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RESEARCH ARTICLE

Polymorphic microsatellite markers demonstrate hybridization and interspecific gene flow between lumbricid earthworm species, *Eisenia andrei* and *E. fetida*

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Abstract

The lumbricid earthworms *Eisenia andrei* (Ea) and *E. fetida* (Ef) have been used as model organisms for studies on hybridization. Previously they have been identified by species specific sequences of the mitochondrial COI gene of maternal origin ('a' or 'f') and the nuclear 28S gene of maternal/paternal origin ('A' or 'F'). In experimental crosses, these hermaphroditic species produce progeny of genotypes Ea (aAA), Ef (fFF) and hybrids (aAF and fFA) originating by self-fertilization or cross-fertilization. To facilitate studies on new aspects of the breeding biology and hybridization of earthworms, polymorphic microsatellite markers were developed based on 12 Ea and 12 Ef specimens and validated on DNA samples extracted from 24 genotyped specimens (aAA, fFF, aAF and fFA) from three laboratory-raised families and 10 of them were applied in the present study. The results indicate that microsatellite markers are valuable tools for tracking interspecific gene flow between these species.

Introduction

The uniformly reddish *Eisenia andrei* (Ea) and striped 'tiger worms' *E. fetida* (Ef) are widely used in toxicology, ecotoxicology, ecology, environmental studies [1, 2] and in biomedicine as a source of bioactive molecules [3, 4], all of which require accurate species identification. However the existence of hybrids was revealed by mixed patterns of electromorphs of esterase activity in laboratory mated specimens [5] and then relicts of past hybridization between Ea and Ef were recognized in natural populations from Scandinavia [6]. Asymmetrical hybridization has been experimentally proven in the progeny of laboratory-paired Ea and Ef virgin specimens delimited by species-specific sequences of the mitochondrial COI gene of maternal origin and

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the 28s rRNA gene of maternal/paternal origin [7, 8]. The first generation offspring of these hermaphroditic species included self-fertilized Ea and Ef specimens and fertile hybrids derived from Ea ova fertilized by Ef spermatozoa, while sterile hybrids from Ef ova fertilized by Ea spermatozoa appeared only among progeny of back-crossed Ea-derived hybrids with Ef parental specimens [7–10]. Although the reproductive capabilities of hybrids was gradually impaired [9], interspecies gene flow was documented by intermediate pigmentation patterns and by the presence of Ea-specific fluorophore not only in hybrids but also in a few Ef specimens [4, 8]. Novel species-specific genetic markers are needed for detailed studies on mechanisms of hybridization and gene introgression in these convenient model species.

Microsatellites are highly polymorphic, very short nucleotide sequences inherited in a codominant way and repeated many times in the nuclear genome [11] that can be used for addressing questions concerning the mating systems, genetic diversity, and population structure of a diverse array of organisms [12]. Since next-generation sequencing (NGS) has become more and more accessible, the identification and characterization of species-specific microsatellites is currently faster and requires less effort.

Polymorphic microsatellite markers have been developed for several earthworm species, including *Aporrectodea longa* [13], *Aporrectodea icterica* [14, 15], *Allolobophora chlorotica* [16, 17], *Hormogaster elisae* [18], *Dendrobaena octaedra* [11], *Amyntas cortices* [19], several *Lumbricus* sp. [12, 20–25], *Drawida gisti* [26], and *Eisenia fetida* [27], but not for *E. andrei*.

Thus, in response to the continuously growing need for new species-specific markers for studies on hybridization, the aim of the present work was to develop polymorphic microsatellite markers for *E. fetida* and *E. andrei* followed by their validation on DNA samples from three families of earthworms genotyped during previous experiments. Our results indicate that microsatellite markers are valuable tools for tracking interspecific gene flow.

