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PRIMER NOTE

Microsatellite loci for the progenetic trematode, *Coitocaecum parvum* (Opecoelidae)

CLÉMENT LAGRUE, JONATHAN M. WATERS, ROBERT POULIN and DEVON B. KEENEY
Department of Zoology, University of Otago, PO Box 56, Dunedin 9015, New Zealand

Abstract

Nine polymorphic microsatellite loci were characterized from the freshwater trematode *Coitocaecum parvum*. This parasite can either reproduce sexually in the definitive host or produce eggs by selfing inside its second intermediate host. Two to 11 alleles per locus were detected in 24 trematode sporocysts and observed heterozygosities ranged from 0.04 to 0.96. These loci will be useful for identifying parasite genetic clones within hosts and testing for effects of relatedness on parasite life history strategy.

Keywords: *Coitocaecum parvum*, enriched library, microsatellite, trematode

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Multi-host life cycles are widely observed in distantly related groups of parasites (Choisy *et al*. 2003). In digenean trematodes, or flukes (phylum Platyhelminthes), the typical life cycle involves three host species. However, several trematode species abbreviate their life cycle by skipping one host (Lefebvre & Poulin 2005). *Coitocaecum parvum* (family Opecoelidae) is an intestinal parasite of the common bully, *Gobiomorphus cotidianus*, and utilizes the snail *Potamopyrgus antipodarum* as first intermediate host (sporocyst stage) and two amphipod species (*Paracalliope fluviatilis* and *Paracorophium lucasi*) as second intermediate hosts (metacercaria stage). Asexual multiplication in the snail host produces clones that infect amphipods. This hermaphroditic freshwater trematode can omit the fish definitive host and produce eggs by selfing (progenesis) inside amphipods (Holton 1984). Because several metacercariae can share the same amphipod, and genetic relatedness may influence parasite life history strategies via kin selection (Frank 1992), it is important to determine if individual parasites within the same host are clones. Here we developed nine microsatellite loci to study the influence of genetic relatedness on the life history strategy of parasites within hosts.

A *C. parvum* subgenomic library enriched for microsatellites was constructed using a modified version of Glenn *et al*. (2000) as described in Keeney & Heist (2003). Snail hosts were collected in Lake Waihola, South Island, New Zealand. Snails were dissected under a microscope; sporocysts were manually separated from snail tissues and rinsed with 0.22-μM filtered water. Genomic *C. parvum* DNA was isolated from 65 sporocysts pooled from two *P. antipodarum* using the QIAGEN DNeasy Tissue kit. DNA was digested with the restriction enzyme *Mbo*I (Promega), made blunt-ended with 1 U of mung bean nuclease (New England Biolabs) per μg DNA, and 5′ dephosphorylated by adding 1 U of calf intestinal alkaline phosphatase (New England Biolabs) to 50 μL of digested DNA. Fragments between 400 bp and 1400 bp were gel extracted and purified using the QIAGEN Gel Extraction Kit. Complementary linkers (linker-F: 5′-CTAAGGCCTTGATCGCAGAAGC-3′ and phosphorylated linker-R: 5′-pGCTTCTGCGATCAAGGCCTTAGAAAA-3′) were ligated to the fragments. Linker-ligated DNA fragments were hybridized to biotinylated $(GT)_{15}$ and $(GA)_{15}$ probes and microsatellite-containing DNA was selectively retained using streptavidin MagneSphere paramagnetic particles (Promega). Retained fragments were amplified with polymerase chain reaction (PCR) using 10 ng microsatellite-enriched DNA, 1 μM linker-F as primer, 2.5 U *Taq* DNA polymerase (Bioline), 200 μM each dNTP, 2 mM $MgCl_2$, and 1× *Taq* buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, 0.01% Tween 20] in a total of 50 μL. Amplification was performed with an Eppendorf Mastercycler gradient thermal cycler and consisted of 4 min at 94 °C, followed by 40 cycles of 45 s at 94 °C, 1 min at 62 °C, and 1 min at 72 °C, and a final 10-min extension at 72 °C. PCR products were digested with *Mbo*I, ligated into a pUC 19 cloning vector (Qbiogene) and used to transform XL1-Blue competent

Correspondence: Clément Lagrue, Fax: +64-3-479-7584; E-mail: lagcl981@student.otago.ac.nz

Table 1 Microsatellite loci developed for *Coitocaecum parvum*. Locus name is followed by repeat motif of cloned allele, primer sequences, primer annealing temperature (T_a), size range of alleles in base pairs (bp), number of alleles, sample size (n), observed (H_O) and expected (H_E) heterozygosities and GenBank Accession no. Significant deviations from Hardy–Weinberg expectations are marked with *($P < 0.05$) and **($P < 0.001$)

