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Microsatellite development and inheritance in the planarian flatworm *Schmidtea polychroa*

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ABSTRACT. We developed four polymorphic microsatellite loci for the hermaphroditic planarian *Schmidtea polychroa* and used them to identify differences in recombination rate in the male and female germ line. DNA isolation protocols were optimized for tissue from adults and hatchlings. The final PCR protocols and profiles yielded repeatable and reliable amplification. Null alleles detected at one locus could be avoided by redesigning one primer. Routine genotyping was established using fluorescent-labeled primers and an ABI 310 automated sequencer. They amplify successfully in a number of populations. The four loci are characterized by extreme within-population polymorphism, with 15 to 20 alleles per locus in a standard sample. All four have been submitted to GenBank. Two loci (SpATT16, SpATT18) appeared to be coupled. From parent-offspring comparisons, the recombination fraction could be estimated, which was significantly different for the male ($c = 0.07$) and female ($c = 0.23$) line. High overall exclusion rates for first (>0.94) and second parent (>0.98) even with three (unlinked) loci demonstrates the suitability of these microsatellites for other applications such as parentage analysis.

KEY WORDS: Platyhelminthes, hermaphrodite, microsatellites, recombination rate, *Schmidtea polychroa*.

INTRODUCTION

Within the last decade, microsatellites have proven to be extremely powerful codominant genetic markers for parentage as well as population studies (QUELLER et al., 1993; JARNE & LAGODA, 1996; HUGHES, 1998). They consist of 'simple sequences', in which short nucleotide motifs (usually 1-6 bp) are tandemly repeated, and which occur throughout the genomes of all eukaryotic organisms (TAUTZ & SCHLÖTTERER, 1994). Variation in the repeat number of the microsatellite motif can easily be detected electrophoretically as fragment length polymorphism following PCR of the microsatellite locus.

Previously described microsatellite loci (RAMACHANDRAN et al., 1997) in the simultaneously hermaphroditic freshwater planarian *Schmidtea* (formerly *Dugesia*) *polychroa* Ball (1974) could reliably be amplified and scored in some of our study populations but not in others. In particular, samples from some of the most important study populations from Northern Italy showed no or poor amplification for those loci. Therefore, a second genomic library was constructed with DNA samples from individuals collected in Lago di Caldonazzo (Trentino, Italy), where many samples for population genetic analyses as well as paternity studies have also been collected.

METHODS

Microsatellite screening and amplification

Screening was restricted to $(ATT)_n$ trinucleotide repeats, because the first screen had shown their high abundance in the genome of *S. polychroa*. Methods used for constructing the library and screening were the same as described in RAMACHANDRAN et al (1997). Fourteen positive clones were isolated and sequenced, all containing repetitive $(ATT)_n$ sequences (GenBank accession nos.

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AF201314-AF201327). We designed PCR primers for amplification of these regions, and successfully achieved amplification in 5 cases, for which the primer sequences are listed in Table 1. Presence of ATT repeats in the PCR products was verified by blotting and hybridizing with a DIG labeled oligonucleotide $(ATT)_6$ probe. Banding patterns of SpATT19 PCR products on 10% PAGE gels did not suggest within- or between-population polymorphism, while the ones obtained from loci SpATT12, SpATT16, SpATT18 and SpATT20 showed considerable allele size variability. All subsequent analyses of those four loci were done with fluorescent-labeled primers (see table 1) and a PE Applied Biosystems ABI310 genetic analyzer. PCR was performed in 10 µL reactions with the following components (final concentrations): $MgCl_2$ (2.5 mM), BSA (0.1%, bovine serum albumin), dNTPs (0.2 mM each), primers (0.4 µM each), Promega® Taq Polymerase (0.003 units/µL), and Promega® DNA polymerase buffer (as recommended in the product information). 0.4 µL of template DNA solution (50-200 ng/µL) was added to 9.6 µL of premix. PCR conditions included an initial 2´ denaturation step at 94°C prior to cycling, and 30-35 cycles of the following temperature profiles: 40´´ at 94°, 1´ primer annealing, and 1´ at 72°C (loci SpATT12, SpATT16, SpATT20), and 50´´ (94°C), 1´ (55°C) for SpATT18.