Materials and methods

Earthworms

Lumbricid earthworms *Eisenia andrei* (Ea) and *E. fetida* (Ef) originally derived from laboratory stocks at the University in Lille (France) were cultured for a decade in the laboratories of the Institute of Zoology and Biomedical Research of the Jagiellonian University (Krakow, Poland), and in parallel for the last five years in the College of Natural Sciences, University of Rzeszów [7, 9, 10, 28, 29]. For several years they were used as convenient models for studies of hybridization of these simultaneous hermaphrodites by genotyping them by species-specific sequences of mitochondrial COI gene ('a' for Ea and 'f' for Ef) and diploid nuclear sequences of 28s rRNA gene of maternal/paternal origin ('A' for Ea and 'F' for Ef) as aAA, fFF and aAF or fFA interspecific hybrids [7–10]. Every genotyped specimen from these experiments was marked by the genetic symbols followed by a unique numerical code and all sequences have been deposited in GeneBank [7, 9, 10, 28] while ethanol-fixed posterior segments were preserved for further use.

Twelve adult specimens of each species, Ea and Ef, derived from Lille, Krakow and Rzeszow laboratory cultures were chosen to create two separate pools of DNA for microsatellite library preparation. Then another 15 specimens per species were used to establish numbers of alleles of potential microsatellite markers. Finally DNA samples from ethanol-fixed posterior segments of 24 earthworms from previous laboratory studies on hybridization [7, 9] were used for a pilot application of microsatellite markers in tracking interspecific gene flow between Ea and Ef.

DNA extraction, enrichment and microsatellite library construction

Genomic DNA was extracted from supravivally amputated tail tips (approx. 50 mg of tissue). Homogenisation was performed by freezing the tail in liquid nitrogen, followed by mechanical homogenisation with a mortar. Afterwards, the genomic DNA was extracted with Universal DNA/RNA/Protein kit (Eurobio, Poland), following the manufacturer's instructions. The quality and quantity of the extracted DNA were checked on the SPECTROstar Nano spectrometer (BMG LABTECH, Germany). Prior to the NGS sequencing, the quality and quantity of extracted DNA were rechecked using a 2100 Bioanalyzer (Agilent Technologies, USA). 100 ng of DNA from each of 12 specimens per species was used to prepare two pools of DNA (one for Ea and one for Ef). The samples were then sent to the GenoScreen company (Lille, France), which prepared a microsatellite-enriched genomic library as described previously [30]. Both pools of DNA were sequenced with Illumina MiSeq flowcell v2 2x250 bp paired-end sequencing kit. Total DNA was mechanically fragmented, and enrichment was performed with probes containing 8 microsatellite motifs: TC TG, ACG, AGG, AAC, AAG, ACTC, ACAT. The QDD v.3 software [31] was used for microsatellite identification from the raw sequences, including all bioinformatics steps from adapter removal from raw sequences, detection of microsatellites, detection of redundancy/possible mobile element association, selection of sequences with target microsatellites and PCR primer design using BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), ClustalW [32] and Primer3 [33] programs. Only perfect di/tri/tetra motifs were kept, with A and B designs (according to QDD internal parameters: regions that do not have multiple microsatellites, nanosatellite, and homopolymers), and a minimum of 20 bp between the primer and the microsatellite.

PCR and data analysis

The PCR reactions were performed in a 10 µL reaction volume (~100 ng genomic DNA, 1X PCR buffer, 6 pmol dNTPs, 37.5 pmol MgCl₂, 0.5 U Taq DNA polymerase, 5 pmol of the forward primer, and 5 pmol of the reverse primer), performed in a Veriti thermal cycler (Applied Biosystems). The thermal profile of the amplification for all markers had an initial denaturation at 95°C for 10 min, followed by 40 cycles of: 30 s at 95°C, 30 s at 55°C and 60 s at 72°C, with a final extension of 10 min at 72°C. Amplicons were separated using automated high-resolution capillary electrophoresis (QIAxcel[®] —Pure Excellence, Qiagen, Germany).

Alleles were sized using a 50–350 bp standard (LI-COR Biosciences), and genotypes were scored using SAGA v.3.3 software (LI-COR Biosciences). The species-specific primer sets were also checked for cross-species amplification under the same conditions as described above.

