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PRIMER NOTE

Microsatellite loci from the trematode *Lecithochirium fusiforme*, a parasite of the European conger eel

R. VILAS,*‡ C. D. CRISCIONE,*§ E. PANIAGUA† and M. S. BLOUIN*†
*Department of Zoology, Oregon State University, 3029 Cordley Hall, Corvallis, Oregon 97331, USA, †Laboratorio de Parasitología, Universidad de Santiago de Compostela, Facultad de Farmacia, 15782 Santiago de Compostela, Spain

Abstract

Seven polymorphic loci containing dinucleotide repeats and one trinucleotide microsatellite were developed for the hemiurid fluke *Lecithochirium fusiforme*, a parasite of the European conger eel *Conger conger*. All parasites that were collected from a single individual host (a total of 54 specimens) were genotyped. The number of alleles ranged from two to eight. The observed and expected heterozygosities ranged from 0.057 to 0.736 and from 0.091 to 0.794, respectively. Hardy–Weinberg deviations were statistically significant for two loci. These markers will be useful for study of parasite transmission patterns and population genetic structure.

Keywords: *Conger conger*, *Lecithochirium fusiforme*, microsatellites, parasites, trematode

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The hemiurid fluke *Lecithochirium fusiforme* is a common parasite of the European conger eel *Conger conger* from north of the Iberian Peninsula. The life cycle of this worm is complex and involves three or four marine hosts. The parasite matures and sexually reproduces in the stomach of the eel. The eggs are released with the faeces and they may be ingested by the first intermediate host (snails of the genus *Gibbula*). Larvae emerging from the snail can infect copepods (second intermediate host). Conger eels become infected upon either ingestion of infected copepods or by feeding on small fishes which previously ingested infected crustaceans (Gibson & Bray 1986). These fish (paratenic hosts) increase the chance of the parasite reaching the definitive host because numerous immature worms can spend a relatively long time encapsulated in their tissues. Paratenic hosts could contribute to increasing the levels of genetic variability within adult infrapopulations (parasites within a single individual eel) because conger eels in the study area are quite sedentary, and both snails and copepods constitute very structured populations. Given the usual diet of the conger eel, infection via paratenic hosts seems very likely (O'Sullivan *et al*. 2004). Vilas *et al*. (2003) studied some potential implications of the presence of this special kind of host on the population genetic structure of *L. fusiforme* by using allozymes as molecular markers. However, allozymes have important limitations in the study of this system because of the relatively small size of the worms (alleles from only two or three different polymorphic loci could be scored in the same specimen), and low variation. More variable markers such as microsatellites will be useful for investigating the influence of a complex life cycle on trematode population genetic structure in a marine environment.

Genomic DNA was extracted from a single fluke collected from a conger eel captured in Ría de Arousa (Galicia, NW Spain), using a standard phenol-chloroform procedure (Sambrook *et al*. 1989). We isolated microsatellites by following the protocol of Criscione & Blouin (2005), which was based in the method published by Cabe & Marshall (2001). A degenerate oligonucleotide-primed-polymerase chain reaction (DOP-PCR) was used to produce genomic fragments with suitable size for cloning (200–2000 bp). Cycling and conditions of the PCR were as in Cabe & Marshall (2001). The DOP-PCR product was enriched with a 5′-biotinylated $(CA)_{12}$ probe. A 3′-inverted dT was added

Correspondence: Román Vilas. Fax: +34 982258641; E-mail: vilasr@usc.es
‡Current address: Departamento de Genética, Universidad de Santiago de Compostela, Facultad de Veterinaria, 27002 Lugo, Spain
§Current address: Department of Genetics, Southwest Foundation for Biomedical Research, PO Box 760549 San Antonio, Texas 78245, USA