TABLE 1

Characteristics of PCR primers for amplification of the four microsatellite loci in *S. polychroa* (FL = fluorescent label used for detection on Abi310; B = length of the primer in nucleotide numbers)

Locus/primer	Name	Nucleotide sequence	FL	B
SpATT12/forward	ATT12U	5'TTAGATTTTGCTGGATGAA 3'	-	19
SpATT12/reverse	ATT12L	5'TTGCCACTGAAATAATAA 3'	TET	18
SpATT16/forward	ATT16U	5'TTGATGAGAAATTATTGAAA 3'	6-FAM	20
SpATT16/reverse	ATT16L	5'CTTGCATTTTGCTCTGATAA 3'	-	20
SpATT18/forward	ATT18U	5'TACATTATTCGCAACAAAA 3'	HEX	19
SpATT18/reverse	ATT18BL	5'TTGGTAAAATCTCTTGAACA 3'	-	20
SpATT20/forward	ATT20BU	5'CCAGGAGATTGACAAAGACT 3'	6-FAM	20
SpATT20/reverse	ATT20L	5'ATGTTTACCACTAAAATTATTG 3'	-	22

Sample origins

Adult individuals collected at various sites from four different localities in Northern Italy provided DNA samples for the characterization of the variability within and between populations at the four loci, which enabled us to evaluate their use for parentage testing and population studies. Mendelian inheritance could be demonstrated using known mother-offspring pairs from our laboratory culture animals (data not shown). In order to study sex-specific linkage, parent-offspring comparisons with suitable variability (heterozygous genotypes in the parental genotype at both loci) were analysed. They allowed calculation of the recombination fraction between the two loci separately for the male and female line. 342 father-offspring pairs from 40 different sperm donors could be used for analysis of the male gametes, and 299 mother-offspring pairs from 41 different mothers for analysis of the female gametes.

DNA isolation

Samples obtained from adults

Tissue for DNA extraction was cut off the posterior part of individuals and stored in pure absolute ethanol at -20°C or -80°C. Tissues were transferred to empty 1.5 ml tubes, air-dried in the open tubes (3 min) before adding 400 µL of DNA extraction buffer (10 mM Tris/HCl, 2 mM EDTA, 10 mM NaCl, 1% SDS, 0.4 mg/ml Proteinase K). Samples were incubated at 50°C for 2 h until the tissue was completely dissolved, followed by heating the mixture to 90°C for 5 min in order to stop Proteinase K activity. After chilling the samples on ice, 200 µL of 4.5 M NaCl solution was added in order to precipitate proteins. After adding 600 µL of Chloroform-Isoamylalcohol (24:1), samples were mixed and shaken for 10 min by inverting the tubes continuously. Aqueous and organic phases were separated by centrifuging at 16000g for 10 min at RT. From the aqueous phase, 500 µL of each sample were transferred to new tubes. DNA was precipitated by adding 500 µL of isopropanol. Centrifugation (16000g, RT) resulted in brownish pellets containing DNA and epidermal pigments of the individuals. The pellets were washed (70% EtOH), air-dried at RT for about 20 min, and redissolved in TE at room temperature.

Samples obtained from hatchlings

Hatchlings provide only very limited material for DNA extraction, and therefore complete individuals were extracted using a commercial DNA extraction kit (Nucleon BACC1, Amersham™). We used the protocol provided for extracting DNA from mammalian blood cells, but with reduced volumes. Hatchlings were transferred to Eppendorff tubes containing 100 µL of *Reagent*

A from the kit, which then were shock-frozen in liquid nitrogen, and stored at -80°C.

RESULTS

Characterization of the four microsatellite loci

All four microsatellite loci were highly polymorphic (Table 2). Locus SpATT16 has only been tried on samples from two populations, while the three other loci could successfully be amplified in all diploid sexual as well as tri- and tetraploid parthenogenetic *S. polychroa* populations that we sampled across Europe (data not shown). Data analyses concerning population differentiation in sexuals, and genetic diversity in parthenogenetic populations will be presented elsewhere. Amplification of the loci failed in two closely related sister species (Benazzi´s biotypes E and F; BENAZZI, 1982).

