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PERMANENT GENETIC RESOURCES

Microsatellite markers for the drought-resistant earthworm *Hormogaster elisae*

M. NOVO,* T. P. VELAVAN,† A. ALMODÓVAR,* H. SCHULENBURG,† D. J.DÍAZ-COSÍN* and N. K. MICHIELS†

**Department of Zoology, Complutense University of Madrid, Madrid, Spain, †Department of Animal Evolutionary Ecology, University of Tüebingen, Tüebingen, Germany*

Abstract

We developed and characterized 10 highly polymorphic microsatellite loci from a simple sequence repeat-enriched genomic DNA library of the earthworm *Hormogaster elisae*. Characterization of these loci using 26 individuals revealed eight to 25 alleles per locus and high levels of heterozygosity. These loci will be used for paternity analysis and population genetic studies.

Keywords: earthworms, *Hormogaster elisae*, microsatellite, paternity

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Hormogaster elisae is an obligate outcrossing hermaphrodite, endemic to the central area of the Iberian Peninsula. This species is adapted to sandy soils that are poor in organic matter. Hence, it can form monospecific populations in poorer soils where other earthworm species cannot survive (Hernández *et al*. 2007), and when conditions are adverse, such as lack of rain and dryness of soil, it can undergo paradiapause (Díaz Cosín *et al*. 2006). All these make *H. elisae* a potential beneficiary of global warming. Molecular markers are essential to understand its population biology as well as the underlying selective processes such as those imposed by temperature gradients or change, migration rates, population isolation due to habitat fragmentation, historical events (e.g. bottlenecks, range expansions), and also mating behaviour (e.g. Avise 1994, 1995). The aim of this study is to develop microsatellite markers for such analyses in the earthworm *H. elisae*. Microsatellite markers have been developed for other earthworm species, such as *Lumbricus rubellus* (Harper *et al*. 2006) and *Lumbricus terrestris* (Velavan *et al*. 2007). Since between-species amplification is not possible, the microsatellites that we describe in this study, are the first for this earthworm species, *H. elisae*.

Microsatellites were isolated from a single individual. This individual was collected in El Molar (Central Spain: 40°44′22.9″N, 3°33′53.1″W), carefully cleaned from any soil debris and stored in ethanol at –20 °C until DNA extraction. A simple sequence repeat (SSR)-enriched genomic library was constructed from this specimen using the method described earlier by Velavan *et al*. (2007). Briefly, 10 μg of total genomic DNA was isolated from tissue using a modified cetyltrimethyl ammonium bromide (CTAB) method as detailed by Schulenburg *et al*. (2001). After digestion overnight at 50 °C in CTAB buffer (2% (w/v) CTAB, 0.1 M Tris-HCl pH 8.0, 0.02 M EDTA, 1.4 M NaCl, 0.1% (v/v) β-mercaptoethanol), containing 5 μL proteinase-K (10 mg/mL), DNA was extracted with two volumes of chloroform:isoamylalcohol (24:1), then precipitated using isopropanol, washed with 70% ethanol, and resuspended in sterile water. Extracted DNA was digested with *Hae*III and *Rsa*I restriction endonucleases (New England Biolabs) and fractionated on a 1.5% agarose gel. Fragments of 0.5–1.5 kb were gel-eluted by GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia) and ligated with *Mlu*I adaptors (Edwards *et al*. 1996). The adaptor-ligated SSR-enriched DNA fragments were then selected by hybridization to biotinylated oligonucleotides [$(GA)_{15}$, $(CA)_{15}$, $(GAA)_{10}$, $(CAA)_{10}$] and captured with streptavidin-conjugated magnetic beads (Dynabeads, DYNAL, Invitrogen). SSR-enriched DNA fragments were cloned into pMOS vector (Amersham Pharmacia) and transformed into competent *Escherichia coli* DH5α cells. A total of 536 recombinant clones were recovered. Plasmids were isolated from 169 clones using QIAprep Spin Miniprep Kit (QIAGEN), and cloned inserts were amplified and sequenced for both

Correspondence: T. P. Velavan, Fax: +49-7071-295634;
E-mail: velavanp@yahoo.com
M. Novo and T. P. Velavan equally contributed to this work.