Results and discussion

Development of microsatellite markers for Ea and Ef

Genetic loci containing simple sequence repeats (SSRs, i.e. microsatellites) can be used as powerful markers in population genetics primarily because they are found throughout the nuclear genome, generally have several alleles per locus, and are inherited in a codominant way [11].

A total of 1 709 140 reads obtained for Ef were assembled into 918733 contigs, whereas 2 094 624 reads obtained for Ea were assembled into 1 140 654 contigs. Overall, 19 758 pairs of primers were designed for Ef and 23 255 for Ea. After that, 3 777 primer pairs for Ef and 4 258 primer pairs for Ea were validated with QDD v3 software. Primer sequence, product size and the sequence of each product are available elsewhere [34].

Out of those, 47 primer pairs per each species, characterized by the largest repeat number, were selected for amplification and polymorphism tests. In samples of Ef, 36 out of 47 loci

Table 1. Characterization of microsatellite loci isolated from *E. andrei* (a) and *E. fetida* (f) used in present paper.

marker	Primer sequence	Repeat motif	Size range (bp)	Dye	N _A	Allele size range	Most frequent allele size	GenBank Accession number
a1	ACCATTGAGTGTAATACATTACTCCA AGCGCTTATGCAGTGATTTAGT	(AAT)17	173	6FAM	6	143–180	180	MW521367
a3	AGCAGAAGATTTGAGGCGGT CTGAGACATCTGCCAGCGAA	(AATG)15	177	VIC	3	143–184	161	MW521370
a5	CACCACGAGTACCAGCTGAA ACGTCATTGGGTTTAATTATCACCT	(ATC)12	171	PET	5	169–193	169	MW521372
a6	CGCTTTGTTCAGAAAGCTGC GCACAGGATATGCTTCAATTGCT	(AATG)11	200	PET	4	193–209	197	MW521373
a7	TTTAGAACAGAAATGAAAGACCGA GCTGGCTTACAATTGCACCA	(AC)11	147	VIC	4	148–166	166	MW521374
f3	CTAATAGTGTGATGTGGCTGCC CAACATCCACAACCCATCTGT	(AATC)16	240	NED	8	203–284	203	MW521357
f4	TCCCTCTGCGTTTCTGACAG CCCAAGCTGGCATGCAAT	(ATC)16	187	6FAM	4	164–194	194	MW521358
f6	ACTGTATCCGTGCATGGCAT CCAATAATCAATTTCTAGAGGTTCCA	(AC)15	185	NED	4	178–202	178	MW521360
f7	ACATCTACGTCACTAGCCCTCT ACTGTTGAACTGAATGCGCTG	(ATC)12	291	NED	3	265–297	297	MW521362
f9	TGATGACAATAATATTGATGGTGACTT AACACTCGAAGAAGGTTAAGCAC	(AGG)11	140	6FAM	5	124–162	124	MW521365

[Repeat motif–repeat motif with a number of repeats, N_A—number of alleles in analyzed population].

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amplified reliably and showed evidence of polymorphism. In samples of Ea, 30 out of 47 loci amplified reliably and showed evidence of polymorphism. These markers were initially tested using 15 Ea and 15 Ef individuals to assess the number of alleles for each marker. Ten microsatellite markers demonstrating the largest variability (5 for Ea and 5 for Ef) were used in the present study because of their potential usefulness in the discrimination between Ea, Ef and hybrid specimens. Their primer sequences, repeat motif, number of alleles, and GenBank accession numbers for sequences are shown in [Table 1](#).

