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Tracking Introgression of Quinazoline M-Fluorophores from *Eisenia andrei* to *E. fetida* via Interspecific Hybrids of these Hermaphroditic Lumbricid Earthworms

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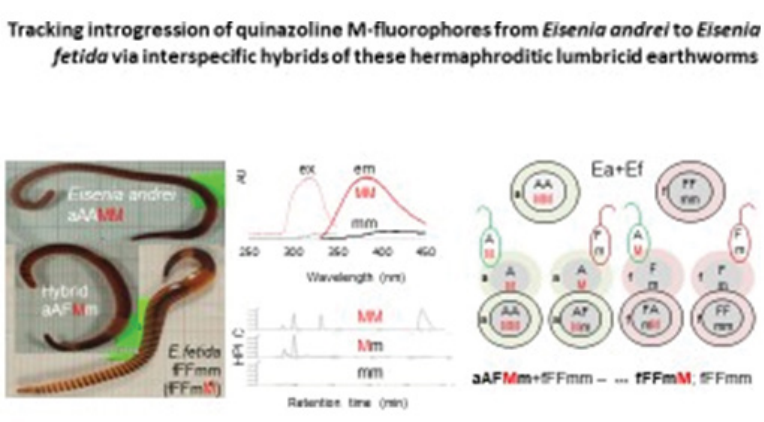
Abstract

Characteristic M-fluorescence detected in earthworm coelomic fluid by spectrofluorometry was originally considered as a taxonomic molecular marker of *Eisenia andrei* (Ea), but later it was detected also in some *E. fetida* (Ef) specimens and interspecific hybrids. It was assumed that M-fluorescence is encoded/controlled by hypothetical dominant/recessive M/m alleles. HPLC analysis revealed quinazoline origin of M fluorescence therefore that method was chosen to re-evaluate hypothetical gene flow from Ea to Ef via interspecific hybrids by parallel spectrofluorometric and HPLC analyses of coelomic fluid samples. These samples were retrieved from Ea (aAA), Ef (fFF), and hybrid specimens (aAF/fFA) genotyped by species-specific sequences of the haploid mitochondrial COI gene of maternal origin and the diploid 28S rRNA gene of maternal/paternal origin. The obtained results confirmed that quinazoline M-fluorophore might be transferred from Ea via Ea-derived hybrids to Ef and then fixed in the Ef species. The origin and biological function(s) of quinazoline M-fluorophore or its derivatives should be discerned.



Graphical Abstract

Highlights

Eisenia andrei (Ea) and *E. fetida* (Ef) are hermaphrodites capable of hybridization

Quinazoline M-fluorescence is detected by HPLC and/or spectrofuorometry

M fluoresence is present in coelomic fluid of almost all Ea and Ea-derived hybrids; M fluorescence is present in coelomic fluid of a few Ef and Ef-derived hybrids

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Hybrids transferred hypothetical gene(s) responsible for M fluorescence from Ea to Ef