TABLE 2

Characteristics of the four microsatellite loci in 13 subpopulations collected from four locations in Northern Italy (Lago di Levico, Lago di Caldonazzo, river Sarca near Arco, Lago d'Iseo). Details of the population studies will be published elsewhere.

Locus	SpATT12	SpATT16	SpATT18	SpATT20
A	30	24	36	28
BP	199	299, 353-365	255-261	220, 226
	226-262	413, 422, 425	346, 388	232-304
	268-314	452-482, 497[a]	397-487	310
R	4-42	47-116[a]	see text	19-49
H_{obs}	0.83	0.61	0.86	0.85
H_{exp}	0.89	0.88	0.89	0.89
N	640	67	613	644

A Allele numbers
BP Allele size range (base pairs)
R Allele size range (repeat numbers)
H_{obs} Observed heterozygosity (mean over 13 subpopulations)
H_{exp} Expected heterozygosity (mean over 13 subpopulations)
N Sample size (number of adults analysed)
a plus 3 alleles > 500 bp

The allele size ranges comprised almost continuous allele arrays consisting of 13-42 (SpATT12), about 50 to 80 (SpATT18, see below), and 19-47 (SpATT20) repeat units (Table 2). SpATT16 showed a highly discontinuous allele size range, with extremely large allele size differences (> 200 bp). Large allele size differences were also found at locus SpATT18. Apart from the pure *ATT* repeat motifs, the repeat region of SpATT18 contains other derived motifs (*ACT*, *ATTT*), and therefore assigning repeat numbers as allele labels would have required an arbitrary definition of what is considered the repeat array. Sequencing of the short alleles showed that they lacked almost the entire core sequence (data not shown).

Unusual banding patterns

Detection of a third allele in offspring samples

Individuals at hatching are full of swallowed yolk cells, some of which are still intact (MARINELLI & VAGNETTI, 1975). Since yolk cells provide an excellent source for maternal DNA, it is likely that hatchling DNA samples can contain small amounts of maternal DNA extracted from yolk cells that are occasionally amplified during PCR. This was concluded from the analysis of 198 offspring samples, for which the maternal genotypes were known, and which had an additional band that was much less intense (by at least one order of magnitude) than the other (one or two) bands defining the genotype. The respective banding patterns were clearly different from the ones obtained from triploid individuals, and therefore did not indicate triploidy. Out of 198 offspring samples with known maternal genotypes, 190 supported the interpretation that the additional weak bands represented the second maternal allele, amplified from small amounts of DNA from yolk cells extracted together with the hatchlings. Eight cases only were not consistent with this hypothesis, probably due to mutation or genotyping errors.

Peak intensity patterns at SpATT12

Locus SpATT12 showed particular peak intensity patterns that depended on the allele size combinations in heterozygotes. The allele frequency distribution is bimodal, with a shorter range (SR) of alleles 15 to 25 repeats long, and a longer one (LR) comprising alleles from 27 to 41 repeats in length. Usually, when two peaks indicate a heterozygous genotype, the peak representing the longer allele appears less intense. This was also the case in SpATT12 when the genotype was either composed of two SR or two LR alleles. However, when an SR allele occurred together with an LR allele, the intensity of the former was much weaker than the one of the LR allele. A more detailed treatment of these particular banding patterns, including pictures of the respective electropherograms, is given elsewhere (PONGRATZ, 2000).

Null alleles

A common problem with microsatellites is the occurrence of null alleles (PEMBERTON et al., 1995). Null alleles are alleles that are not amplified through PCR. They are often caused by a mutation within the primer region, which prevents proper annealing under stringent conditions, and inhibits or completely prevents

amplification (Callen et al., 1993). Samples with one null and one amplified allele appear as homozygous genotypes. This can result in significant heterozygote deficiency. Null alleles can impair the use of a microsatellite locus for paternity when they remain unnoticed. In practice, the best way to detect null alleles is by following inheritance of the alleles in known parent-offspring combinations (Callen et al., 1993). Indications for the presence of null alleles have been found at SpATT16 (N=66) where a significant heterozygote deficiency was observed (H_{obs}=0.61, H_{exp}=0.88 ; χ^2 = 37.18 ; P < 0.001). With genotype comparisons of known parent-offspring pairs it could be confirmed that some genotypes that appear as homozygotes, because only one allele is seen, must have a second allele that is not or only poorly amplified and cannot be detected.