Efficacy of microsatellites for investigation of hybridization and gene flow

Offspring of Ea+Ef pair. *Eisenia* sp. earthworms are simultaneous hermaphrodites, thus each specimen produces ova and spermatozoa. In contrast to Ea and Ef from Spain, capable of uniparental reproduction by self-fertilization [35], these species from French, Polish, and Hungarian laboratory stocks are unable to self-fertilize when cultured in isolation but the presence of conspecific or closely related partner (like Ea, Ef and their hybrids) induces copulatory behavior [10]. As described earlier [7], copulating partners exchange sperm that moves from the male pores along the external body grooves to the partner's spermatheca; at this stage, spermatozoa from a single individual may be admixed with the partner's spermatozoa and thus both auto- and allo-spermatozoa may be stored in spermathecas of copulating partners. Earthworms separate after copulation and after several days the clitellum of each individual produces a mucous tube forming an oval capsule (cocoon) harvesting the individual's ova and both auto- and allo-spermatozoa, thus ova within the same cocoon may be either self-fertilized or cross-fertilized. Thus, the progeny of interspecific Ea+Ef pairs is expected to have both a new generation of pure Ea or Ef specimens and interspecific hybrids derived either from Ea ova fertilized by Ef spermatozoa (aAF), and Ef ova fertilized by Ea spermatozoa (fFA) ([Fig 1A](#)). Contrary to such expectations, over a 5-year experiment using laboratory pairings of virgin Ea