Introduction

Hermaphroditic lumbricid earthworms from *Eisenia sp*., popularly known as 'red worms' *Eisenia andrei* (Ea) and striped 'tiger or brandling worms' *Eisenia fetida* (Ef), are valuable model species for studies on immune system relying primarily on cells and molecules present in their coelomic cavity, e.g. [1-5]. Earthworms with intermediate pigmentation patterns, e.g. [6-7], and the presence of Ea-specific M-fluorescence in coelomic fluid of some striped earthworms [8-9] strongly suggested hybridization between Ea and Ef; that has been already proven by biochemical profiles of tissue extracts and coelomic fluid of *Eisenia* sp. [10] and the results of genetic analyses of field-sampled earthworms from Scandinavia [11]. The existence of fertile interspecific hybrids between Ea and Ef from Polish and French laboratory stocks has been proven during controlled laboratory mating of three successive generations of genotyped specimens. They were delimitated as aAA (Ea), fFF (Ef), aAF (hybrids from Ea ova) or fFA (hybrids from Ef ova) by species-specific 'a' or 'f' sequences of mitochondrial COI gene of maternal origin and nuclear 'A' or 'F' sequences of 28S rRNA gene of maternal/paternal origin [7, 12-14]. Hybridization patterns established during such genotyping have been confirmed by recent analysis of microsatellite markers in DNA samples [15]. During studies on progeny of controlled mating of Ea+Ef pairs it turned out that coelomic fluid of nearly all aAA specimens and aAF hybrids exhibited the M-fluorescence, while vast majority of fFF or fFA were M-negative with only a few M-positive exceptions [12-13]. Analysis of genealogy of investigated specimens led us to the assumption that the hypothetical M-fluorophore might be encoded/controlled by a hypothetical gene with a dominant 'M' and recessive 'm' allele being responsive for M-positivity in 'MM' and 'Mm' animals, while earthworms with two recessive 'mm' alleles were M-negative. The same analysis revealed that M/m alleles segregate independently from the nuclear AA, FF or AF alleles [12]. The name of M fluorophore was originally taken from 4-methylumbelliferyl-β-D-glucuronide (MUGlcU) as originally suggested by [16], but later the factor/s present in coelomic fluid of Ea was identified as SP-8203 compound composed of two quinazoline-2,4-dione rings connected by spermine linker [17-18] and its derivatives [18]. Very distinct HPLC chromatograms of coelomic fluid pooled from one hundred adult Ea specimens have opened a new opportunity of looking for quinazoline M-fluorophores in earthworm coelomic fluid [18]. The main aim of present investigations was to re-evaluate hypothetical gene flow from Ea to Ef via interspecific hybrids by parallel spectrofluorometric and HPLC analyses of coelomic fluid samples retrieved from genotyped Ea (aAA), Ef (fFF), and hybrid specimens (aAF/fFA). It turned out, that both methods of detection of quinazoline M-fluorescence combined with earthworm genotyping by both mitochondrial and nuclear sequences are useful for tracking the gene flow.

Materials and Methods

Earthworms

Eisenia andrei (Ea) and *Eisenia fetida* (Ef) lumbricid earthworms were obtained from three different sources: 1) laboratory cultures in the Lille University (France); 2) wastewater treatment plant in Kluczbork (Poland); and 3) seminatural manure and compost cheap localized close to moist forest in Jasienczyk (Poland). These earthworms were reared for generations in the Institute of Zoology and Biomedical Research of the Jagiellonian University, Krakow, Poland, since 2011. Upon arrival to Krakow laboratory, the earthworm specimens with most distinct and discriminatory coloration patterns were genotyped using species-specific DNA sequences of mitochondrial COI gene 'a' (for Ea) or 'f' (for Ef) from amputated tail tips [6, 8-9] and their progeny cohorts were reared in boxes filled with soil from a commercial supplier (PPUH Biovita, Tenczynek, Poland) marked by the name of species and its origin and fed ad libitum on mixed diet comprised of wheat flour, boiled/dried/powdered tea, nettle (*Urtica dioica*) and dandelion (*Taraxacum officinale*) leaves, and powdered mice food pellets. During present experiments, 62 earthworms were used as a source of coelomic fluid. Among them, 44 specimens from Lille were involved in studies on reproductive activity of subsequent generations of hybrids and their offspring [7, 12-13], while 18 specimens were progeny of earthworms originally collected from seminatural field populations, 9 Ef from Jasienczyk (J) and 9 Ef from Kluczbork (K). Pigmentation patterns were photographically documented with the DSL camera (Sony SLT-A58).

