# Isolation and characterization of microsatellite loci from the tapeworm *Schistocephalus solidus*

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*Schistocephalus solidus*, a pseudophyllidean cestode parasite, is an outstanding model organism for studying the evolution of hermaphroditism, as it is a simultaneous hermaphrodite that appears to reproduce by self- and cross-fertilization. Learning more about, for example, sex allocation, selfing and out-crossing strategies or decisions in the hermaphrodite's dilemma requires determination of selfing and out-crossing rates and the extent of sperm competition (Jarne & Charlesworth 1993). A necessary prerequisite for such studies are high-resolution, co-dominant molecular markers that allow assignment of paternity and the assessment of levels of heterozygosity. In this report, we describe the isolation of five microsatellite DNA loci in *Schistocephalus solidus* and the development of appropriate PCR primers. These are, to our knowledge, the first microsatellite primers for any cestode species.

Aliquots (75 μg) of genomic DNA from three individuals from Bochum (Germany) were isolated using the Genomic Tip Kit from Qiagen (Basel, Switzerland). The DNA was sent to the Genetic Identification Services (GIS, http://www.genetic-id-services.com; Chatsworth, California, USA), to develop two enriched microsatellite genomic libraries, one containing inserts with $(CT)_n$ repeats and the other containing $(CA)_n$ repeats. The inserts (cut with *Hin*dIII) were cloned into dephosphorylated pUC18 cut with *Hin*dIII. The libraries (stored as plasmid DNA) were used to transform *E. coli* DH5α competent cells. Individual bacterial clones were picked, suspended in 20 μL sterile water and the insert DNA was amplified by polymerase chain reaction (PCR) using primers complementary to the regions flanking the polycloning site (5′-ACGACGTTGTAAAACGACGGCCAG-3′ and 5′-TTCACACAGGAAACAGCTATGACC-3′, respectively). PCR products were isolated for sequencing using 'Qiaquick' PCR purification columns (Qiagen), and eluted into 30 μL of distilled water. Insert DNA samples were subject to dideoxy sequencing using the ABI BigDye ready reaction kit (PE Biosystems) and the M13 universal forward (5′-TGTAAAACGACGGCCAGT-3′) and reverse (5′-ACAGGAAACAGCTATGACC-3′) primers. Sequencing reactions were resolved on urea–5% polyacrlamide gels and the gels were analysed on an ABI 377 DNA Sequencer (PE Biosystems). Primers for amplification of the microsatellite loci were designed with the help of primer 3 (Rozen & Skaletzky 1996–1997). Genomic DNA of two individuals each from various geographical locations including Loirston (near Aberdeen, UK), Belfast (UK), Perth (UK), Inverness (UK) and 15 individuals from Bochum (Germany) was isolated as follows: approximately 1 mg of tissue was suspended in 100 μL solution containing 10 mM Tris, 2 mM EDTA, 0.01% Triton X-100 and 20 μg Proteinase K (Sigma). The solution was incubated for 2 h and then heated at 95 °C for 10 min. PCR reactions were carried out in a 10 μL volume containing: 1 μL of extract, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.5 U of Amplitaq Gold DNA polymerase (PE Biosystems), 2.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM of locus-specific fluorescent-labelled forward primer (fluorescent dyes were 6-FAM and HEX) and non-labelled reverse primer (see Table 1). PCR cycling parameters were the following: 10 min at 95 °C, 30 cycles of 95 °C for 30 s, 15 s at 57 °C and 75 s at 72 °C, followed by a final step of 72 °C for 7 min. Fluorescent PCR fragments were separated on a 5% polyacrylamide gel and visualized on an ABI 373 DNA Sequencer.

All five loci were polymorphic and their characteristics are summarized in Table 1. For the Bochum population

**Table 1** Characterization of five microsatellite loci in the tapeworm *Schistocephalus solidus*

| Locus | Primer (5′ to 3′) | PCR product size of the sequenced allele (bp) | Observed allele sizes (bp) | Observed allele numbers | Repeat structure | $H_O$* | GenBank accession no. |
|---|---|---|---|---|---|---|---|
| SsCTA22 | F:TGATCCCAACCCTACTGCTG | | | | | | |
| | R:GCACAAGTCACCGTCCCTG | 152 | 146–160 | 8 | $(CT)_{14}$ | 0.73 | AF247830 |
| SsCAB6 | F:GTTGGTGATGGTCGAGAAAG | | | | | | |
| | R:GAATGTGTGATTTTCAGGGAAC | 120 | 96–130 | 10 | $(CA)_{11}$ | 0.33 | AF247829 |
| SsCTB24 | F:ACGCAGTCCGAGTTATACCG | | | | | | |
| | R:CGGTGGTCTGATTGTGAATG | 188 | 162–196 | 9 | $(CT)_{21}$ | 0.33 | AF247831 |
| SsCA25 | F:CGCAATAAGGTTGGATCGTC | | | | | | |
| | R:TAAATCACGCGCAAGTCATC | 170 | 132–194 | 8 | $(CA)_{19}$ | — | AF247832 |
| SsCA58 | F:GGAGTCAGACATACCGGGTG | | | | | | |
| | R:GAAAAGGTCGTGCTTCAACC | 169 | 143–179 | 8 | $(CA)_{26}$ | 0.2 | AF247833 |

*Observed heterozygosity ($H_O$) is only given for the Bochum population ($n$ = 15).