to the probe in order to avoid possible amplification artefacts in the enrichment (Koblížková *et al*. 1998). Hybridization of the probe to the genomic fragments and capture with streptavidin-coated magnetic particles (Promega) followed Criscione & Blouin (2005) except that a single enrichment was done. PCR fragments were cloned using Invitrogen's TOPO TA Cloning Kit. Screening of colonies containing potential repeats was as in Cabe & Marshall (2001). A total of 24 positive colonies were isolated (8% of the screened clones). Of these, 18 colonies were sequenced. No PCR artefacts were observed and all sequences contained at least one microsatellite locus. A clone had two closely linked microsatellite loci (*LFU14A5A* and *LFU14A5B*); one of them is a trinucleotide repeat that is linked to the most polymorphic locus, a perfect CA microsatellite (Table 1). Because the two loci are separated by just 262 bp, one would not normally use both in studies of population genetic structure. However, we include both loci in the identification of potential genetically identical parasites that can result from asexual amplification. After removing replicates and clones with short flanking sequences, primers for 13 different loci were designed using the software OLIGOTECH 1.0 (http://www.oligosetc.com/analysis.php). The eight loci (7 clones) that were found to be polymorphic in a prescreening (an analysis of four parasites from different hosts) were scored for a set of 54 specimens of *L. fusiforme* which constituted a whole infrapopulation. Parasites were identified following Gibson & Bray (1986). The specific identification was confirmed by examination of permanent preparations. Because this trematode is a hermaphroditic endoparasite and only adult specimens were analysed, there is some chance of both contamination from outcrossing matings and host tissue. In order to avoid potential contamination from fertilized eggs in the uterus and stored sperm in the seminal receptacle (anterior to the ventral sucker), solely the posterior end was used for extractions. Moreover, we set control reactions using extracted DNA from *C. conger*. These fish controls were negative. Worm tissue of each individual was placed in 200 µL of 5% Chelex containing 0.2 mg/mL of proteinase K, incubated for 2 h at 56 °C, and boiled at 100 °C. PCR amplification was performed in a 25 µL vol. containing 4 µL of extraction supernatant, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer and 0.5 U *Taq* DNA polymerase (Promega). Amplification was carried out in an MJ Research PTC-200 thermocycler programmed for: 3 min at 95 °C, followed by 35 cycles of 45 s at 94 °C, 30 s at the annealing temperature (Table 1) and 45 s at 72 °C. This was followed by a further 7 min at 72 °C. PCR products were run on ABI PRISM 3100 Genetic Analyser using GENESCAN software and scored with GENOTYPER (Applied Biosystems). LIZ500 size standard was used for all loci.

Allelic diversity and average observed and expected heterozygosity were computed with GENETIX version 4.04 (Belkhir *et al*. 2004). GENEPOP version 3.4 (Raymond & Rousset 1995) was used to test Hardy–Weinberg equilibrium (HWE) and genotypic linkage disequilibrium. We found two individuals with the same multilocus genotype that might be result of clonal reproduction. Assuming diploidy and random mating, the probability of this observation

Table 1 Microsatellite primer pairs for *Lecithochirium fusiforme*

Locus	Primer sequence (5′–3′)	T_a (°C)	Repeat motif	Clone size (bp)	N_A	H_O	H_E	HWE *P*-value
LFU18B10	^{P}CTGAGCTTACCTCGATTAT TCCGCGTCTGAAAATTGAA	54	$(CA)_7$	165	2	0.358	0.360	1.000
LFU18A5	^{N}AGTTGCCTCACCATTCTC TGCGGTTGGTAGCTTGAT	54	$(CA)_7$	223	3	0.094	0.091	1.000
LFU20D1	^{V}TTTCCGGAGTCAATTGCTA CTTTCAAAGAGTACTGCCA	61	$(CA)_6$	143	4	0.528	0.676	0.096
LFU14F11	^{F}GGTTACACATACTTGGAC GCGTGTATAAACTCAATTAC	55	$(GC)_3(CA)_5$-CC-$(CA)_2$	115	3	0.057	0.091	0.008
LFU18B8	^{P}TTCATGGAGCTTGCAGAA ACTGAAAGATGTCAAGTTG	54	$(CA)_4$	133	4	0.340	0.349	0.512
LFU18E2	^{N}GGCTGTTGTTGTCTTCCA TCCAGAAAGTGAAACAAGCT	60	$(CA)_9(TA)_7$	173	4	0.641	0.669	0.638
LFU14A5A	^{N}GATAACTTACTTGACCTCAG GGAGATTAGCCAAGCGAT	54	$(CA)_{11}$	190	8	0.736	0.794	0.037
LFU14A5B	^{V}AACTGGATGCCCCTGATT AGATGTTTTGCAACGAGAGT	55	$(TGC)_6(TGT)_4$	116	5	0.566	0.508	0.901

T_a, annealing temperature; N_A, total number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; HWE, test for Hardy–Weinberg equilibrium. ^{P,N,V,F} refer to the 5′ fluorescent-labelled primer 6-FAM, PET, NED, VIC. GenBank Accesion nos DQ413187–DQ413193.

and its statistical significance value were calculated using MLGSIM (Stenberg *et al*. 2003). Results confirmed that it is very likely those individuals are clones ($P < 0.001$). After removing one of the clones from the infrapopulation, only loci *LFU14F11 (*$P < 0.01$) and *LFU14A5A* ($P < 0.05$) showed deviation from HWE (Table 1). A total of three pairwise comparisons showed a significant genotypic disequilibrium ($P < 0.05$). Two loci in HWE (*LFU18B8* and *LFU14A5B*) showed significant genotypic association ($P < 0.01$). After Bonferroni correction over the eight loci, HWE deviations observed were not statistically significant. After correction for multiple tests, genotypic linkage was not significant for any pair, including the two closely linked loci (*LFU14A5A* and *LFU14A5B*). The adjusted significance threshold for HWE and genotypic linkage tests were $P < 0.006$ and $P < 0.002$, respectively. Low frequency null alleles may explain the deficiency of heterozygotes detected at some loci. However, given the complexity of the life cycle, we cannot rule out other possible locus-specific causes for the deviation observed such as the Wahlund effect. Although microsatellites reported showed a relatively low variation they will be useful in fine-scale population genetic structure studies of *L. fusiforme*.

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