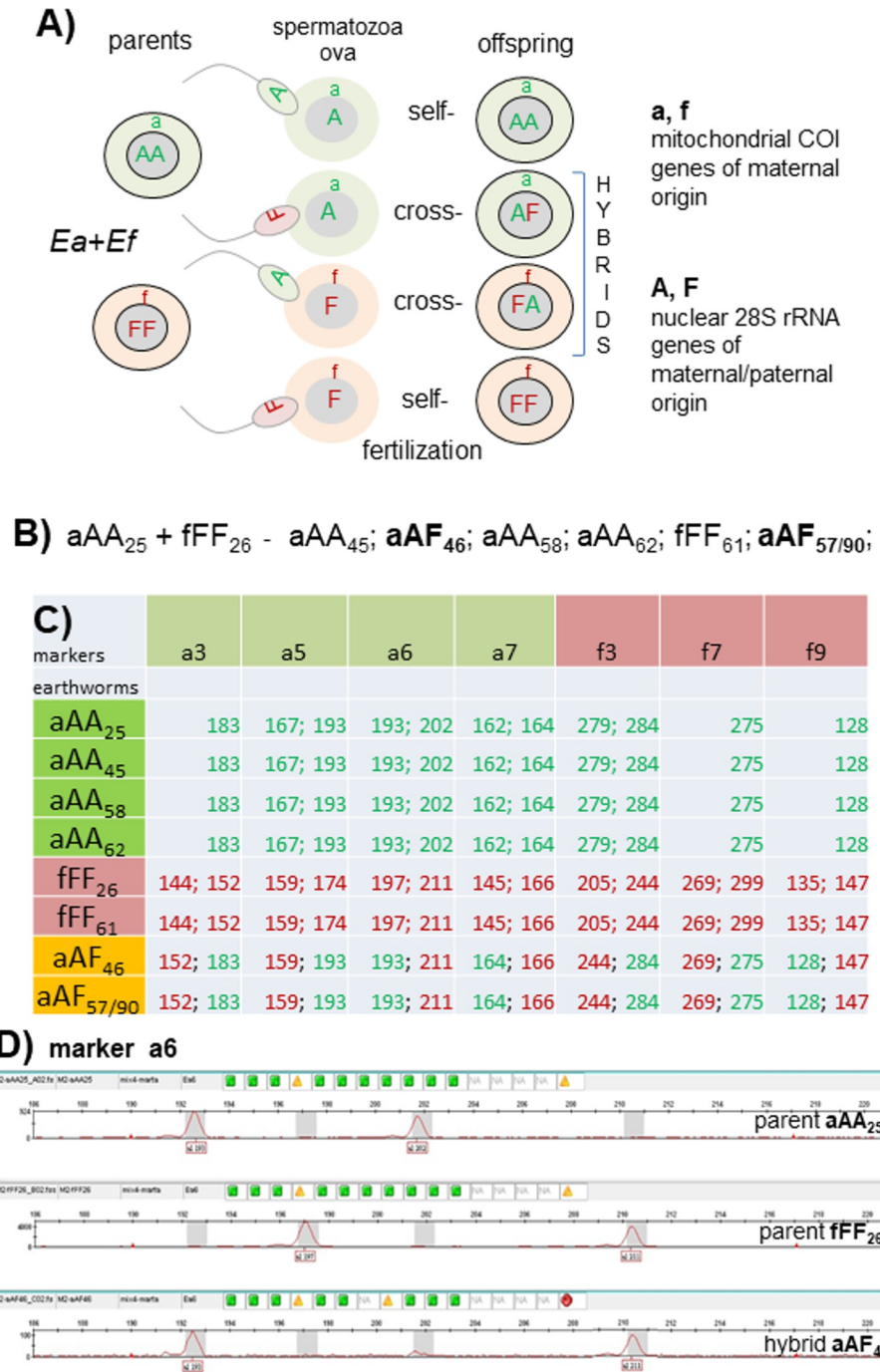


Fig 1. Scheme of mating of EaxEf earthworm pairs. (A) The germline cells of parental earthworms aAA and fFF, their gametes ('aA' or 'fF' ova and 'A' or 'F' spermatozoa) and self-fertilization or cross-fertilization resulting in zygotes of the pure Ea (aAA) and Ef (fFF) species or their Ea-derived aAF or Ef-derived fFA hybrids (the latter were lacking in our experiments); (B) Offspring of aAA+fFF earthworms from previous experiments; each earthworm marked by genotype followed by unique numerical code [7]; (C) Data concerning selected microsatellite markers (numbers indicate allele sizes) in particular members of this family; (D) Examples of visualization of a6 microsatellite marker sizes of the parental specimens aAA25 and fFF26 and hybrid aAF46. The Y-axis shows fluorescence intensity of PCR products while the X-axis shows the size of particular alleles.

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and Ef partners, first generation offspring included relatively common aAA, fFF, and aAF specimens, whereas fFA hybrids were not detected [7–10], as exemplified in Fig 1B.

In this paper, ethanol-fixed samples from the parental specimens aAA25+fFF26 and some of their offspring from experiments performed in 2018 [7] were analyzed using seven microsatellite markers, i.e. a3, a5, a6, a7, f3, f7, f9 (Fig 1C) visualized on an example of the marker a6 on Fig 1D). The results were fully concordant with data obtained using the COI/28S markers, showing that three aAA offspring share all microsatellites with the aAA25 parent, one fFF offspring shares alleles with the fFF26 parent, and two offspring specimens (aAF46 and aAF57/90) are interspecific hybrids possessing one nuclear allele from Ea and the second from Ef. It is worth noting that microsatellite analysis cannot clearly determine whether the hybrids were derived from Ea or Ef ova, thus COI gene was necessary to discriminate this.

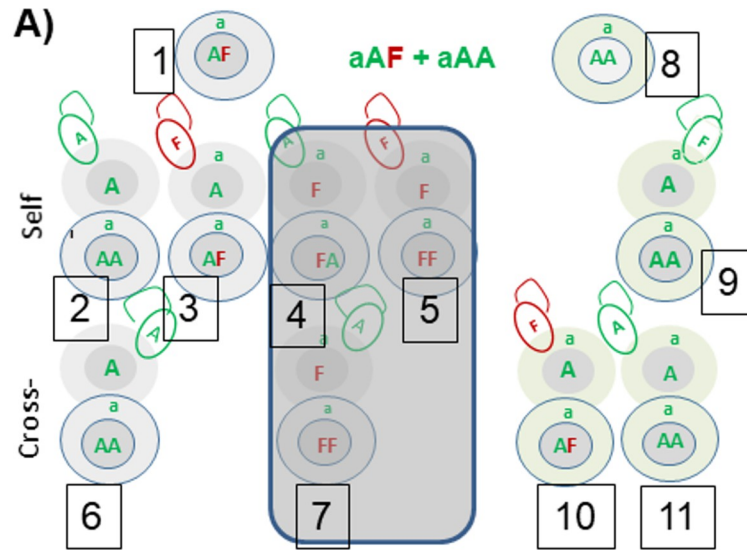
Offspring of back-cross aAF+aAA. Each aAF hybrid (numbered as 1 in Fig 2A) produces two kinds of ova, 'aA' and 'aF' and two kinds of spermatozoa, 'A', and 'F'. Thus these hybrids may give four types of progeny: aAA, aAF, aFA, and aFF by self-fertilization (numbered 2–5 in Fig 2A). The aAA partner (number 8) produces only 'aA' ova and 'A' spermatozoa, thus may give the aAA offspring by self-fertilization (number 9). However, neither aAF nor aAA specimens reproduced in isolation but initiated production of fertile cocoons only in the presence of a closely related partner [10]. Cross-fertilized 'aA' ova of the Ea partner give pure Ea specimens (number 11) and aAF hybrid (number 10) (Fig 2A). Cross-fertilized 'aA' ova of the hybrid give aAA specimens (number 6), while 'aF' ova give aFA hybrids (number 7). However, 'aF' ova may be incompatible due to cyto-nuclear mismatch, and their vitality might be strongly impaired [36–38], thus mitonuclearly incompatible ova and potentially incompatible resulting fertilized cells are shadowed (numbers 4, 5, and 7 on Fig 2A).