Linkage

Parent-offspring comparisons as well as disequilibrium analyses of field samples did not show indications for linkage between pairs of loci except for SpATT16 / SpATT18. The loci are not strictly linked, but allelic combinations for those loci among the offspring show significant deviations from the ones expected under random association. The two loci therefore appear to be on the same chromosome. There was a significant difference between observed allelic associations in the gametes and the ones expected under random segregation of alleles for both male and female line. A χ^2-test revealed highly significant differences between the frequencies of coupled and recombined genotypes between male and female gametes (Table 3). We estimated the recombination fraction $c_{SpATT16/SpATT18}$ between loci SpATT16 and SpATT18 (Weir, 1996, p.230). For two loci (A, B), c_{AB} can vary between 0 (no recombination between loci = strict linkage) and 0.5 (random segregation of alleles from the two loci). The mean estimate for $c_{SpATT16/SpATT18}$ was 0.06 for the male line, and 0.23 for the female line, which means that 6% and, respectively 23% of gametes bear recombined two-loci genotypes.

TABLE 3

Frequencies of coupled and recombined two-loci haplotypes at SpATT18 and SpATT16, and the recombination fraction ($c_{SpATT18, SpATT16}$) estimated from them. The frequencies between male and female gametes are significantly different (χ^2 = 36.28 ; P < 0.01)

Genotypes	Male	Female
Coupled	320	230
Recombined	22	69
Total	342	299
$c_{SpATT18, SpATT16}$	0.06	0.23

DISCUSSION

Potential of the four microsatellites as genetic markers

The four microsatellite loci described here showed reliable and consistent amplification for *S. polychroa* and have been applied to population samples of different origins. They were highly polymorphic within local populations, with 15-20 alleles in standard population samples. SpATT12, SpATT18, and SpATT20 represent suitable markers for both parentage analysis and population genetic questions. Care has to be taken when using SpATT16 due to nonamplifying or undetectable alleles. Whether SpATT16 can be applied has to be decided for each study and population separately.

There was evidence from mother-offspring comparisons that some offspring samples contained remnants of maternal DNA from ingested yolk cells that was amplified during PCR but appeared much weaker than the true alleles. This has to be taken into account when analysing offspring sample banding patterns.

In general, the high polymorphism present in the diploid sexual study populations makes the four microsatellite loci ideal markers for individual identification, and identification of parents and offspring. Individuals collected from the same subpopulation showed sufficiently high polymorphism to allow successful parentage assignment even when several candidates for maternity and paternity were present (Pongratz, in prep.). In contrast to previously studied allozymes (Pongratz et al., 1998), the microsatellite loci can be used for studies of population differentiation on a very small scale. For polyploid, parthenogenetic populations they can provide a better resolution of the clonal structure (unpublished data), and they can also be extremely useful in the analysis of hybridization processes in mixed sexual/parthenogenetic populations.

Sex-specific linkage

We found significant differences in the recombination fraction between male and female line for one pair of loci. Sex differences in recombination have been documented in a variety of taxa by studying chiasma frequencies in spermatocytes and oocytes (review in Burt et al., 1991), but across species no conclusive relationship between sex and chiasma frequency could be observed (Burt et al., 1991). A higher chiasma frequency (which results in a higher recombination rate) in oocytes compared to spermatocytes was also observed in the planarian *Dendrocoelum lacteum* (Jones & Croft, 1989). The higher recombination rate between loci SpATT16 and SpATT18 may indicate the same general trend in *S. polychroa*, although detailed recombination studies in humans revealed that the male-female recombination ratio can vary significantly over short regions (Robinson & Lalande, 1990). Certain regions with higher female

recombination rate can occur, while other regions on the same chromosome show higher male recombination rates (ROBINSON & LALANDE, 1990; ZOGHBI et al., 1990). Therefore, a general conclusion regarding sex differences in chiasma frequencies and recombination rates, should not be drawn from the analysis of only two loci.

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