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Microsatellite marker development in the protozoan parasite *Perkinsus olseni*

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ABSTRACT: The analysis of an enriched partial genomic library and of public expressed sequence tag (EST) resources allowed the characterization of the first microsatellite loci in the protozoan parasite *Perkinsus olseni*. Clonal cultures from laboratory isolates derived from infected clams *Ruditapes decussatus* (from Spain), *R. philippinarum* (from Spain and Japan), and *Austrovenus stutchburyi* (from New Zealand) were used for the characterization of 12 microsatellites. Low variation was detected at most loci, with the number of alleles at polymorphic loci ranging from 2 to 7 (average 3.20 ± 0.51) and gene diversity from 0.11 to 0.79 (average 0.40 ± 0.07). Preliminary results show that (1) isolates of *P. olseni* are diploid cells, and (2) multiple infections can occur within a single host. Eight of the loci analyzed successfully cross-amplified in the congeneric species *P. mediterraneus*. These microsatellite markers will be useful to analyze in detail the population genetic structure of *P. olseni*, crucial for the efficient management of this parasitic disease.

KEY WORDS: Microsatellites · *Perkinsus olseni* · Genetic diversity · Clams · Diploidy · Protozoan · Parasite

Perkinsus olseni is a protozoan parasite with a genome size around 28 megabases (Mb) organized in 9 chromosomes (Teles-Grilo et al. 2007) that has been blamed for high mortalities of venerid clams in Portugal, Spain, and Korea (Azevedo 1989, Santmartí et al. 1995, Park et al. 1999). The species was originally described parasitizing abalones *Haliotis ruber*, though its host range also includes several species of clams and oysters. This parasite has been reported in a broad geographic range, including Oceania (Australia and New Zealand), East Asia (Korea, Japan, China, and Thailand), South America (Uruguay), and Europe (Portugal, Spain, France, and Italy). The first occurrence of the parasite in Italy, Portugal, and Spain was reported in the 1980s. *P. olseni* most likely originated in the Pacific Ocean and was introduced into Europe and South America through Manila clam *Ruditapes philippinarum* transfers (Hine 2001, Villalba et al. 2004).

Microsatellite loci have been characterized in few species of protozoan, mainly human parasites belonging to the genera *Plasmodium* (Karunaweera et al. 2007), *Trypanosoma* (Balmer et al. 2006), *Leishmania* (Rougeron et al. 2010), *Sarcoystis* (Asmundsson et al. 2006), *Toxoplasma* (Ajzenberg et al. 2002), and *Cryptosporidium* (Feng et al. 2000). These markers have proved to be useful in the genetic characterization of parasite populations, which has led to a more efficient management of the disease. For example, microsatellites of *P. falciparum* have been use to identify virulence genes, the detection of natural selection of

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phenotypes associated with resistance to drugs, and the demonstration of a variety of population genetic structures with important epidemiological implications (Su et al. 2007).

Here we report the isolation and characterization of microsatellite loci for the first time in *Perkinsus olseni*. Our goal was to develop a set of highly variable genetic markers for analyzing population structure and life cycle of this parasite in future work. For this, we followed 2 different approaches. First, a microsatellite-enriched genomic library from *P. olseni* was developed using the relatively quick and inexpensive FIASCO protocol (Zane et al. 2002). Total genomic DNA from 10^7 cells of a culture derived from a highly infected clam *Ruditapes decussatus* was extracted using the DNAzol reagent® (Invitrogen Life Technologies™) according to the manufacturers' instructions, and 250 ng DNA were simultaneously digested with *Mse*I and ligated to *Mse*I amplified fragment length polymorphism (AFLP) adaptor. A single biotinylated probe containing an $(AC)_{17}$ repeat motif, coupled with magnetic bead separation technology, constituted the basis of the enrichment protocol. PCR products above 200 bp were ligated into pGEM-T Easy Vector (Promega) and transformed into JM109 high efficiency competent cells (Promega) as described by Pardo et al. (2006). A total of 576 white transformant clones were picked and re-grown overnight into microtiter plates with 120 µl of freezing medium (final concentration: 36.0 mM K_2HPO_4, 13.2 mM KH_2PO_4, 0.4 mM $MgSO_4$, 1.7 mM Na_3-citrate, 6.8 mM $(NH_4)_2SO_4$, 4.4% [v/v] glycerol) plus ampicillin at 37°C. Plasmids were purified using the DirectPrep 96 Miniprep kit (Qiagen) and sequenced following the ABI Prism BigDye™ Terminator v3.1 Cycle Sequencing Kit protocol in an ABI 3730xl sequencer (Applied Biosystems). In a second approach, we screened the GenBank EST resources of this species (98 sequences; Ascenso et al. 2009) to track microsatellites motifs. The microsatellite search was conducted using the program Sputnik (http://espressosoftware.com/sputnik/index.html). All sequences were clustered to avoid redundancy using CAP3 (http://seq.cs.iastate.edu/) with default parameters.

