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MICROSATELLITE LETTERS

Development and characterization of microsatellite loci in the entocommensal *Malacobdella arrokeana* (Nemertea: Bdellonemertea), from Patagonia (Argentina) and cross-amplification in 34 nemertean species

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Abstract Microsatellite loci (26 in total) were isolated for the first time for the entocommensal nemertean *Malacobdella arrokeana*, using 454 GS-FLX Titanium pyrosequencing. We developed conditions for amplifying these markers in 8 multiplex and 5 individual reactions. One to 14 alleles were detected per locus across 25 samples analyzed from San Matías Gulf, Patagonia (Argentina). For the 25 polymorphic loci, observed and expected heterozygosities ranged from 0.125 to 0.920 and 0.119 to 0.890, respectively; five loci deviated from Hardy–Weinberg equilibrium. Limited distribution and host specificity of *M. arrokeana*, which only inhabits *Panopea abbreviata*, have endangered this nemertean given the unregulated commercial exploitation of its host. These useful markers provide data for future conservation strategies. Cross-amplifications were also tested in 34 nemerteans species, representing the major lineages in this phylum. In total, 18 of the 26 loci (from one to 11 per species) gave clear allelic profiles.

Keywords Nemertean · Population structure · Patagonia · 454 pyrosequencing

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The family Malacobdellidae (Nemertea: Bdellonemertea) comprises a single genus, *Malacobdella*, with 6 species, all of which inhabit the mantle cavity of bivalves (Ivanov et al. 2002). *Malacobdella arrokeana* Ivanov et al. (2002) lives commensally within the mantle cavity of the geoduck *Panopea abbreviata* (Valenciennes, 1839). It is the only *Malacobdella* species reported in the southern Atlantic Ocean, and indeed, the entire southern hemisphere. Previous work analyzing two mitochondrial (COI, 16S) and one nuclear (ITS) DNA marker in *M. arrokeana* showed no genetic structure between four nemertean populations, which were mainly distributed in separate, well-characterized gulfs of northern Patagonia (Alfaya et al. 2013). Contrasting hypotheses were raised to explain this lack of genetic structure (such as larval dispersion, selection pressure and lack of molecular marker resolution). Therefore, to validate these hypotheses and provide conservation guidelines for this species, more information related to its population genetic structure is needed. Hence, for the first time in nemerteans, we have isolated microsatellite markers for *M. arrokeana* using 454 GS-FLX Titanium pyrosequencing.

Malacobdella arrokeana specimens were collected inside *P. abbreviata* clams inhabiting the San Matías Gulf (41°37.5′S, 65°1.25′W), northern Patagonia. DNA was extracted from tissue using the BioSprint 15 DNA Blood Kit (Qiagen), according to manufacturer's protocol. Genomic libraries were constructed at the Cornell Evolutionary Genetics Core Facility (EGCF, USA) using the same biotinylated repeat probes and conditions as described in López-Márquez et al. (2013) for *P. abbreviata*.

A total of 17,273 contigs (from 28,922 sequences) were obtained and analyzed with MSATCOMMANDER (Faircloth 2008), which searched for simple repeats with a minimum of 8 perfect repeats for di- (809 contigs) and

