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PRIMER NOTE

Ten polymorphic microsatellite loci for the trematode *Gymnophallus* sp.

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Abstract

Ten polymorphic loci were isolated and characterized from the intertidal New Zealand trematode *Gymnophallus* sp., a common parasite of the abundant local bivalve *Austrovenus stutchburyi*. Observed heterozygosities ranged from 0.17 to 0.96, and three to 23 alleles were detected in 24 trematode metacercariae. These loci are currently being used to investigate the molecular ecology of this species within the bivalve host.

Keywords: bivalve, *Gymnophallus* sp., microsatellites, parasite, trematode

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Digenetic trematodes are ubiquitous in intertidal communities, and their presence can greatly influence the diversity and functions of these ecosystems (Mouritsen & Poulin 2002). Because of this, it is worthwhile to develop molecular markers to investigate various aspects of their ecology. A trematode recently identified as belonging to the *Gymnophallus* genus (F. Cremonte, personal communication) is commonly found infecting the New Zealand cockle, *Austrovenus stutchburyi*, an abundant bivalve living on the intertidal mudflats of South Island, New Zealand. At certain locations, prevalence of infection by this parasite can approach 100%. It is most likely that *Gymnophallus* sp. has a typical trematode three-host life cycle with alternating stages of asexual and sexual reproduction as it has been reported for other species of *Gymnophallus* (Stunkard & Uzmann 1958; Campbell 1985). Its first intermediate host (asexual sporocyst stage) is currently unknown but is probably the tellinid bivalve *Macomona liliana*, which occur in sympatry with *A. stutchburyi*. Cercariae are released from this host into the environment and infect cockles, the second intermediate host (metacercariae stage). The life cycle is completed when infected cockles are eaten by the definitive host, the oystercatcher *Haematopus ostralegus*, where the worm reaches sexual maturity.

Our protocol is modified from Perrin & Roy (2000). Total genomic *Gymnophallus* sp. DNA was isolated from approximately 6000 metacercariae pooled from 50 *A. stutchburyi* collected from Company Bay, South Island, New Zealand using QIAGEN DNeasy Tissue kit. Genomic DNA was digested using the restriction enzyme *Mbo*I (New England Biolab) and fragments 400–1200 bp in length were ligated to a pUC19 *Bam*HI/BAP cloning vector (Qbiogene). DNA inserts were amplified using the polymerase chain reaction (PCR) with M13 primers. PCR (50 μL) consisted of 5 μL of DNA ligation, 0.6 μM of each M13 primer, 1.25 U *Taq* DNA polymerase (Bioline), 200 μM of each dNTP, 1.5 mM $MgCl_2$ and 1× *Taq* buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, 0.01% Tween 20]. PCR amplification was performed on an Eppendorf Mastercycler gradient thermal cycler and consisted of 5 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C and a final extension for 10 min at 72 °C. PCR products 400–1200 bp in length were annealed to biotinylated $(GA)_{12}$ and $(GT)_{12}$ probes (Invitrogen) (incubation consisted of 5 min at 94 °C, followed by 1 min at 55 °C, and 10 min at 72 °C) and microsatellite-containing DNA was retained using Streptavidin MagneSphere Paramagnetic Particles (Promega). Microsatellite-enriched DNA was purified using a High Pure PCR product purification column (Roche) and 5 μL was used in a PCR with M13 primers as described previously. The microsatellite-containing fragments were digested with *Mbo*I and ligated to pUC19 *Bam*HI/BAP cloning vectors which were used to transform *Escherichia coli* XL1-Blue competent cells (Stratagene). A total of 1376 colonies were picked and grown in 96-well microtitre plates. To screen for microsatellites, colonies were transferred to Biodyne B nylon membranes (Pierce) and probed with γ-^{32}P-dATP radiolabelled $(GA)_{12}$ and $(GT)_{12}$ oligonucleotides.

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Table 1 Microsatellite loci developed for *Gymnophallus* sp. Locus name is followed by repeat motif of cloned alleles, primer sequences, primer annealing temperature (T_a), size range of alleles in base pairs (bp), number of alleles, number of amplified individuals (n), observed (H_O) and expected (H_E) heterozygosities and GenBank Accession no. (Accession no.)