Table 1 Details of the microsatellite markers developed in the study

Locus	Repeat motif	Primer sequence (5′–3′)	Tag*	Size range (bp)	N	NG	NA	H_O	H_E	HWE *P* value	PIC	GenBank Accession no.
HEM07	$(CT)_4TT(CT)_6$ $CA(CT)_{12}TT(CT)_4$	F: CTGTTCTCCGTGACTTCGAG R: CAGGGAGTCAGACAGGCAGT	FAM	136–192	26	20	19	0.69	0.92	0.0038	0.90	AM902182
HEM114	$(GT)_{69}$†	F: TGTGCCTGTGCGCCTGTGTG R: GGGGAGGAGGGATGGGACAAGAG	HEX	154–258	26	14	9	0.52	0.80	0.0028	0.76	AM902183
HEM128	$(GT)_{45}$	F: GCACAACTTCATAGCGCCATCGAC R: TCCCAATTGCTCGGCGCCATC	FAM	188–300	26	10	8	0.77	0.66	0.6082	0.61	AM902184
HEM129	$(GT)_{18}GA\ (GT)_{98}$	F: CCATGCCATATCCTGCGTCCAACA R: AGAAATACTGTAGACACGCACACC	FAM	280–432	26	10	8	0.65	0.61	0.8723	0.57	AM902185
HEM155	$(GT)_{68}$†	F: TGTCCTCACCCCAACTACCATAAGC R: ACGACGGCGATGCAGCAAATATT	HEX	232–434	26	20	25	0.62	0.98	0.000	0.95	AM902186
HEM185	$(CA)_{11}$	F: AAAGGACTTAAGCACTGACACTCGAACA R: CATCTTGATCCACAGTGTTGCCATATAC	FAM	94–192	26	15	12	0.92	0.88	0.0007	0.85	AM902187
HEM188	$(CT)_{24}$	F: CCGGGAGCCTCATGCAACAG R: CCGATAAACTCAGAAAAACGCATAAACT	FAM	200–310	26	20	16	0.58	0.93	0.0000	0.90	AM902188
HEM193	$(GT)_{24}GGGTAG$ $(GT)_{15}GG(GT)_4$	F: CAGTTATGTATGTGTTTTGCGTGGGTGTA R: CAAAGAGAGCTCCGCCAGTTACGTAGAC	FAM	130–156	26	12	8	0.33	0.79	0.0000	0.72	AM902189
HEM194b	$(GTCTCT)_4$	F: GCCCCATCCCCGCTTCTTTGTAT R: GCGCACCAAAATAAAGCCACACTAGTA	HEX	142–244	26	12	10	1.00	0.82	0.0565	0.77	AM902190
HEM215	$(CAA)_4AAA(CAA)_4$ $AAA(CAA)_4AAA(CAA)_4$	F: AGTTCGTGTATGTTTGTCGGTTCTTA R: GGGCGCTTAAGAAACCTGGAGA	FAM	180–348	26	16	10	0.70	0.82	0.1610	1.00	AM902191

*fluorescent label at the 5′-end for forward primers.
†microsatellites contain interruptions among repeats.
N, number of individuals analysed; NG, number of genotypes obtained per locus; NA, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; HWE, Hardy–Weinberg equilibrium; and PIC, polymorphism information content.

strands using M13 universal primers and a commercial sequencing service (MWG Biotech). One hundred and fourteen clones having SSR motifs were manually identified. Primers were designed for 18 clones that had repeat regions > 18 bp in length, using the program GENETOOL version 1.0 (http://www.doubletwist.com). For clones HEM114 and HEM155, highly interrupted imperfect GT repeats were observed. Fluorescent labels were added to the 5′ end of the forward primer (Table 1).

After optimization of PCR conditions, the utility of the different loci as genetic markers was tested on a panel of 26 individuals obtained from a single population of El Molar locality of Madrid in central Spain. PCR amplifications were carried out in 20 μL reaction volumes with 5 ng of genomic DNA, 1× PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl; Invitrogen), locus-specific $MgCl_2$ concentration (Table 2), 2 mM of dNTPs, 2 pM of each primer and 1 U *Taq* DNA polymerase (Invitrogen) on a Master Cycler EP Gradient (Eppendorf). Thermal cycling parameters for all tested loci are summarized in Table 2. Amplified products were first checked on a 1.5% agarose gel and then analysed on the ABI 3130*xl* automated DNA sequencer (Applied Biosystems), following the manufacturer's instructions. Resolved PCR products were precisely sized using Genescan Rox 500 size standard and GENEMAPPER 3.7 software (Applied Biosystems), in order to calculate the number, range and distribution of amplified microsatellite alleles. Population genetic parameters were calculated using ARLEQUIN version 2.0 (Schneider *et al*. 2000) and polymorphism information content (PIC) using an online tool (http://ibgwww.colourado.edu/~pshaun/gpc/mpic.html) (Purcell *et al*. 2003). Tests for linkage disequilibrium using Fisher's exact test were conducted in GENEPOP version 3.4 (Raymond & Rousset 1995) with default Markov chain parameters.

Ten microsatellite loci proved to be highly polymorphic and informative. Analysis of linkage disequilibrium yielded two significant cases (HEM 07 vs. HEM 128, $P = 0.00007$; HEM 114 vs. HEM 193, $P = 0.0204$) out of 45 pairwise comparisons. One of the comparisons (HEM 07 vs. HEM 128) remained significant even after Bonferroni correction (critical significance level of $\alpha = 0.0011$), indicating that the other eight loci are unlinked. The proportion of missing data for the locus HEM 155, HEM 193, HEM 215 are 0.19, 0.07 and 0.07, respectively, suggesting the presence of null alleles. Hardy–Weinberg equilibrium (HWE) was tested using the Markov chain algorithm and Fisher's exact test in ARLEQUIN version 2.0 (Schneider *et al*. 2000). Six loci (HEM07, HEM114, HEM155, HEM 185, HEM188 and HEM193) showed large differences between observed and expected heterozygosities (Table 1). Possible reasons for these significant departures from HWE are presence of null alleles and/or inbreeding and population substructuring. As *H. elisae* individuals exist in clusters, inbreeding could explain this departure from HWE. More details for these loci, i.e. locus designation, repeat motifs, primer sequences,