Table 1 Characterization of 26 microsatellite loci in *Malacobdella arrokeana*

Locus name	Primer sequences 5′–3′	Repeat motif	Clone size	Alleles range	PCR reaction	Na	Ho	He	NAF	GenBank Access. number
Ma3	F: 6FAM-AGTCCAATTGTGTAGTAGAT R: AATTCTTTTAGGAAATTAAC	$(CT)_{14}$ CC $(CT)_4$	249	251–271	1	7	0.520	0.710	0.111	KF434343
Ma6	F: PET-TATGCTGTGAAAAATATGGGCT R: TGTTTTCTAACATTTTGAATTCCTG	$(GGTA)_8$	153	147–179	2	6	0.920	0.708	0	KF434344
Ma7	F: VIC-CAACCTCGTTGAAAAATTCTA R: TCGGGGTATTTAAAAGGTCTTC	$(AATG)_8$	127	118–158	3	10	0.333	0.706[a]	0.218	KF434345
Ma8	F: 6FAM-ACCAAATTATGATTGGATG R: AAAATTACAATCTAACGCAG	$(AGTT)_9$	189	188–220	4	6	0.640	0.746	0.060	KF434346
Ma12	F: NED-TCGCTAGCGCCACAATTC R: AAACCTAATCACAATGACTACAATGG	$(ACT)_{11}$	115	111–129	5	7	0.680	0.672	0	KF434347
Ma15	F: PET-AGTTCCACGTGTAAGACTC R: TGACTACGGTACACCAAA	$(TGTC)_6$	130	139–143	6	2	0.250	0.330	0.060	KF434348
Ma16	F: VIC-GACAACCTAACGATGTATTC R: GGATTCATCACCTCACAG	$(GAT)_{10}$	121	112–148	7	6	0.320	0.350	0.022	KF434349
Ma17	F: PET-TAAGTTGATTCGTCTTTCTC R: GATGACGTAAATAATATCAGAG	$(CAT)_{15}$	282	260–302	8	10	0.640	0.726	0.050	KF434350
Ma18	F: VIC-ACATCACTGTCAGGATTTAC R: GAATATTCAAGGGAATCAC	$(TAC)_{11}$	317	317–341	8	7	0.680	0.741	0.035	KF434351
Ma20	F: PET-TACTCTCGTATTCCAGACC R: GACGAGTAATAAGTGTTTGC	$(ACG)_7$	352	360	5	1	0	0	0	KF434352
Ma21	F: NED-ATGTCTAATAAAACGCAATC R: GTTACTCACCGTCGTATCT	$(GGA)_9$	281	263–293	9	4	0.280	0.252	0	KF434353
Ma22	F: 6FAM-ACTTTGCTAAGGAAATGTTA R: GATAAAGTAACATCGTCGAA	$(CAT)_8$ CGTAAC $(CAT)_6$	199	205–211	3	3	0.320	0.274	0	KF434354
Ma24	F: 6FAM-CATCAGCTTACCGCAGAC R: ATTCAGACACGCTGGATT	$(AAT)_4$ TAT $(AAT)_7$	153	158–173	10	3	0.125	0.119	0	KF434355
Ma27	F: PET-GTTTAAAGAAACAATAAATGC R: TTGACTACGTGTGTATATGG	$(GTAGT)_{11}$ TTAGN $(GTAGT)_3$	151	113–166	11	14	0.792	0.890	0.052	KF434356
Ma28	F: 6FAM-ATTATAGTCCCTCCGAGAC R: GTAGGATTGATTCTTTCAGA	$(TAC)_{11}$ T $(TAC)_4$ $(CAC)_2$ $(TAC)_{15}$	166	99–228	5	13	0.720	0.882[a]	0.086	KF434357
Ma29	F: PET-AATGCATCATATTTCACAC R: CTTCGTTTGTTCACTAGAAT	(CCA) $(CTA)_2$ $(CCA)_2$ $(CTA)_2$ $(CCA)_8$	179	171–195	7	9	0.440	0.737[a]	0.171	KF434358
Ma30	F: 6FAM-GACTGGACTTTCTCCATT R: ACTTCCTATGAACAGTAACG	$(CTA)_2$ CAA $(CTA)_5$ CCA $(CTA)_8$	163	151–196	6	13	0.458	0.873[a]	0.221	KF434359
Ma33	F: VIC-CATATTTTCCATCCAACTAA R: TAATCTACAAAGACGGACAT	$(ACT)_{10}$	165	172–181	1	4	0.583	0.520	0	KF434360
Ma34	F: PET-GAGTCACCTCTTCGATAAC R: TCATACTGTTTTATTCGACTC	$(AC)_{11}$	221	224–230	1	4	0.800	0.685	0	KF434361

