCATTTCAGCCCTTAC*				
SSR33	F: ATTCTGAGTCCGTACAATCCTC**	$(GA)_{18}$	362	57	JN050985
	R: GCTAAAATTTCTCTCTCGTCTTG				
SSR9	F: GAACTCCCCTTATGATTTAGATTGA**	$(CT)_{14}G(TC)_{10}$	227	59	JN050986
	R: ATAAGACCTTGAGGCTGCTG				
SSR22	F: GAAGAGAGGGTGCGAAGAA*	$(AG)_{10}$	244	56	JN050987
	R: AACTTCTTTCTTTCTCTTGATTCC				
SSR49	F: TGGATGTTGAGTTATCTGTTCA	$(GT)_{20}...(AG)_{18}$	199	55.4	JN050988
	R: CCACCAAGCACTTTTTATCA*				
SSR42	F: GCGCGCTTTTTAGAACACT**	$(GA)_{10}…(GA)_{12}$	265	55.4	JN050989
	R: AAGACAAGCCCTAGAATGGA				
SSR43	F: GGAGATCTATAACGGGGTTG**	$(TC)_{13}$	339	55.4	JN050990
	R: GCCGATAACCAGACCATTAG				
SSR56	F: TGAGTCGAGTCGATTTACCA*	$(GT)_7$	198	63	JN050991
	R: GACGGTGGCTCTGTAACTCT				
SSR51	F: CATACCCAAAAACCCTTTCA*	$(GT)_{10}$	242	61	JN050992
	R: ACCCTCACAAACCGACACAT				

Note: T_a = annealing temperature.
* Denotes FAM-labeled primer.
** Denotes HEX-labeled primer.

from Elution 2 were cloned using the TOPO-TA cloning kit (Invitrogen, Carlsbad, California, USA). A total of 395 colonies were screened using the universal SP6/T7 primer combination. Colonies producing insert sizes larger than 300 bp were sequenced using the DYEnamic ET dye terminator sequencing kit (GE Healthcare, Baie-d'Urfé, Québec, Canada) on an Applied Biosystems model 377 automated DNA sequencer (PE Biosystems, Foster City, California, USA).

Sixty-two sequences contained equal to or greater than six dinucleotide repeats with sufficient flanking regions within which primers could be designed. Each candidate microsatellite locus was tested in five individuals from five different populations for amplification and polymorphisms. Amplifications were performed in 25 μL reactions containing 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs (Invitrogen, Burlington Ontario, Canada), 0.1 μL forward and reverse primers, 0.5 μL DMSO, and 0.1 U JumpStart *Taq*DNA polymerase (Sigma-Aldrich, Oakville, Ontario, Canada). PCR conditions were 94°C for 4 min, followed by 36 cycles of 20 s at 93°C, 50 s at specific annealing temperatures (Table 1), 50 s extension at 70°C, with a final extension step of 30 min at 72°C. Amplicons of expected length were purified and sequenced (following the same protocol as described above). Labeled primers (forward or reverse, labeled HEX or FAM; see Table 1) were ordered for 11 loci with variable repeat numbers.

These 11 primer pairs revealing a polymorphism in two or more individuals were chosen for a larger screening of 72 individuals from 11 populations of *C. americana* (Table 1 and Appendix 1) and amplified separately under the same optimal conditions described previously. Fragments were also genotyped separately (by locus) on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, California, USA) at the Centre for Applied Genomics at The Hospital for Sick Children (Toronto, Canada). Allele sizes were initially estimated using GENEMAPPER (Applied Biosystems), but all electropherograms were examined manually before assigning final genotypes.

Characteristics of these 11 polymorphic loci are summarized in Tables 1 and 2. Exact tests for Hardy–Weinberg equilibrium were performed in GENEPOP 4.0.10 (Raymond and Rousset, 1995; Rousset, 2008). The computer programs ARLEQUIN (Excoffier and Lischer, 2010) and FSTAT version 2.9.3.2 (Goudet, 1995) were used to calculate linkage equilibrium among loci within *C. americana*. The number of alleles as well as observed and expected heterozygosity were estimated using the software GDA (Lewis and Zaykin, 2001). At the population level, the mean number of alleles per locus was 1.339. An average of 4.182 alleles per locus was observed. A significant deviation from linkage equilibrium was observed for the pairwise locus combination of SSR9–SSR10. The microsatellite loci SSR6 and SSR33 were the most polymorphic, with seven and eight alleles, respectively. Expected heterozygosity ranged from 0.158–0.778 while observed heterozygosity ranged from 0–0.286. These low levels of observed heterozygosity in *C. americana* are expected given the life history of the genus and would suggest a selfing mode of pollination as proposed by Baird and Riopel (1986).

TABLE 2. Results of the initial primer screening in population of *Conopholis americana*.

Locus	*A*	H_o	H_e
SSR6	7	0.167	0.680
SSR10	4	0.000	0.356
SSR27	5	0.000	0.667
SSR33	8	0.113	0.778
SSR9	4	0.028	0.378
SSR22	3	0.000	0.287
SSR49	6	0.286	0.426
SSR42	3	0.000	0.213
SSR43	2	0.000	0.293
SSR56	2	0.000	0.179
SSR51	2	0.000	0.158

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity.

CONCLUSION

We developed 11 microsatellite loci that show variability at the population level in *C. americana*. These are the first microsatellite DNA markers for the genus, and will function as primary tools to quantify levels of genetic variation and patterns of population structure in this holoparasitic species. Although they have not been tested yet, the microsatellite markers described here are also likely to be extendable to the other two species within this genus, making them useful not only to draw conclusions

about mating systems and postglacial migration of *C. americana* in eastern North America, but also for population, conservation, systematics, and phylogeographic studies of other *Conopholis* species.

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APPENDIX 1. DNA accession numbers, collection locality of the specimens, and geographic coordinates for sequences of *Conopholis americana* used in this study.

DNA accession no.	No. of individuals	Locality[a]	Geographic coordinates[b]
AC.FL.JS	2	Marion Co., Florida, USA	29°10′N, 81°42′W
AC.NC.L	10	Madison Co., North Carolina, USA	35°44′N, 82°51′W
AC.IN.CCF	7	Clarke Co., Indiana, USA	38°32′N, 85°49′W
AC.IL.KSP	9	Vermillion Co., Illinois, USA	40°07′N, 87°44′W
AC.PA.MSF	9	Franklin Co., Pennsylvania, USA	39°55′N, 77°26′W
AC.MA.MN	14	Hampshire Co., Massachusetts, USA	42°18′N, 72°30′W
SS.05.79	3	Huntsville, Madison Co., Alabama, USA	34°43′N, 86°35′W
SS.09.37	2	Cheboygan Co., Michigan, USA	45°33′N, 84°40′W
AC.FL.SF	5	Alachua Co., Florida, USA	29°44′N, 82°26′W
AC.WI.DL	6	Sauk Co., Wisconsin, USA	43°24′N, 89°42′W
AC.VA.TRP	5	Fairfax Co., Virginia, USA	38°57′N, 77°09′W

Note: in column DNA accession: AC = Alison Colwell; SS = Saša Stefanović.

[a] Geographic areas where the specimens were collected.

[b] Approximate geographic coordinates for the localities from which the specimens were obtained.