

PRIMER NOTE

Twelve variable microsatellite loci for the North American medicinal leech, *Macrobdella decora*

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Abstract

We isolated and characterized 12 microsatellite loci for the North American medicinal leech, *Macrobdella decora*. *Macrobdella decora* is abundant in central and northern North American freshwater systems. The resulting microsatellite library, the first for any species of leech or any clitellate annelid, demonstrates the efficacy of this type of analysis on unexploited leech populations and should serve as a baseline for comparison with other species, notably the commercially important and threatened European medicinal leech, *Hirudo medicinalis*.

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Hirudo medicinalis, the European medicinal leech, historically has been the leech species most often used for medicinal purposes, particularly those involving microsurgical procedures, and for the study of anticoagulants present in their saliva. Once abundant, its range has been drastically reduced due to over-harvesting and *H. medicinalis* has protected status across Europe (Sawyer 1981; Elliott & Tullett 1992). To satisfy the demand from medical institutions, there is also an *H. medicinalis* captive breeding industry (e.g. Bishop 1992). The North American medicinal leech, *Macrobdella decora*, is abundant and widespread across its range and, although possessing potent platelet aggregation inhibitors (Munro *et al.* 1991), is not similarly exploited for commercial purposes. Both species are freshwater leeches in the family Hirudinidae. Both have similar habitat preferences and feed on similar prey items (typically frogs or fish and surreptitiously mammals) and both, although aquatic, deposit egg case cocoons on land with the attendant possibilities for interpopulation dispersal.

Preliminary attempts to examine genetic variability in these medicinal leeches were frustrated by lack of variation in mitochondrial loci. Microsatellite data for these species would provide an opportunity to examine population structure, genetic diversity across ranges and tracking of gene flow whether due to natural dispersal or clandestine

introduction. Moreover, the highly diverse *M. decora* may serve as a relatively unimpacted baseline species against which over-exploited wild or captive-bred populations of *H. medicinalis* can be compared. The ease of access to substantial numbers of individuals from multiple populations in North America renders *M. decora* the obvious starting point to examine the viability of these investigations.

We developed the first microsatellite library for any leech species (and presumptively the first for any clitellate annelid) by following and adapting the protocol written by Dr Travis Glenn at the University of Georgia. DNA was isolated from one *M. decora* individual collected from Broadwing Lake (Algonquin Park, Ontario, Canada) using the DNeasy Animal Tissue Kit (QIAGEN). Tissue from the caudal sucker was used to prevent any bloodmeal contamination. Genomic DNA was digested with *RsaI*, ligated to an SNX linker and hybridized to a mixture of di- and trinucleotide biotinylated probes (i.e. GT, CAC, CTC and CAG). After hybridization to the repeat probes, the linker-ligated leech DNA was bound to streptavidin-coated (biotin-attracting) Dynabeads® (Dynal Biotech) and collected using a magnet. Following polymerase chain reaction (PCR) enrichment with the SNX forward primer, microsatellite-rich fragments were cloned using the Topo TA cloning kit (Invitrogen), transformed into *Escherichia coli* OneShot cells and grown on ampicillin selective plates. Rather than probe colonies negative for β -galactosidase activity (white)

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Table 1 Characteristics of 12 microsatellite loci for *Macrobodella decora*

Locus	Primer (5'–3')*	GenBank Accession no.	Optimal annealing temp. (°C)	Total no. of alleles	Product size and localities present	Core repeat	Heterozygosity by locality					
							o		m		v	
							H _O	H _E	H _O	H _E	H _O	H _E
MDA11	F: CGATGCTTTTGGTCTGTAGC R: CTTGTTTCGTTTCACGCCG	AY576618	53	6	254ov, 257m, 259o, 263o, 268m, 274om	GT	0.43	0.58	0.18	0.53	0	0
MDA02	F: GGGTTCGGATTTTGTATTC R: CTCGGCGATGAAGAGAIT	AY576612	56	3	137o, 142o, 146omv	GT	0.11	0.29	0	0	0	0
MDA03	F: TGTGAGTGTATGATGAATGTATG R: CGTTACGGATGGAGAGATG	AY576619	54	5	178om, 181mo, 184mo, 187ov, 196v	CAT	0.66	0.5	0.33	0.35	0.29	0.24
MDA06	F: CGAAGCAGTAGTCAATCCG R: TCCAACCTCCCTTCTCAOCTG	AY576611	56	8	213o, 214mo, 216o, 217m, 225o, 226m, 231m, 235m	GAT	0.8	0.64	0.54	0.79		
MDB06	F: TCCTTTCATTTCCGCCGACG R: CCTTTACCTTTCCTTTTCTTAGG	AY576614	54	5	198mov, 209mov, 214m, 218m, 220o	AC	0.5	0.45	0.75	0.54	0.8	0.48
MDC02	F: CGTCGTCACTTGTGTACTG R: GCCTTTCAGCGAATTCAGG	AY576610	52	4	155m, 179m, 196m, 202v	TGA			0.42	0.35	0	0
MDD07	F: AACATCGTCACTGTCACCG R: TCTTCGTGTGCCGACCAAGTG	AY576616	56	5	190v, 205o, 209o, 215ov, 227v	AG	0	0.67	0.18	0.31	0.2	0.34
MDE02	F: CAACACCATCAACCAGTCCC R: TGCAGTAGTAGAGGCTTGTG	AY576617	54	5	110v, 119mov, 134o, 138m, 141mv	ACC	0.18	0.17	0.64	0.47	0.63	0.57
MFF06	F: CGAAGGGAACAACCACATAGC R: AGAAGCAACAACCGCACTCC	AY576613	55	7	182o, 188mov, 190v, 198mov, 204mo, 208o, 220mo	CA	0.66	0.72	0.33	0.57	0	0.63
MDF08	F: TGTGTGTGTTTCTGCTCTAC R: TTTTCGTACCTTGGTTGGC	AY576620	56	3	183mov, 195mv, 211m	ACCACCACT	0.14	0.13	0.55	0.59	0.33	0.28
MDG03	F: TCACCAGGAAAGGTATGGC R: GCAGAAGCACCCGAGAAACAAG	AY576609	54	8	121o, 127mv, 134o, 136mov, 138o, 140o, 142m, 147m	AG	0	0.77	0	0.78	0.25	0.47
MDM33	F: TAAACCCCTATAAAGGAACA R: TTTTCGGACATTTGCTCTTT	AY576615	50	2	222m, 227m	ATG			0	0.49		