Table 1 continued

Locus name	Primer sequences 5′–3′	Repeat motif	Clone size	Alleles range	PCR reaction	Na	Ho	He	NAF	GenBank Access. number
Ma35	F: NED-TATTCTTTCTTGATTCTTGG R: AGTATATCGTCTCCACTGC	$(TC)_{10}$	236	233–251	12	5	0.625	0.707	0.048	KF434362
Ma36	F: VIC-CACAAATCCTACTCAAGGT R: CAAATATGATTTTTGTCTGC	$(TA)_{10}$	101	100–106	2	4	0.560	0.678	0.070	KF434363
Ma38	F: VIC-GATAACAGCATTAGAAGGAC R: AAGGAAAGAGGGAATTAGTA	(TA) TAA $(TA)_9$	239	227–249	11	6	0.560	0.506	0	KF434364
Ma39	F: PET-CCAAGGAGGCTATATGACT R: ACTATTCGTCTGTCTGGTCCA	$(AG)_{10}$	178	178–190	3	5	0.680	0.658	0	KF434365
Ma41	F: NED-GGAATTGTAAATCATTCGC R: CACACTCTGAATTGGTGAG	$(AG)_9$	362	366–374	1	6	0.458	0.702	0.143	KF434366
Ma42	F: 6FAM-ACGTGTTTCAATGAGAAAT R: AAAGATGCTCATTGTCTATC	$(GT)_{14}$	214	206–220	7	6	0.360	0.695[a]	0.198	KF434367
Ma44	F: NED-AGCTATAATATCCAGTGAAGA R: GAATTGCCGAGTATAAAGT	$(AC)_{10}$	108	98–112	11	6	0.480	0.572	0.058	KF434368

Primer sequences, repeat motifs, clone size (base pairs, bp, without pig-tail), alleles size range (bp). To facilitate genotyping, reverse primers were pig-tailed with 5′-GTTTCTT-3′. PCR reaction indicates multiplex (loci with the same number in this column were amplified and genotyped together) or individual reactions. GenBank accession numbers for each microsatellite

F Forward, *R* Reverse, *Na* number of alleles, *Ho* observed heterozygosity, *He* expected heterozygosity, *NAF* Null allele frequency (Brookfield method)

[a] Significant deviation from Hardy–Weinberg equilibrium

trinucleotides (1678) and 6 repeats for tetra- (104) and pentanucleotides (46), focusing on tri- and tetra-repeat motifs. Of these contigs, 50 were chosen for primer design. Twenty-six of these microsatellites markers produced clear electropherogram patterns and were selected for multiplex PCR optimization and genotyping, performed using eight specimens from five different populations.

Multiplex PCRs were performed in a total volume of 10 µl, with between 1 and 4 ng of DNA and 1X Qiagen Multiplex PCR Master Mix (Qiagen), using conditions previously determined for *P. abbreviata* (López-Márquez et al. 2013).

