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Isolation and characterization of eleven polymorphic microsatellite markers for the earthworm *Aporrectodea longa* (Ude)

Heiko Strunk [a], Axel Hochkirch [b], Michael Veith [b], Thomas Hankeln [c], Christoph Emmerling [a,*]

[a] Department of Soil Science, Trier University, Campus II, Behringstraße, 54296 Trier, Germany
[b] Department of Biogeography, Trier University, 54286 Trier, Germany
[c] GENterprise Genomics, J.-J.-Becherweg 30a, 55128 Mainz, Germany

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ABSTRACT

Microsatellite markers are a promising tool for accurate estimation of population structure, dispersal and gene flow among populations. We isolated and characterized eleven new polymorphic microsatellite loci for the earthworm *Aporrectodea longa* (Ude), a widespread Palaearctic species. Polymorphism was tested in 31 individuals collected from one population and ranged from three to 15 alleles per locus. Observed heterozygosity ranged from 0.308 to 0.828, expected heterozygosity varied between 0.380 and 0.891. To the best of our knowledge, the presented microsatellite markers are the first for the species *A. longa*. Cross-species amplification tests indicated that the presented markers are not usable for other earthworm species.

Earthworms constitute more than 90% of the soil invertebrate biomass and play a major role as decomposers of organic matter in soils [3,9]. They can be classified into three major functional categories: epigeic, endogeic and anecic species. This classification mainly considers the preferred habitat within the soil matrix and the burrowing behaviour of the species [1]. The species *Aporrectodea longa* (Ude) has an exceptional position within lumbricids, because it comprises ecological behaviour patterns of both, endogeic and anecic life forms. Thus, it can be referred to as an endo-anectic life form [5]. This earthworm is common in the Palaearctic, where it is usually found in grassland and cultivated soils. *A. longa* is a hermaphroditic, diploid species with a karyotype of 36 chromosomes [12].

Land-use changes, such as the current increase of mono-cultivation of energy crops will probably affect the population connectivity of several biota, including earthworms. In many regions mono-cultivation led to a restructuring of the landscape and a fragmentation of habitats. Previous studies showed, that the cultivation of various energy crops markedly affected litter decomposition and mineralization [4], and earthworm population [6]. We primary used microsatellites to study population structure affected by different soil management, and in more detail, to study the effects of habitat fragmentation on genetic differentiation, diversity and gene flow among populations of the earthworm *A. longa*. Microsatellites have been shown as the most suitable markers for such questions [14]. This is caused by their high mutation rate and neutrality. To our knowledge, no microsatellite markers have been developed for *A. longa* to date. We used this species because of its importance in our investigation area. However, microsatellite markers have been previously developed for other earthworm species, such as *Lumbricus terrestris* [15], *Lumbricus rubellus* [8], *Eisenia fetida* [13] and *Allolobophora chlorotica* [2].

For the isolation of microsatellite loci, a genomic DNA library was constructed from 15 μg of genomic DNA (DNeasy Blood & Tissue Kit, Qiagen). DNA was sheared by nebulization, 1.5–2.5 kb sized fragments were separated electrophoretically, purified by electroelution and end-repaired by using *Escherichia coli* DNA polymerase I Klenow fragment. The fragments were blunt end-ligated into pUC 18 vector plasmids cut with SmaI and then transformed into DH10B *E. coli* host cells via electroporation. In order to identify fragments containing typical microsatellite DNA sequence motifs, we picked insert-containing clones onto gridded nitrocellulose filters (Schleicher & Schuell, BA85) for colony filter hybridization [7]. Clones (n = 18,000) were screened for microsatellites using seven different radioactive labelled synthetic

* Corresponding author. Tel.: +49 (0) 651 201 2238; fax: +49 (0) 651 201 3809.
E-mail address: emmerling@uni-trier.de (C. Emmerling).

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oligonucleotide probes ($(CA)_{15}$, $(GA)_{15}$, $(AAT)_{10}$, $(AAG)_{10}$, $(ATG)_{10}$, $(GGC)_{10}$,$(GATA)_6$). Following autoradiographic detection, 2 × 96 hybridizing clones were selected, their plasmid DBNA was isolated by the standard alkaline lysis method and sequenced by dye terminator cycle sequencing (Applied Biosystems) on an ABI 3730 capillary sequencer. Candidate microsatellite loci ($n = 42$) containing the longest, uninterrupted repeats were selected by visual sequence inspection and further tested on initially 3 individuals for yielding distinct and heterozygous PCR products.