Genotyping

Supravitally amputated tail tips were fixed in 80% ethanol and used for DNA extraction, PCR amplification and sequencing by the methods described earlier [7]. Tail tips of *Eisenia* sp. earthworms efficiently regenerate [9] thus for some specimens amputation was repeated. On a basis of species-specific sequences of mitochondrial COI gene of maternal origin ('a' or 'f') and nuclear 28s rRNA gene of maternal/paternal origin ('A' or 'F'), earthworm genotypes were as follow: 'aAA' for Ea, 'fFF' for Ef or f2FF for the second lineage of Ef called Ef2, and aAF or fFA/f2FA for their hybrids. First two letters ('aA', 'fF' or 'f2F') indicate genotypes of ova, while the third letter indicates genotypes of spermatozoa; for example, the earthworms genotyped as aAF derived from 'aA' ovum of the Ea parent fertilized by 'F' spermatozoon of Ef parent.

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Coelomocyte-containing coelomic fluid

Retrieval of coelomocyte-containing coelomic fluid was performed individually from all 62 earthworms by the method described earlier [12]. In short, after overnight depuration on moist filter papers, earthworms were immersed in 3 mL 0.9% Natrium chloratum (Kutno, Poland) and electrostimulated for 30 sec with a mild electric current (4.5V) for induction of coelomic fluid extrusion through dorsal pores. After fluid extrusion the earthworms were returned to their original boxes. For disruption of extruded coelomocytes, samples of coelomic fluid from particular earthworms were lysed by Triton (T) for spectrofluorometric analysis or disrupted by freezing-thawing (F) that is useful for both spectrofluorometry and HPLC analyses. A few control samples were divided into two parts for quantitative comparison of the results obtained by T and F methods.

Triton lysates

As described previously [12], one mL of the extruded coelomocyte-containing coelomic fluid was supplemented with 20uL of Triton (Sigma-Aldrich) and shaked for 20 min on Elpon Laboratory Shaker type 358S to dissolve cellular components. Then samples were adjusted with PBS to 2 mL and final 1% Triton lysates were analyzed using Perkin-Elmer Spectrofluorometer LS50B.

Freeze-thaw disruption

As described previously [18], the solutions were treated twice by freeze-thaw cycles in liquid nitrogen. After centrifugation (5000*g* x 10 min) the sediment was discarded, while the supernatants from 47 earthworms were divided into two parts, one of them used for HPLC and the second for spectrofluorometry.

Spectrofluorometry

Fluorescence spectra were measured using Perkin-Elmer LS50B spectrofluorometer. As previously described [12], emission spectra of quinazoline M-fluorophores were recorded between 340 and 480 nm (excitation at 320 nm) while excitation spectra between 260 and 360 nm (emission at 380 nm). In the M- positive samples, intensity of excitation and emission peaks were almost identical. As an additional control, fluorescence spectra of M-fluorophore were compared in samples prepared by the T or F methods performed on fluids from the same earthworms.

HPLC

As described in detail previously [18], HPLC was performed using Acclaim C30 reverse-phase column (4.6 x 250 mm, 5 µm) developed 0.1% formic acid/ acetonitrile (82.5/17.5 v/v) at the flow rate 1.2 ml/min and 100 µl injection loop. Absorption at 320 nm and fluorescence at 320/390 nm excitation/emission was monitored.

Results and Discussion

Detection of quinazoline M-fluorophores by spectrofluorometric and HPLC analyses

As described in our previous papers [12], extruded coelomic fluid of the M-positive (Mp) and M-like positive (Mlp) earthworms exhibited distinct fluorescence spectra with an excitation peak at 314-320 nm (λem= 380 nm) and an emission peak at 370-380 nm (λex= 320 nm), that are absent in M-negative (Mn) earthworms. Maxima of emission and excitation were almost identical in particular M-positive samples being either relatively high (Mp), relatively low (M-like positive, Mlp) or absent in M-negative (Mn) specimens, being hypothetically controlled/encoded by MM, Mm, or mm alleles (Figure 1A). Maxima of excitation/ emission peaks in both parts of the sample divided into equal parts processed either by Triton lyses or freeze-thaw disruption method were very similar (S1), thus the results obtained by these two methods may be considered as equivalent. The representative HPLC chromatograms of coelomic fluid extracts from *Eisenia* sp. earthworms are shown on Figure 1B. The first (top) chromatogram in Figure 1B, showing several fluorescent compounds with the most distinct peak (called the compound 6) at the longest retention time, is the most similar to the chromatogram from pooled 100 specimens of *E. andrei* [18] used for analysis of chromophores responsible for the fluorescence, i.e. substance SP-8203 (containing quinazoline) and its derivatives, hypothetically encoded/ controlled by the dominant 'MM' alleles. In the third (bottom) chromatogram in Figure 1B only minuscule fluorescent peaks are found showing that the extract has been derived from the earthworm without M-fluorescence, hypothetically possessing two recessive 'mm' alleles. Chromatogram in the middle part of Figure 1B has small peak 6, but distinct peaks at shorter retention times, hypothetically coming from 'Mm' alleles (Figure 1B).