*M-13 (CAGCAGTTGTA AAAACGAC) tail attached to the 5' end of the forward primers.

H_E: expected heterozygosity; H_O: observed heterozygosity; o, Ontario; m, Michigan; v, Vermont.

for repeats, 576 white colonies were picked, recultured in broth and purified with the QIAprep 96 Turbo Miniprep Kit (QIAGEN) and sequenced directly with M13 and T7 primers and BigDye on an ABI 3700 automatic sequencer.

Thirty-nine clones with microsatellite regions of good length and with substantial flanking regions were identified with SEQUENCE NAVIGATOR (Applied Biosystems) and GENEJOCKEY II (BioSoft). Di-, tri- and tetranucleotide repeats were most common but larger repeats, including a nonamer, were observed. Primer design was accomplished with MACVECTOR 6.5.3 (Oxford Molecular). Twelve primer pairs (Table 1) successfully amplified *M. decora* individuals from Ontario, Michigan or Vermont. Rather than use each forward primer with a fluorescent label, we adapted the M13-tailed microsatellite protocol of Boutin-Ganache *et al.* (2001) in which each forward primer sequence is 5'-augmented with an M13 forward sequence (CACGACG-TTGTAACGAC). This augmented primer is then used in combination with fluorescently labelled forward M13 in amplification reactions. Thus, AmpliTaq Gold (Perkin Elmer) PCR reactions used equal concentrations of three primers: forward M13 fluorescent primer, 5'-augmented microsatellite forward primer and unmodified microsatellite reverse primer. Reactions consisted of 1 µL DNA, 0.5 µL of each 10 µM primer, 2.5 µL of 25 mM MgCl₂, 2.5 µL PCR Buffer II (Perkin Elmer), 2.0 µL of a 10 µM dNTP mixture, 0.13 µL AmpliTaq Gold and 15.37 µL H₂O for a total volume of 25 µL. The thermal profile was 94 °C for 10 min followed by 40 cycles of 94 °C for 30 s, the primer-specific annealing temperature (see Table 1; however, 50 °C was successful for all 5'-augmented primer combinations) for 30 s and 72 °C for 45 s followed by a 7-min extension at 72 °C.

Amplification reactions were run on an ABI 3700 automated DNA sequencer using ABI GENESCAN and analysed with GENOTYPER 2.1 (Perkin Elmer). Individuals from three populations were examined for distribution of alleles and measures of heterozygosity (Table 1). The population localities were: Broadwing Lake (Algonquin Park, Ontario, Canada), collected and preserved in 95% ethanol in July 2002; a small pond near Douglas Lake (Cheboygan County, Michigan), collected and preserved in 95% ethanol in June 1998 and an unnamed pond on Horseshoe Road (Chester, Windsor County, Vermont), collected in August 2003 and maintained live. Sixteen individuals were used from the Ontario and Michigan populations although only eight

were available from the Vermont population. Amplifications of MDA06, MDC02 and MDM33 were inconsistent in the Vermont and Ontario populations possibly indicating null alleles and probably precluding their use for measures of heterozygosity, for example, at least until fresh material can be examined. Furthermore, the deficit in observed heterozygotes compared with the number estimated (e.g. MDG03, MDA11, and MDD07) may reflect null alleles, small sample sizes or deviations from equilibrium attendant with the small isolated bodies of water for source populations in Michigan and Vermont. Further work screening more individuals from these populations, from larger water sources and from more populations across the species' range should determine the effects of null alleles, as well as sample and habitat size. Nonetheless, Table 1 illustrates some alleles useful for the examination of gene flow as well as private alleles that can be used to diagnose individuals from different populations. In order to address inter-specific variation, all of the microsatellite primer pairs were tested without success on *M. ditetra* from Georgia.

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