The unlabelled primer pairs were first tested with 10 individuals taken from different sampling sites situated in an agricultural used area near Trier, Rhineland-Palatinate, Germany (49°49′2.47″N, 6°43′10.77″E). Earthworms have been sampled from agricultural soils by hand-sorting and subsequently cleaned with tap water and stored in a box filled with soil. The individuals were then starved for 2 days, cleaned of soil remnants (depurated) and stored in pure ethanol.

DNA extraction was performed with the DNeasy Blood & Tissue Kit (Qiagen). Optimal annealing temperatures were determined using gradient PCRs. PCR product length and specificity were checked by agarose gel electrophoresis and ethidium bromide staining. Loci that amplified successfully were then 5′ end-labelled either with FAM, HEX or TAMRA fluorescent labels (Table 1). For eleven primers, we were able to develop reliable PCR conditions, ten of which could be combined in three multiplex reactions using the Qiagen Multiplex PCR Kit. We then tested the 19 loci positive by these criteria in 31 adult individuals of *A. longa* collected from the same area (see above).

These primer combinations were Multiplex 1 (H09_Alo_3f, H07_Alo_5_2, D12_Alo_5), Multiplex 2 (C04_Alo_6_2, C06_Alo_6) and Multiplex 3 (C04_Alo_6, C10_Alo_1f, A08_Alo_6, B02_Alo_4f, E04_Alo_6). Reaction volume per PCR was 10 μl, consisting of 1×PCR Buffer (3 mM $MgCl_2$), a final concentration of 0.2 μM per primer, dNTP's, HotStarTaq Polymerase and 100 ng genomic DNA as template. PCR conditions were as follows: initial denaturation at 95 °C for 15 min, followed by 35 cycles (30 s denaturation at 94 °C, 90 s annealing at 57–61 °C (Table 1), 90 s extension at 72 °C) and a final extension step at 72 °C for 30 min. The remaining locus (E05_Alo_5) had to be amplified in a single PCR reaction as it did not amplify when combined with other primers. PCRs for this primer pair were performed with a HotMasterMix (5Prime GmbH, Hamburg; Germany). Final concentrations in a 10 μl PCR reaction contained 0.2 U/10 μl Taq DNA Polymerase, 45 mM KCl, 2.5 mM Mg^{2+}, 200 μM of each dNTP, 100 nM per primer and 50–100 ng of genomic DNA. Cycling conditions were: initial denaturation at 94 °C for 2 min followed by 35 cycles (30 s denaturation at 94 °C, 30 s annealing at 57 °C, 30 s extension at 65 °C) and a final extension step at 65 °C for 10 min. All reactions were run in a Biometra T Gradient Thermocycler. PCR products were analyzed on a DNA sequencer (GE Healthcare Life Sciences MegaBACE 1000) and scored using Fragment Profiler 1.2 with default settings (Amersham Biosciences). We used Micro-checker [10] to test for scoring errors, such as null alleles, large allele dropout or stutter bands. Population analyses were executed with GenAlEx – Genetic Analysis in Excel Version 6.4 [11].

The eleven polymorphic microsatellite loci had between three and 15 alleles (Table 1). Observed heterozygosity ranged between 0.308 and 0.828, while the expected heterozygosity varied between 0.380 and 0.891. Two loci (A08_Alo_6: $p < 0.01$, $DF = 10$, Chi = 26.542; E04_Alo_6: $p < 0.001$, $DF = 3$, Chi = 28.388) showed significant departure from Hardy–Weinberg-Equilibrium. Subsequent tests showed evidence for the existence of null alleles at three loci, such as D12_Alo_5 with a probability according to Oosterhout et al. [10] of 0.139, 0.1352 (A08_Alo_6) and 0.2112 (E04_Alo_6), respectively. These null alleles are likely to be the main reason for the deviations from the Hardy–Weinberg-Equilibrium. No evidence was found for large allele dropout or stutter bands. The high variability of the microsatellite loci suggests that the developed marker system will have a sufficient degree of polymorphism for population genetic studies.