Locus	Repeat motif	Primer sequences (5′–3′)	T_a (°C)	Range (bp)	No. of Alleles	n	H_O (H_E)	Accession no.
G1C10	$(AC)_9$	F: TATCTTCGTTTGTTGATTTGC R: CCGCTGTGTTTAGTGCTAC	52	60–84	7	24	0.38* (0.74)	EF128434
G3A4	$(GT)_8$	F: TGGAGTATTTTTCTAGTCGG R: CTCAAAGCTGTGCATTATTA	49	85–99	5	24	0.71 (0.78)	EF128435
GYM6b	$(CT)_7(GT)_6$	F: CATCACTGTCCGACCAAC R: CTGGCAATCTCATTTGTAAG	49	114–124	6	24	0.75 (0.69)	EF128436
GYM8	$(GT)_8$	F: CCGCACAACAAGCAGACC R: GCTTTGTCTCATTAGAGTGCCC	64	86–94	4	24	0.17 (0.16)	EF128437
GYM9	$(CA)_6$	F: CCCGAATTGATTGTCAGTG R: CCTATGTTGAGTGCTTGTTTG	63	76–80	3	24	0.67 (0.78)	EF128438
GYM11	$(AC)_8$	F: GATTGCAGACAGTCGGCG R: CAAGGTCAAATAGAGTGAAGCTG	64	93–107	6	24	0.71 (0.62)	EF128439
GYM12	$(GT)_6(C)(GT)_6$	F: GTTTTGACAGGCACACCGTTG R: GACAGAATGCCCAGGTGCAG	58	84–107	10	24	0.79 (0.84)	EF128440
GYM14	$(CTCA)_{18}$	F: GACAACGACCATTCACAACG R: GCCTGTATCCCACAAGGG	60	77–135	19	24	0.96 (0.93)	EF128441
GYM15	$(GT)_6(AAC)_6$	F: CACCGGCAGAGCAAGCAAAG R: GTTGTTCACCGTTTCCGTCATTC	58	123–135	7	24	0.36* (0.65)	EF128442
GYM16	$(CATA)_{13}(CATA)_6$	F: GATTCCGCTTCAGTTTCC R: CAACCTATGTAAATCCGAACTC	58	97–211	23	24	0.5* (0.96)	EF128443

*Significant deviation from Hardy–Weinberg equilibrium.

Positive clones were identified on exposed autorad films and amplified with M13 primers as described previously. The PCR products were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and resolved with an ABI PRISM 3730 Genetic Analyser (Applied Biosystems). PCR primers were designed using the OLIGO software package (Molecular Biology Insights).

Successful primer amplification and optimal annealing temperatures were determined using DNA from three individual *Gymnophallus* sp. metacercariae from three *A. stutchburyi*. Individual metacercarial DNA was extracted in 600 μL of 5% Chelex containing 0.1 mg/mL proteinase K, incubated at 60 °C for 4 h and boiled at 100 °C for 8 min. PCRs (10 μL) consisted of 3 μL of DNA extraction (concentration approximately 3 ng/μL), 0.6 μM of each primer, 0.5 U *Taq* DNA polymerase (Bioline), 200 μM of each dNTP, 3 mM $MgCl_2$ and 1× *Taq* buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, 0.01% Tween 20]. PCR amplification was performed on an Eppendorf Mastercycler gradient thermal cycler and consisted of 4 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at a gradient of 44–66 °C and 45 s at 72 °C. PCR products were resolved in 9% nondenaturing 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). Successfully amplified loci were tested for polymorphism using the optimal annealing temperature with DNA from 24 individual *Gymnophallus* sp. metacercariae isolated from seven *A. stutchburyi*. DNA extractions and PCR were performed as described for primer screening. Polymorphic loci were tested for deviation from Hardy–Weinberg equilibrium allele frequencies and all pairs of loci were tested for genotypic disequilibrium using GDA 1.1 (Lewis & Zaykin 2001). Alpha significance levels were adjusted using the sequential Bonferroni approach (Rice 1989).

One hundred and twenty-five colonies were positive for GA or GT microsatellites out of the 1376 colonies screened (9.1%). Of the positive colonies, 79 were sequenced, 17 primer pairs were designed and 10 polymorphic loci were developed. Loci possessed three to 23 alleles and observed heterozygosities ranged from 0.17 to 0.96 (Table 1). After Bonferroni correction, the initial adjusted significance thresholds for Hardy–Weinberg equilibrium and genotypic linkage tests were $P < 0.005$ and $P < 0.001$, respectively. Three loci (G1C10, GYM15 and GYM16) were found to deviate significantly from Hardy–Weinberg expectations with fewer heterozygotes than expected. Genotypic disequilibrium was not found to be significant for any pairs of loci.

These markers are currently being used to detect the presence of multiple copies of the same clonal lineage within individual cockles, assess its implications for intraspecific competition, and answer other questions regarding the molecular ecology of *Gymnophallus* sp.

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References

Campbell D (1985) The life cycle of *Gymnophallus rebecqui* (Digenea: Gymnophallidae) and the response of the bivalve *Abra tenius* to its metacercariae. *Journal of the Marine Biological Association of the United Kingdom*, **65**, 589–601.

Lewis PO, Zaykin K (2001) *GENETIC DATA ANALYSIS: Computer Program for the Analysis of Allelic Data*, Version 1.0 (d16c). Available from URL: http://hydrodictyon.eeb.uconn.edu/people/plewis/software.php (Accessed on 20 March 2007).

Mouritsen KN, Poulin R (2002) Parasitism, community structure and biodiversity in intertidal ecosystems. *Parasitology*, **124**, S101–S117.

Perrin C, Roy MS (2000) Rapid and efficient identification of microsatellite loci from the sea urchin, *Evechinus chloroticus*. *Molecular Ecology*, **9**, 2221–2223.

Rice WR (1989) Analyzing tables of statistical tests. *Evolution*, **43**, 223–225.

Stunkard HW, Uzmann JR (1958) Studies on digenetic trematodes of the genera *Gymnophallus* and *Parvatrema*. *Biological Bulletin*, **115**, 276–302.

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