A total of 237 out of 429 good-quality sequences (>100 bp; PHRED score > 20) from the enriched library contained short tandem repeats, and 198 were unique. A total of 11 tandem repeat motifs were detected in 98 expressed sequence tag (EST) sequences from GenBank, which clustered in 4 unique sequences. PCR primers flanking microsatellites that contained ≥8 repeats in dinucleotide and ≥4 repeats in tri- and tetranucleotide motifs were designed using the online primer design software Primer3 (http://frodo.wi.mit.edu/primer3/primer3_code. html). Twenty-six primer pairs could be designed from the enriched library, and 8 of them successfully amplified the target regions. All primers designed within the 4 unique EST sequences correctly amplified the targets. The lack of amplification of some primer pairs from selected sequences is a common observation in these types of studies and has been related with the low quality of sequences and/or inappropriate primer design (Pardo et al. 2007). Annotation of the 12 loci was performed using BLASTX against NCBI 'nr' (nonredundant protein database) database, which searches proteins using a translated nucleotide query (http:// blast.ncbi.nlm.nih.gov/Blast.cgi?). Homology expectation (*E*)-values (see http://www.ncbi.nlm.nih.gov/BLAST/tutorial/Attschul-1.html#head2 for specific definition) $<10^{-5}$ were considered for gene annotation. Five of the 12 screened loci showed *E*-values $<10^{-5}$ (Table 1).

Polymorphism at each locus was determined in parasites isolated from a variety of hosts species obtained from different localities: carpet shell clams *Ruditapes decussatus* from Ría de Arousa, Ría de Pontevedra (both bays in Galicia, NW Spain) and Río Carreras (Huelva, SW Spain); Manila clams *R. philippinarum* from Delta de l'Ebre (Catalonia, NE Spain) and Japan, and clams *Austrovenus stutchburyi* from New Zealand. *In vitro Perkinsus olseni* cultures were established from the gills of one clam from each Iberian Peninsula site (Ría de Arousa, Ría de Pontevedra, Río Carreras, and Delta de l'Ebre) using the culture medium JL-ODRP-2A (Casas et al. 2002a). The PCR procedure described by Casas et al. (2002b) was used to confirm that the cultured cells corresponded to *P. olseni* by analysing ca. 5×10^6 cells from each culture.

Table 1. BLAST search information about microsatellite-containing sequences. *E*-value: expectation value

Locus	Accession no. hit	*E*-value	Information
Pol-USC7	EER19832.1	8×10^{-6}	Hypothetical protein Pmar-PMAR006724 [*Perkinsus marinus* ATCC 50983]
Pol-USC8	EER08584.1	2×10^{-21}	Hypothetical protein Pmar-PMAR017637 [*Perkinsus marinus* ATCC 50983]
Pol-USC9	EER16958.1	1×10^{-15}	Serine/threonine-protein kinase rio1, putative [*Perkinsus marinus* ATCC 50983]
Pol-USC11	BAG85335.1	9×10^{-61}	Adhesion related protein[*Perkinsus olseni*]
Pol-USC12	EER04606.1	6×10^{-45}	Hexokinase-7, putative [*Perkinsus marinus* ATCC 50983]