Furthermore, the developed markers have been tested for possible cross-species amplification in four other species. These tests have been performed with worms of the species *Aporrectodea caliginosa*, *A. chlorotica*, *L. terrestris* and *L. rubellus*. For every species 20 individuals of different locations in the area of Trier have been used. The analysis showed that PCR could be successfully

Table 1
Properties of the developed microsatellite markers for the earthworm species *Aporrectodea longa* (Ude) with locus name, repeat motif, fluorescence label at 5′ end (Tag), primer sequence, Plex no. (MP: Multiplex, SP: Singleplex), annealing temperature, allele size range, most common allele, number of alleles per locus, observed heterozygosity (H_O) and expected heterozygosity (H_E). H_E values which are marked with an asterisk are significantly different from Hardy–Weinberg-Equilibrium.

Locus	Repeat motif	Tag	Primer sequence (5′–3′)	Plex No.	Annealing temperature	Allele size range	Most frequent allele size	Number of alleles	H_O	H_E
H09_Alo_3f	$(ATG)_6(ACG)_5(ATG)$	FAM	F: ACCAATCCCGCTATTCTCATG R: ACTCCAAGCCGATCACGTG	MP 1	57	236–251	242	5	0.813	0.625
H07_Alo_5_2	$(CGCT)_5$	HEX	F: CGTGCCTAAGAGTTTTCGGC R: TCGCACCAATCGAACGCCA	MP 1	57	248–256	256	3	0.344	0.466
D12_Alo_5	$(TTA)_8$	TMR	F: TTTCCGAGGTACATACGATCC R: CCGTAGCTTCAGAGGCAC	MP 1	57	200–207	201	5	0.500	0.688
C06_Alo_6	$(TTA)_{30}$	HEX	F: CCTTGGCTACAGAGAGGTAG R: ACTTTCGGTCACAATTCAGCG	MP 2	57	244–316	295	12	0.828	0.849
C04_Alo_6_2	$(ATT)_{23}$	FAM	F: CCATCTCGGGCATAGTGTCA R: GCAGTCTGTGCATTCGTCAT	MP 2	57	322–361	328, 331	11	0.679	0.803
E05_Alo_5	$(CAT)_3(CAG)_6$	FAM	F: TTTCGACGAGTAGCAGAGGA R: AACCCAGATCACGTATGGTC	SP 1	57	214–220	214	3	0.407	0.394
C04_Alo_6	$(TTA)_{29}$	TMR	F: CCTTGGCTACAGAGAGGTAG R: ACTTTCGGTCACAATTCAGCG	MP 3	61	212–260	221	15	0.813	0.891
C10_Alo_1f	$(GTT)_{12}(GAT)_{32}$	HEX	F: GAGTTGGTCCTAAATTCCTTTCG R: TTGTTTATGTTGCGGCTGTGTC	MP 3	61	280–319	295	10	0.781	0.842
A08_Alo_6	$(TCA)_{33}$	FAM	F: CAAGAGCCAATGGACCTGC R: CAACAAGGCATGTGTTTACAGG	MP 3	61	248–275	251	5	0.469	0.660 *
B02_Alo_4f	$(GA)_5(ACAG)_{14}$	TMR	F: ATCAAGATCCACCAGCGGTG R: GGGCCGTAAATCTGTTTCGAC	MP 3	61	315–343	321	7	0.333	0.380
E04_Alo_6	$(AGAC)_{18}$	FAM	F: AATGAGCCTCCCAGTACAACG R: AATCTGGCTATTGCGGACTTC	MP 3	61	182–215	212	3	0.308	0.595 *

conducted using DNA of those species. Unfortunately, *A. chlorotica*, *L. terrestris* and *L. rubellus* showed only monomorphous alleles by use of the tested markers. No differentiations were found in *A. caliginosa,* with the exception of H07_Alo5_2 which showed heterozygous and homozygous loci as well as different allele sizes. Hence, we suggest, that the presented markers should only be used for investigation of *A. longa* population structure.

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