The offspring of aAFap274/361+aAA362 pair from previous experiments [9] encompassed both 'pure' Ea specimens and the next generation of Ea-derived hybrids (Fig 2B). Each of the pure specimens may be derived by four different genotype combinations (numbers 2; 6; 9; 11), while each hybrid by another four different combinations (numbers 3; 4; 7; 10) (Fig 2A). The microsatellites applied here have helped reduce the numbers of such possibilities (Fig 2C). Microsatellite marker a1 is heterozygous in parental aAF274/361 hybrid (with alleles 150 and 163) while a1 is homozygous in aAA362 parent (allele 144 only). All investigated hybrid offspring (aAF379, aAF347 and aAF444) are heterozygotes with allele 144 inherited from Ea and 163 from the hybrid. This combination of alleles fits the examples numbered as 10 and 7 (the latter less probable) in Fig 2A.

Among Ea offspring of the investigated pair, only aAA390 specimen originated by self-fertilization of the Ea parent as they share allele 144 of marker a1 (9 from Fig 2A). The two remaining aAA391 and aAA443 specimens inherited allele 144 from the Ea parent and allele 150 from the hybrid, as illustrated by 11 in Fig 2A. However it cannot be excluded that allele 150 was inherited from the hybrid and allele 144 from the Ea parent, as illustrated by 6 in Fig 2A.

The same family was analyzed by the marker f4 (Fig 2C) that is heterozygous in both parents, with allele 198 being shared by both of them, and the results were even less discriminatory as the origin of all Ea offspring and aAF444 hybrid might be explained by the four possibilities. However, the possible combinations revealed by f4 marker were also shared with the more discriminatory marker a1, thus the latter were considered as most probable for this family (last green column of Fig 2C). Thus the marker a1 was much more valuable for investigation of this particular family than the f4 marker, but further markers are needed to precisely discriminate the origins of some particular earthworms, including discerning between possibilities 6 or 11.

The presence of microsatellite marker (e.g. allele 150 of a1 marker) both in the hybrid parent (1) and in the aAA genotype numbered as 11 originating from Ea-derived ova fertilized by