Table 2 Thermal cycling parameters for different loci used in the study

Loci	Mg++ (in mM)	Initial denaturation	Cycle denaturation	Annealing temperature	Cycle extension	Final extension	No. of cycles
HEM07 HEM194b	1.5	94 °C, 1 min	94 °C, 30 s	60 °C, 15 s	72 °C, 15 sec	72 °C, 3 min	30
HEM114 HEM129 HEM185	2.5	94 °C, 15 min	94 °C, 30 s	55 °C, 90 s	72 °C, 1 min	60 °C, 30 min	35
HEM155	2.5	94 °C, 15 min	94 °C, 30 s	57 °C, 90 s	72 °C, 1 min	60 °C, 30 min	35
HEM128 HEM188 HEM193 HEM215	1.5	94 °C, 5 min	94 °C, 1 min	60 °C, 60 s	72 °C, 1 min	72 °C, 7 min	35

Mg++, magnesium ion concentration used for PCR amplifications.

allele attributes, PIC estimates and GenBank Accession numbers, are summarized in Table 1.

In conclusion, the markers developed here have the degree of polymorphism and reliability that is required for earthworm paternity analysis and population genetics.

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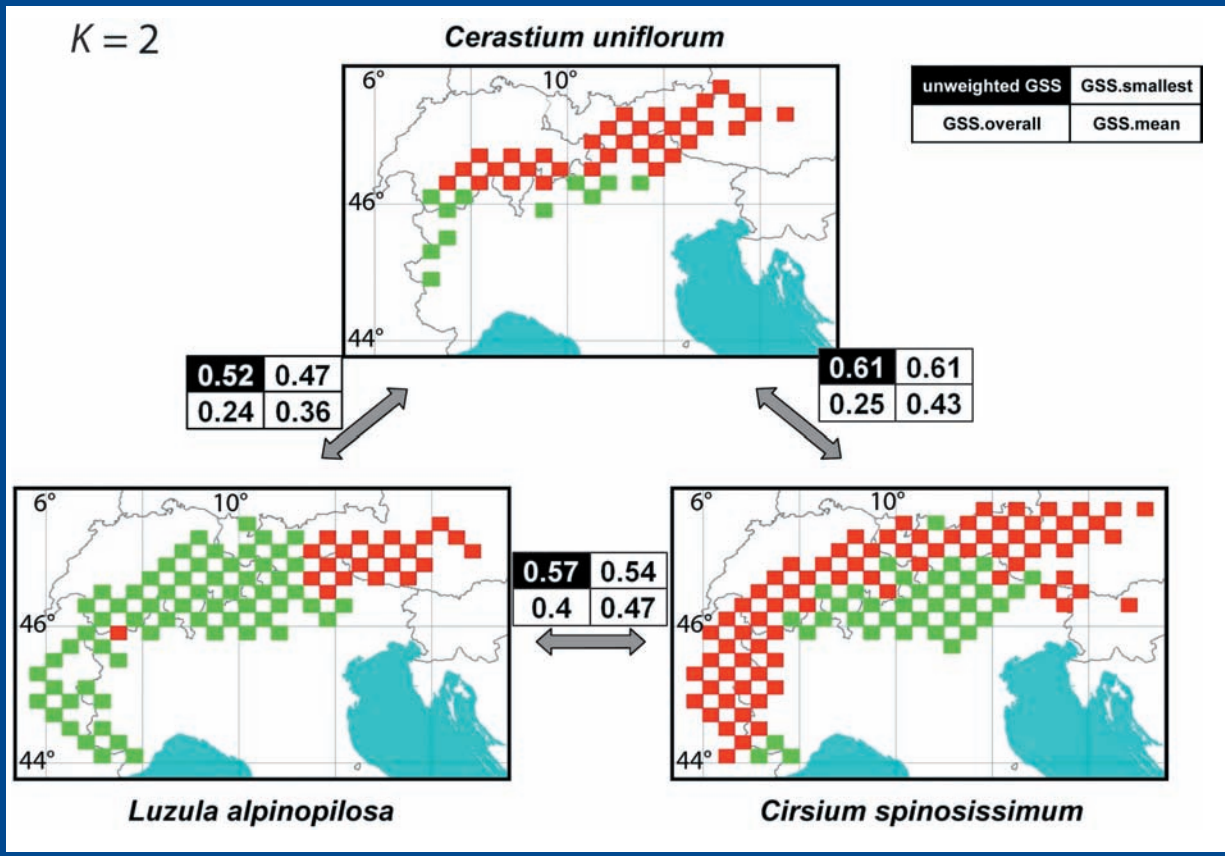

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