The *in vitro* cultures were cloned by limiting dilution plating in 96-well culture plates, and 3 monoclonal derivatives of each isolate culture were expanded. Thus, 3 clonal cultures were produced per host from each location on the Iberian Peninsula, allowing investigation into whether infections with multiple strains of *P. olseni* could occur in one individual host. Additionally, 3 *P. olseni* clonal cultures derived from 3 different *R. philippinarum* clams from Japan and 3 clonal cultures deriving from 3 *A. stutchburyi* clams from New Zealand were provided by C. F. Dungan (Maryland Department of Natural Resources, Cooperative Oxford Laboratory). This rendered a total of 18 clonal cultures in the study to estimate genetic diversity. DNA from 10^7 cells of each clonal culture was isolated with DNeasy Blood & Tissue kit (Qiagen). PCR was carried out in a total volume of 15 µl containing approximately 30 ng of DNA, 1 µM of each primer (the forward was labelled), 0.1 mM of dATP, dTTP, dCTP, dGTP, 1.5× PCR Gold Buffer, 1.5 mM $MgCl_2$, and 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR was performed in a PTC-200 thermal cycler (MJ Research) as follows: an initial denaturation at 95°C for 10 min followed by 35 cycles at 94°C for denaturation during 45 s, 50 s at the specific annealing temperature (Table 1), and 50 s at 72°C for extension. A final extension step was performed at 72°C for 10 min. An initial assessment of microsatellite amplification efficiency and polymorphism was conducted in a subsample of 5 clonal cultures by doing the PCR with unlabelled primers and running the product on 2.5% agarose gels stained with ethidium bromide and visualized under UV light. Those microsatellites which produced bands of expected size were further analyzed on an ABI 3730xl automatic sequencer (Applied Biosystems), the forward primer of each pair being 5′ fluorescently labelled (Applied Biosystems) (detailed in Table 2). Genotyping was

Table 2. Characteristics of the 12 novel microsatellite loci in 18 clonal cultures of *Perkinsus olseni* and cross-amplification in 6 clonal cultures of *P. mediterraneus*. *T*: temperature; EST: expressed sequence tag; –: no amplification

Locus	GenBank no.	Primer sequence (5′–3′)	Repeat unit	Origin	*T* (°C)	Size range (bp)	Primer-label	No. of alleles	Gene diversity	*P. mediterraneus* size range (bp)
Pol-USC1	GU460358	CGAGCAGCAACTACAACAGC ACGATGTCAAACGCAGACAA	$(TAC)_6$	Enriched library	58	99–102	6-FAM	3	0.29	64–73
Pol-USC2	GU460359	TTTGCCGCGTCTTAGGTACT ACCAGCGAAAGACAGACACA	$(GT)_{28}$	Enriched library	57	95	NED	1		–
Pol-USC3	GU460360	TTGAACGGTACACAGCGAAA GCCTTCCCTATAACTTCCAACC	$(GT)_{38}$	Enriched library	58	224–244	NED	3	0.33	273
Pol-USC4	GU460361	GAGGGAGGTGGTGAAGATGA GCTGCTGCTCGTCTGTAATG	$(ATG)_{11}$	Enriched library	58	98–104	VIC	3	0.48	98
Pol-USC5	GU460362	TAGGCTGATCGTCGGGTATC AACAGCCACAACATCACCAC	$(GTT)_{25}$	Enriched library	58	177–248	PET	2	0.29	219
Pol-USC6	GU460363	ACAAGCCTACCCACCGTGTT CTGCATCAGTCCCTCGCTAT	$(GT)_{22}$	Enriched library	55	264–275	VIC	2	0.11	–
Pol-USC7	GU460364	AACACGCAGTAGCCCAAGAG TGGCATAAAGAAAGGAGGTGA	$(CTT)_5$, $(GTT)_{14}$, $(CAG)_{13}$	Enriched library	58	432–454	6-FAM	7	0.79	–
Pol-USC8	GU460365	AGTTCCTCCTGCTCCGTACA GCTGATGCCTCTCAAGTGC	$(GCA)_{16}$	Enriched library	58	271–288	PET	5	0.75	282
Pol-USC9	GU460366	ACCCAGCTCCTCTTCCATCT ACAGCATTGCATCATCCTCA	$(TTA)_4$, $(CTG)_4$, $(TCA)_6$	EST database	58	312	6-FAM	1		–
Pol- USC10	GU460367	CTCTCGGTAATGGCGTCAAC CAGGCACTTCGTCAGCAAA	$(GAT)_4$	EST database	59	214–225	VIC	2	0.29	225–237
Pol- USC11	GU460368	GCTTCTCGAATGCTCTACGC CGAGTCCTTACCCGTCCAGT	$(GCC)_5$	EST database	58	281–290	NED	3	0.51	287
Pol- USC12	GU460369	ACCGTTATCCTCAGCGTTTG GCCGAGGTACTCAACTAGGG	$(GTG)_4$	EST database	57	237–240	PET	2	0.16	237