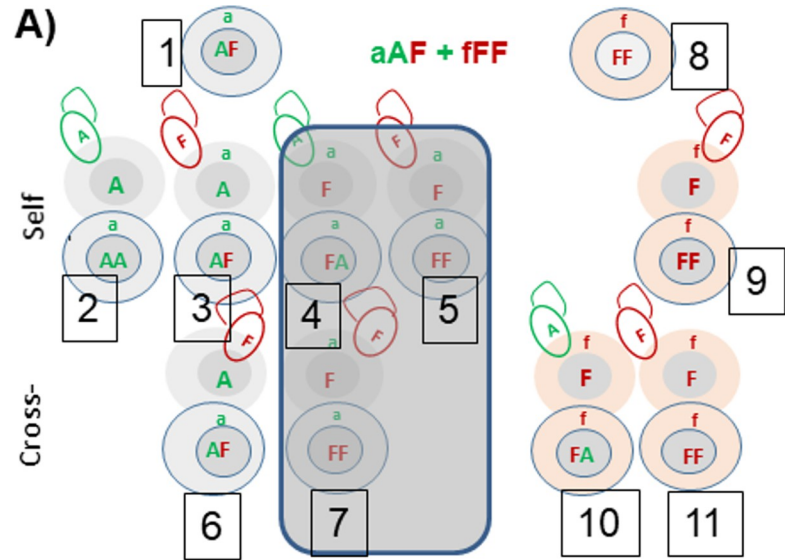
**B) aAF_{ap274/361} + aAA₃₆₂ - aAF₃₇₉; aAF₃₄₇; aAA₃₉₀;
aAA₃₉₁; aAA₄₄₃; aAF₄₄₄**

C) markers earthworms	a1	Fig. 2A	f4	Fig. 2A	FIG. 2A
aAF_{ap274/361}	150; 163	1	194; 198	1	1
aAF ₃₇₉	144; 163	10; (7)	174; 194	10; (7)	10; (7)
aAF ₃₄₇	144; 163	10; (7)	174; 194	10; (7)	10; (7)
aAF ₄₄₄	144; 163	10; 7	194; 198	10; (7); 3; (4)	10; (7)
aAA₃₆₂	144	8	174; 198	8	8
aAA ₃₉₀	144	9	174; 198	9; 11; 6; 2	9
aAA ₃₉₁	144; 150	11; 6	198	11; 6; 9; 2	11; 6
aAA ₄₄₃	144; 150	11; 6	198	11; 6; 9; 2	11; 6

Fig 2. Considerations on the results of mating the aAF hybrid with aAA parental specimen. (A) Back-cross of aAF + aAA pair; symbols as on Fig 1. (B) Offspring of pair aAF+aAA (the example taken from [9]). (C) Allele sizes of a1 and f4 microsatellite markers in particular specimens from the above family and putative identification with genotypes 1–11 in Fig 2A (in yellow for particular markers and in green as most probable).

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hybrid-derived spermatozoa is indicative of gene transfer from Ef to Ea. This might be exemplified by hypothetically polymorphic genes responsible for Ef-specific striped body pigmentation pattern of the ‘tiger’ fFF grandparent mated with aAA earthworm, through the aAF parents (1) to some Ef-like earthworms (11) with mixed pigmentation pattern but genotyped as aAA by species-specific sequences of COI/28S genes [7, 8]



B) aAF₄₈+fFF₄₇ - fFA₁₀₈; fFA₁₄₃; fFF₁₅₈; fFF₁₄₄; fFF₁₆₀; fFF₁₆₁

C) markers earthworms	a5	Fig. 3Aa	f6	Fig. 3Aa	Fig. 3Aa
aAF₄₈	159; 167	1	177; 204	1	1
fFA ₁₀₈	167; 174	10	177; 177	10	10
fFA ₁₄₃	167; 174	10	177; 177	10	10
fFF₄₇	157; 174	8	177; 194	8	8
fFF ₁₅₈	159; 174	11	177; 194	9; 11	11
fFF ₁₄₄	157; 174	9	177; 194	9; 11	9
fFF ₁₆₀	157; 167	11	177; 204	11	11
fFF ₁₆₁	157; 174	9	177; 194	9; 11	9

Fig 3. Considerations on the results of mating the aAF hybrid with fFF parental specimen. (A) Back-cross of aAF +fFF pair; symbols as on Fig 1. (B) Offspring of aAF+fFF pair (the example from [7]). (C) Allele sizes of a5 and f6 microsatellite markers in particular specimens from the above family and putative identification with genotypes numbered 1–11 on Fig 3A (in yellow for particular markers and in green as most probable).