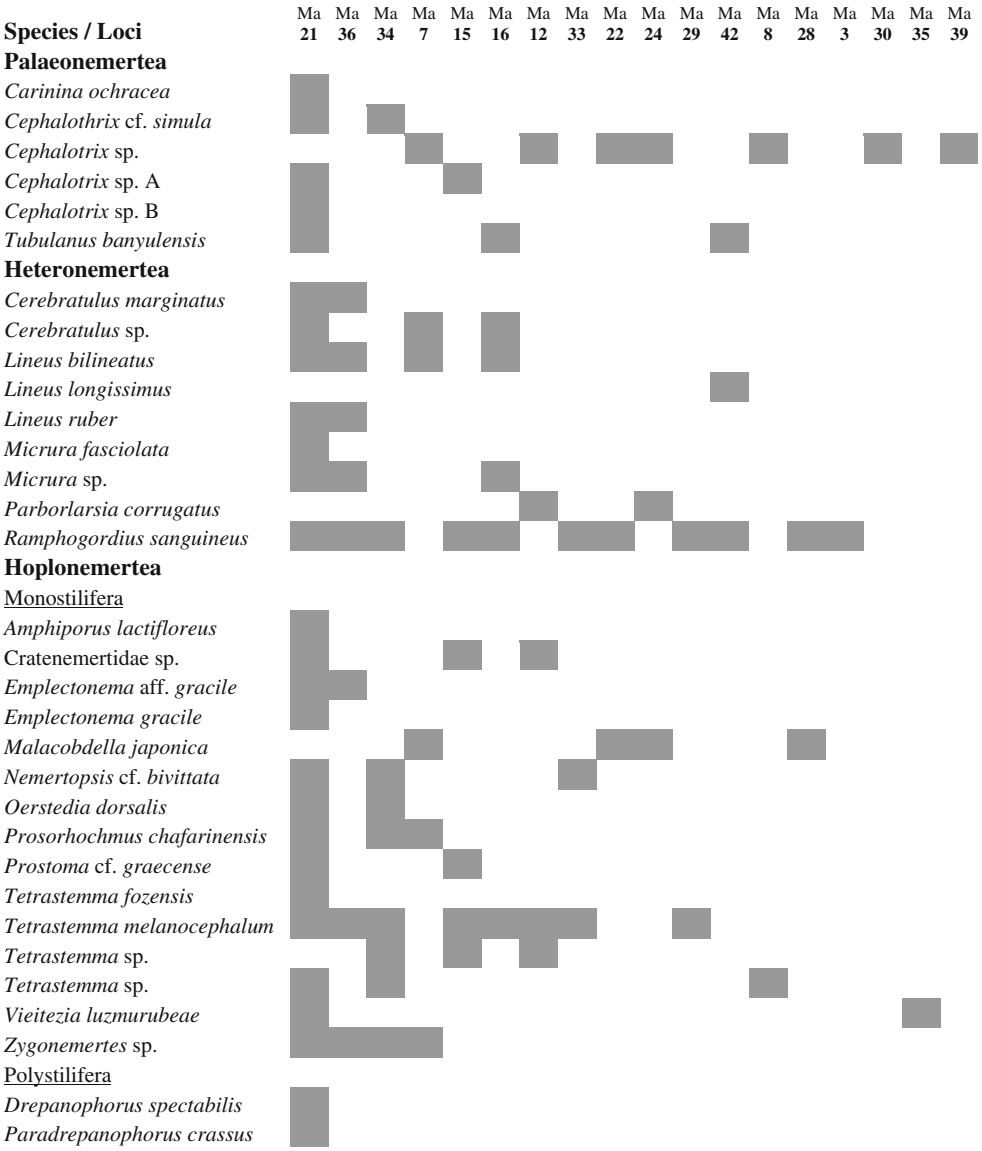
Fluorescently labeled PCR products were run on an ABI PRISM 3730 DNA Sequencer (Applied Biosystems) with the GeneScan500 internal size standard and analyzed with the GeneMapper software (Applied Biosystems). Of the 26 microsatellites tested (GenBank Accession nos. KF434343 to KF434368), one was monomorphic (Ma20) in the San Matías Gulf population. The remaining 25 loci were deemed informative for further analysis. Number of alleles (Na), observed (Ho) and expected (He) heterozygosities, tests of Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were calculated with GENEPOP (Raymond and Rousset 1995); data were reviewed for null alleles and scoring errors using MicroChecker (Van Oosterhout et al. 2004).

A total of 166 alleles (2–14 alleles per locus) were detected across the 25 loci in the 25 genotyped specimens (Table 1). Observed and expected heterozygosities ranged from 0.125 to 0.920 and 0.119 to 0.890, respectively (Table 1). After sequential Bonferroni correction, five significant deviations from HWE were found, probably due, in part, to the existence of null alleles (estimated frequencies from 0.08 to 0.22); none of the loci showed linkage disequilibrium.

Cross-amplification tests of 34 species, representing all current nemertean orders, resulted in at least one positive amplification for all but two species (*Cephalothrix* cf. *filiformis* and *Micrura purpurea*). Eight of the 26 loci for *M. arrokeana* were not amplified in the other nemerteans (Ma6, Ma17, Ma18, Ma20, Ma27, Ma38, Ma41, and Ma44), and the other 18 provided interpretable profiles for at least one (usually locus Ma21), but up to 11 loci per species (Table 2).

These polymorphic microsatellite loci, together with the analysis of its host, will be used to study genetic structure, kinship and ecological traits in *M. arrokeana*, providing important information about infestation, genotype distribution and patterns of coevolution.

Table 2 List of species tested for cross-amplification. Loci with interpretable profiles among the 26 loci analyzed are indicated by grey blocks



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