($n = 15$), observed heterozygosity was low for three loci (SsCTAB6, SsCTB24 and SsCA58). A significant excess of homozygotes, and hence a positive inbreeding coefficient ($f = 0.30$), was found in the 15 individuals of the Bochum population using the AMOVA procedure implemented in Arlequin 3.1 (Schneider *et al*. 1997). This indicates that individuals of this hermaphroditic species exhibit selfing. However, due to the small sample size, it was not possible to test for the presence of null alleles (Van Treuren 1998). No significant linkage disequilibrium could be detected between any locus pair using the software Arlequin 3.1 ($P > 0.04$ in all six comparisons for the Bochum population, Bonferroni correction not applied). Ten individuals of the Bochum population (66%) exhibited three PCR fragments for the locus SsCA25, indicating the presence of a third allele, possibly due to gene duplication. Overall observed heterozygosity in the samples from the UK ranged between 0.88 and 0.62. A third gene copy at the locus SsCA25 was not observed in those worms. We also tested the identified primers in two other cestodes, *Diphyllobothrium latum* and *Taenia solium*. However, PCR amplification was not successful. The high polymorphism of our markers in *S. solidus* suggests that they will be useful for paternity assessment, the estimation of selfing rates and the study of gene flow and population structure.

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# Polymerase chain reaction primers for polymorphic microsatellite loci in the invasive toad species *Bufo marinus*

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Originally from South America, the cane toad (*Bufo marinus*) was deliberately introduced to various Atlantic and Pacific islands, and to Australia, early this century as a biological control (reviewed in Easteal 1981). Because of its well known introduction history and likelihood of strong founder events and demographic flushes, the cane toad provides a good empirical model for studying the population genetics of nonequilibrium systems. Surveys of enzyme variation in the introduced populations in Australia enabled an assessment of some of the genetic consequences of colonization processes (Easteal 1988). However, low level of polymorphism observed from enzyme markers limited resolution. Thus, the development of microsatellite markers in *B. marinus* represents a significant progression in the study of this invasive species.

Two libraries enriched for microsatellites were constructed and screened for $(GT)_n$ repeats following the protocols of Armour *et al*. (1994) and Paetkau (1999). Following the former protocol, 8000 clones were probed, from which 21 positives were picked and sequenced. Eleven of those clones had flanking regions of at least 20 bp and a core sequence of at least 10 repeats, and hence selected for primer design. Following the latter protocol, 400 clones were obtained, 100 sequenced, and 18 selected for primer design. Of the 29 clones selected, preliminary genotyping tests indicated that 17 loci were not suitable as they produced dubious amplification patterns. Two *B. marinus* populations, British Guyana (source population) and Gordonvale (introduced population in Australia), were genotyped with the remaining 12 loci. The primer and core sequences of those loci are provided in Table 1.

Extractions of DNA from individuals (usually a toe section) were performed following Estoup *et al*. (1996). Polymerase chain reactions (PCRs) contained: 1–2.5 mM $MgCl_2$ (see Table 1), 0.17 mM of each dNTP, 1 × PCR buffer [100 mM Tris (pH 8.8)], 1% Triton X-100, 500 mM KCl, 160 μg/mL (BSA), 0.07–0.14 μM of fluorescent labelled primer (Table 1), 0.25 μM of unlabelled primer, and 0.2 units of DyNAzyme DNA polymerase (Finnzymes). PCRs were performed in a GeneAmp PCR System 9700 thermal cycler using the following programme: one cycle of 94 °C for 1 min 45 s; five 'touchdown' cycles: 94 °C for 15 s, 1 °C drop per cycle to a final annealing temperature of 57 °C or 52 °C (Table 1) for 20 s, 72 °C for 10 s; 27 cycles of 94 °C for 15 s, 57 °C or 52 °C for 20 s, 72 °C for 1 s; and a final hold of 72 °C for 2 min. Genotypes were scored using ABI PRISM Genotyper 2.0 software (Perkin Elmer).

All 12 loci were polymorphic with an observed number of alleles per population ranging between one and 10, and heterozygosities between zero and 0.909 (Table 1). Exact tests performed using GENEPOP 3.1 (Raymond & Rousset 1995) with a correction for multiple comparisons (sequential Bonferroni procedure, Rice 1989), detected significant deviation from Hardy–Weinberg equilibrium and linkage disequilibrium for the loci BM102 and BM128. As the sex of the individuals was known for the Gordonvale (Australia) sample, we were able to detect that both these loci were sex linked. This finding