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PRIMER NOTE

Isolation and characterization of microsatellite loci from the planarian *Dugesia polychroa* (Schmidt) (Platyhelminthes: Tricladida)

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The genomes of many species have been shown to contain loci composed of tandemly repeated short DNA sequences known as microsatellites or simple sequence motifs (Tautz & Renz 1984). Allelic variation in copy number of the repeat unit renders these loci polymorphic, making them convenient markers for evolutionary studies, including the analysis of population structure (Bruford & Wayne 1993; Schlötterer & Pemberton 1994), mating systems and paternity (Queller *et al*. 1993). We developed microsatellite markers for the free-living fresh water planarian *Dugesia polychroa* (Platyhelminthes: Tricladida) to study the population structure and mating system of these simultaneously hermaphroditic worms characterized by internal fertilization. *Dugesia polychroa* has two reproductive biotypes, sexual diploids and pseudogamous parthenogens, which can interbreed to form interbiotype hybrids (Benazzi & Benazzi Lentati 1976). We developed microsatellites that can be amplified by PCR and used to assay limited amounts of DNA obtained from individual worms. This is the first report on the isolation and characterization of microsatellite loci from a platyhelminth.

Genomic DNA from ≈ 100 worms from the Ammersee, southern Germany, was isolated using a guanidium isothiocyanate method designed for organisms with high nuclease and mucopolysaccharide levels (Garcia-Fernandez *et al*. 1993). Three genomic libraries were constructed. The first was made by digesting total genomic DNA with *Eco*R1 and cloning the resulting fragments into dephosphorylated pUC18 digested with *Eco*R1. The second library was constructed from size selected fragments according to Rassmann *et al.* (1991) and cloned into dephosphorylated pUC18 digested with *Sma*1. Ligation products of both digestions were used to transform *E. coli* XL1-blue cells (Stratagene), which were plated on selective LB media and cultured overnight. Approximately 8000 colonies were transferred into 96-well microtitre plates and replica plated on to nylon membranes (Hybond-N +, Amersham). Colonies were screened with the digoxigenin (DIG) end-labelled (Boehringer Mannheim) synthetic oligonucleotides $(AC)_8$, $(AG)_8$, $(ASA)_6$, $(GACA)_4$, $(GATA)_4$, $(SAAA)_4$, $(SAAT)_4$, $(SATT)_4$ and $(CGCACA)_3$. Oligonucleotides with identical hybridization temperatures were grouped together (maximum three probes) and used in a single probe cocktail. One clone *DpGATA1* was isolated from the *Eco*RI pUC18 library. In a second round of screening using $(AT)_8$ and $(ATT)_6$ as probes, we isolated two $(ATT)_n$ clones containing 28 and 47 repeats, respectively, from the size-selected *Sma*I library.

In addition, a third library was constructed using the charomid vector 9–36 (Saito & Stark 1986). This vector was chosen because it accepts large inserts (up to 10 kb) and uses a recombinant deficient *E. coli* host N554M in which highly repetitive sequences are stable. Approximately 1 μg of genomic DNA was partially digested with *Eco*RI and ligated into the *Eco*RI site of the vector. The resulting recombinants were packaged *in vitro* using Gigapack 11 Packaging extracts (Stratagene) following the manufacturer's instructions. Approximately 3000 colonies of the resultant library, having insert sizes ranging from 3 to 7 kb, were screened with the same probes as previously mentioned. Two clones were recovered with the same $(GATA)_{39}$ microsatellite as above, suggesting that such repeats are rare in the genome. About 30% of our clones contained ATT repeats, indicating a frequency of approximately one ATT repeat for every 18.5 kb of the genome. Although A + T rich microsatellite sequences have been found in lower organisms such as the slime moulds, fungi, and protists (Field & Wills 1996), as well as in plants (Powell *et al*. 1996), ATT microsatellites have not been reported in such high frequencies from any organism.

Eleven clones with strong hybridization signals were selected from the charomid library in order to subclone the smaller positive fragment into *Sma*I digested pUC 18. Eight of the subclones contained ATT microsatellites which varied in repeat number from 28 to 126. One clone

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Table 1 Primer sequences and characteristics of *Dugesia polychroa* microsatellite loci. Number of alleles and size range refer to the length of observed PCR product

Locus	Repeat array	Primer sequences (5′–3′)	T_a	No. of ind.	No. of alleles	Size range (bp)	% heterozygotes	EMBL Accession number
DpGATA1	$(GATA)_{39}$	ATCCACCCTTTATTTTACAT CATATTGATTTAGTTTGAA	50	176	20	250–494	34	X92189
DpATT1	$(ATT)_{28}$	GCCTCTCTTTTTAATAA TACACATACAATAAAATCC	55	26	2	146–184	0	X92191
DpATT2	$(ATT)_{44}$	GCTCAACAACTCGCAAGGG GGCATAACAATTTCACACAGG	60	56	12	29–351	30	X92192
DpATT4	$(ATT)_{45}$	ATCCCCACAAATATATCTTA TCACATTATCCTACCCCA	55	129	12	182–301	21	X92194
DpATT7	$(ATT)_{28}$	CCTACATTAACCCATTTG TGATGATGACAGGAAAACT	50	92	18	205–256	28	X92197
DpATT8	$(ATT)_{45}$	TCCCCACAAATATATCTTA CCTGTCACATTATCCTACC	50	73	6	189–202	1	X92198
DpATT9	$(ATT)_{35}$	TGGGGAAGAACATTGCTA AAATAAYCTGTGKGGAAAT	55	30	25	231–356	77	X92199
DpAT/AG1	$(AT)_{27}(AG)_{16}$	CCCAAAATGAAATGTAAAG CAAAAACGAATAACATAAATA	55	57	24	186–296	61	X92201

Six microsatellites (EMBL Accession No. X92190, X92193, X92195, X92196, X9200 and X92202) that could not be PCR amplified are excluded

contained three noncontiguous ATT repeats of 32, 10 and 29 units, respectively; one clone a heterogeneous microsatellite with two contiguous regions of the dinucleotide repeats $(AT)_{27}$ and $(AG)_{16}$, and one clone had an $(AG)_{25}$ repeat. PCR primers were designed from the flanking sequences for 12 of these microsatellites (two clones were rejected because in one the flanking region was too short and in the other the sequence was ambiguous) and one primer of each pair was 5′ end-labelled with a fluorescent phosphoramidite dye (6-FAM, TET or HEX, Applied Biosystems). PCR reactions (25 μL total volume) contained ≈ 50–100 ng of genomic DNA, 10 pmol of each primer, 1 × buffer, 2.5 mM $MgCl_2$, 0.2 mM each of dNTPs and 0.75 U of *Taq* polymerase (Promega). Reaction conditions were as follows: an initial denaturation step of 120 s at 92 °C followed by 35 cycles of 30 s at 92 °C, 60 s at the annealing temperature (Table 1) and 60 s at 72 °C (except for locus *DpATT2* which has a synthesis step at 60 °C). All amplifications were performed in a GeneAmp PCR System 9600 thermal cycler (Perkin Elmer) and PCR product lengths were analysed on an ABI 310 Automated Sequencer (Applied Biosystems).

Eight of 12 microsatellites could successfully be amplified from genomic DNA (Table 1). High variability was observed at these loci and heterozygotes were found for all loci examined except *DpATT1* and *DpATT8*. Individuals from several localities including both diploid sexuals and polyploid parthenogens were screened. Numbers of alleles varied from two (*DpATT1*) to 25 (*DpATT9*). We are currently using these polymorphic markers to study population structure and mating systems of both reproductive modes of this hermaphroditic species.

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