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Offspring of back-cross aAF+fFF. Among offspring of back-cross aAF+fFF (Fig 3A), cross-fertilized ova of aAF hybrids may give a new generation of aAF hybrids (6) and (unique) mitonuclearly-incompatible aFF specimens (number 7) very rare in our previous experiments [9] while cross-fertilized ‘fF’ ova of the fFF specimens can give fFA hybrids fertilized by ‘A’ spermatozoa from the aAF parent (number 10) and fFF specimens (number 11) fertilized by hybrid’s ‘F’ spermatozoa (Fig 3A). Such family is exemplified here by progeny of aAF48+fFF47

specimens from previous experiments [7] that gave two Ef-derived hybrids fFA and four fFF specimens (Fig 3B) among offspring. Analysis of distribution of a5 and f6 alleles was fully consistent with the only possibility of the origin of Ef-derived fFA hybrids, i.e. cross-fertilization of 'fF ova by aAF-derived 'A' spermatozoa (10) while new generation of aAF hybrids (6) was absent among progeny of this family (but they were present among offspring of other backcrossed aAF+fFF pairs from previous studies [9]).

There are two possibilities of the origin of fFF specimens among offspring of an aAF+fFF pair, i.e. either self-fertilization (9) or cross-fertilization of 'fF' ova by hybrid-derived 'F' spermatozoa (11). The a5 microsatellite marker, being heterozygous in both parental species, has shown unequivocally that fFF144 and fFF161 originated by self-fertilization (9) while fFF158 and fFF160 came from cross-fertilization of 'fF' from the fFF parent by hybrid-derived 'F' spermatozoa (11). In the case of the heterozygous f6 marker, allele 177 was present in all members of this family thus more genotypic combinations were possible, but only those shared by both markers (a5 and f6) are considered a likely explanation of the origin of fFF offspring (last column of Fig 3C).

These results might shed more light on the transfer of putative genes responsible for fluorescence of coelomic fluid, being a fingerprint of aAA specimens [39]. Fluorescence is present in most aAF hybrids and in further backcrosses with fFF specimens (devoid of fluorescence), it seems to be acquired by rare fFA and fFF earthworms [4, 8], a phenomenon worthy of additional investigation.

Microsatellite markers and earthworm reproduction

Application of microsatellite markers to laboratory-paired virgin aAA+fFF, aAF+aAA and aAF+fFF earthworms and their progeny have fully confirmed previously postulated genotypic combinations of their offspring [7–10]. Moreover, we demonstrate that self-fertilization is not the only pathway for the production of a new generation of pure aAA and fFF specimens, since cross-fertilization of parental specimens significantly contributes to this process. We show that cross-fertilization resulted in interspecific gene flow from pure-species grandparents (P generation) through interspecific fertile aAF hybrids (F1 generation) to 'pure' aAA or FF specimens (F2 generation), inheriting the grandparents' markers. This genotyping strategy might provide a means for linking some of microsatellite markers with physiologically important genes and, i. e. help to explain previously described inheritance patterns of body pigmentation or production of some fluorescent markers [4, 7, 8]. On the other hand, Novo et al. [18] revealed that microsatellite markers are a valuable tool for studies on paternity of the earthworm *Hormogaster elisae* after copulation with three successive partners. Microsatellite markers applied to field-sampled earthworms from various localities might reveal signs of past hybridization and thus shed more light on the evolution of these species [6, 16].

In conclusion, the novel polymorphic microsatellite markers for *E. fetida* and *E. andrei* are potentially useful for the analysis of genetic diversity, population structure and dispersal including past and present hybridization events in natural populations and are valuable in laboratory studies on the controlled reproduction and gene flow between these two hermaphroditic species. *E. fetida* and *E. andrei* may be considered as potential ring species [40] attractive for studies on speciation within the *Eisenia* complex [41, 42] due to their capacity for hybridization, which is increasingly being appreciated as a potent driving force of evolution [e.g. 43].

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Formal analysis: Marta Jaskulak, Maxime Pauwels.

Funding acquisition: Barbara Plytycz.

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