Locus	Repeat motif	Primer sequences (5′–3′)	T_a (°C)	Range (bp)	No. alleles	n	H_O (H_E)	Accession no.
Cpa-3	$(AG)_8$	F: GTTTAGTCAGGCACTGTAGC R: GTAAGATACCTTGGACCGATG	60	73–77	3	24	0.96** (0.62)	DQ789050
Cpa-4	$(GT)_9(AT)_4$	F: CAAGACAAACTGAGGACGC R: ATAAGAGCATTGGGAGGGG	62.5	95–99	3	24	0.79* (0.57)	DQ789051
Cpa-8	$(CT)_7(CT)_{20}(GT)_8$	F: CATCGTGCTTGAGATATACTACG R: GTGAGTCGGGCTGGTGAAG	67	112–144	11	24	0.04** (0.90)	DQ789052
Cpa-12	$(GA)_{13}$	F: CATTTTCCAATTCTAACGAGTG R: CCCTATTCCTTTTGACCTCTC	50	131–169	8	24	0.13** (0.86)	DQ789053
Cpa-18	$(AC)_8(AC)_2(AC)_3$	F: GCGTATAAAAGATTAACAAAGGG R: CTCCAATGTGCTTGCTTCC	62	84–86	2	21	0.10 (0.18)	DQ789054
Cpa-19	$(CT)_{20}$	F: GAAACCAGATTGCGTATCC R: GTATCAAGTTTACCGTTACAGAAG	61	76–116	9	24	0.04** (0.82)	DQ789055
Cpa-26	$(AC)_7$	F: GATTACGCAACTCATTCCAG R: CATCAACGCTTTATGTTCC	60	113–127	6	24	0.08** (0.81)	EF088680
Cpa-28	$(GT)_{10}$	F: CCATTTGACATTGAATTGCG R: CATCGTATGAGGGTGAAATACC	55	59–65	2	24	0.04** (0.40)	EF088681
Cpa-29	$(AC)_{10}$	F: GCTTGGAATGAGTGATAACAC R: GTTCCCTATGGTAAGTCAGC	55	62–64	2	24	0.04** (0.40)	EF088682

cells (Stratagene). Individual colonies were transferred to Biodyne B nylon membranes (Pierce) and probed with ^{32}P radiolabelled $(GA)_{12}$ and $(GT)_{12}$. PCR of positive clones was performed as described above using M13 forward and reverse primers and amplified products were sequenced with M13 primers using the BigDye Cycle Sequencing Kit (Applied Biosystems) on an ABI 3730 Genetic Analyser (Applied Biosystems). Primers were designed for repeat-containing sequences using the OLIGO software package (Molecular Biology Insights).

Primer amplification and optimal annealing temperatures were tested by screening six *C. parvum* adult worms isolated from two *G. cotidianus*. PCR amplifications (10 μL) contained approximately 10 ng genomic DNA, 0.6 μM of each primer, 0.5 U *Taq* DNA polymerase (Bioline), 200 μM each dNTP, 3 mM $MgCl_2$ and 1× *Taq* buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, 0.01% Tween 20]. Thermal cycling consisted of 4 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at gradient of 46–66 °C and 45 s at 72 °C, and a final 4-min extension at 72 °C. PCR products were resolved in 9% non-denaturing polyacrylamide gels using cloned fragments as size standards with a 10-bp DNA ladder and visualized by staining gels in 0.1× SYBR Green I (Invitrogen). Amplified loci were tested for polymorphism as described for primer screening using sporocysts from 24 *P. antipodarum*. Polymorphic loci were tested for deviations from Hardy–Weinberg equilibrium allele frequencies and all pairs of loci were tested for linkage disequilibrium using GDA 1.1 (Lewis & Zaykin 2001). Alpha significance levels were adjusted using the sequential Bonferroni approach (Rice 1989) for multiple pairwise tests.

One thousand five hundred and thirty-six colonies were screened for microsatellites and 435 colonies (28%) were positive for GA/GT repeat DNA. One hundred and seventy-one clones were sequenced and 31 primer pairs were designed and tested. Nine polymorphic loci were characterized and possessed two to 11 alleles and observed heterozygosities of 0.04–0.96 (Table 1). Eight out of nine loci deviated significantly from Hardy–Weinberg expectations with more (Cpa-3 and Cpa-4) or fewer (Cpa-8, Cpa-12, Cpa-19, Cpa-26, Cpa-28 and Cpa-29) heterozygotes than expected. Significant heterozygote deficiencies observed at six loci reflect selfing rather than null alleles as all individual sporocysts amplified at these six loci. Selfing is a facultative strategy in this species (Holton 1984; Lefebvre & Poulin 2005) that is expected to cause inbreeding. No locus pairs were in significant disequilibrium after Bonferroni adjustment of alpha. These markers are currently being employed to investigate the effect of genetic relatedness on parasite life history strategy.

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