carried out using the GeneMapper 4.0 software (Applied Biosystems), with GenScan 500LIZ as the internal size standard. Non-amplification of these microsatellites was observed in non-infected hosts used as negative controls.

Estimates of genetic diversity (allele number and gene diversity; Nei 1987) were obtained with the program Genetix version 4.05 (Belkhir 2004) using the 18 clonal cultures (Table 2). Amplification was observed at all clonal cultures from the Iberian Peninsula but failed in 4 (Pol-USC2, Pol-USC4, Pol-USC6, and Pol-USC7) and 1 (Pol-USC6) loci at the clonal cultures from Japan and New Zealand, respectively. Polymorphisms were observed among the samples analyzed for this study at 10 of the 12 microsatellite loci. Two loci were monomorphic for these samples, one locus derived from the enriched genomic library and the other one from the EST database. The number of alleles of polymorphic loci ranged from 2 at several microsatellites to 7 at Pol-USC7 (average 3.20 ± 0.51). Gene diversity ranged from 0.11 (Pol-USC6) to 0.79 (Pol-USC7) (average 0.40 ± 0.07). Private alleles were observed in clonal cultures from Japan (6) and New Zealand (7). The presence of heterozygotes at *Perkinsus olseni* clonal cultures indicates that cells of this parasite are diploid and suggests that sexual reproduction could be occurring in its life cycle. These results are preliminary due to the sample size managed and will need to be confirmed in further investigations. Also, most individual hosts from the Iberian Peninsula (75%) showed different genotypes in at least 2 clonal cultures, suggesting that isolate cultures initiated from a single infected clam may comprise more than one strain of *P. olseni*. These observations are in accordance with those by Reece et al. (2001) in the congeneric species *P. marinus* and showed the utility of these microsatellites to detect multiple parasite infections in individual mollusks. All polymorphic loci showed strong and significant departures from Hardy-Weinberg expectations due to heterozygote deficit. Deviations may be the result of the Wahlund effect, which is likely due to the large geographic range of the sample, and/or of inbreeding. Deviations detected at all loci do not support an explanation based on null alleles. We also evaluated the cross-amplification of these loci in 5 clonal cultures of *P. mediterraneus* deriving from infected European flat oyster *Ostrea edulis* from Maó (Menorca, Balearic Islands, Spain). Isolate cultures were established from oyster hearts, and clonal cultures were produced as described for *P. olseni*. The ranges of $MgCl_2$ (1.5 to 2 mM) and temperature (48 to 60°C) were considered for PCR optimization. Eight out of the 12 loci tested were successfully amplified in *P. mediterraneus*, but only 2 showed size range differences with *P. olseni*, thus limiting their use as diagnostic interspecific markers (Table 2). The markers developed in the present work will be useful for analyzing in detail the population genetic structure of *P. olseni*. This knowledge is crucial for the efficient management of the parasitic disease.

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