Individual chromatograms of samples from each of 47 *Eisenia* sp. earthworms genotyped as aAA, aAF, fFF/ f2FF, and f2FA are included in the Supplementary Figure (S2). All but one of 14 aAA specimens are M-positive and hypothetically possess two dominant 'MM' alleles, but the one specimen is obviously M- negative (with two 'mm' alleles) (S2A). All of 11 Ea-derived aAF hybrids are M-positive, but hypothetically seven of them are encoded by 'MM' alleles and four by 'Mm' (S2B). All but two of 16 fFF/f2FF specimens are M-negative with 'mm' alleles; one of M-positive fFF seem to be evidently M- positive with 'MM' alleles, and the second one seems to possess 'mM' alleles (S2C). Both HPLC and spectofluorometry show that two out of six Ef derived f2FA hybrids are M-positive with 'mM' alleles, and remaining four f2FA earthworms have none of M-derived peaks at 320/380 (exc./em.); these four f2FA earthworms hypothetically possess two recessive 'mm' alleles despite of some distinct HPLC peaks appearing at

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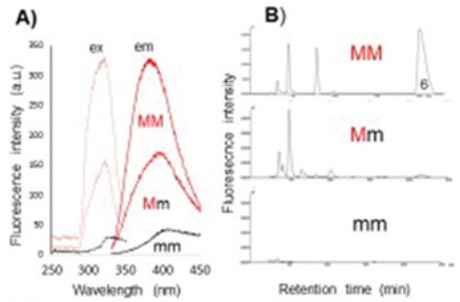

HPLC/ Mp/Mp	Numbers of earthworms				
	aAA	aAF	fFA	fFF	total
MM	13	7	0	1	21
Mm	0	4	2	1	7
mm	1	0	4	14	19
Total	14	11	6	16	47

Figure1: Quinazoline M-fluorescence in *Eisenia andrei* (aAA), *E. fetida* (fFF) and their hybrids (aAF and fFA) being either MM homozygotes, mm homozygotes or Mm heterozygotes in respect of hypothetical gene responsible for M-fluorescence. A) Fluorescence excitation (ex) and emission (em) spectra. B) HPLC spectra. C) Numbers of earthworms of hypothetical MM, Mm, and mm genotypes/phenotypes.

short retention times on their chromatograms, that probably derive from fluorophores other than the M-fluorophore [18] (S2D). The main numerical data are included in the inset (table) of Figure 1C.

Phylograms of investigated specimens

Phylogram of 47 earthworms arranged on the basis of mitochondrial COI gene of maternal origin contains 25 specimens on the clade derived from *E. andrei* ova (14 aAA and 11 aAF), and 22 from *E. fetida* ova, with two distinct sublines, 5 Ef1 (fFF) and 17 Ef2 (f2FF). Similar clades of phylograms of *Eisenia* sp. earthworms obtained from Polish and French populations were constructed during our previous experiments, e.g. [7, 13-14]. During present studies, all but one aAA specimen (arrow at Ea clade) and all Ea-derived aAF hybrids were M-positive, while most Ef earthworms were M-negative. The "aberrant" M-negative Ea earthworm (arrow at Ea clade of Figure 2A) with putative genotype aAAmm appeared for the first time during our long-lasting investigations on M-fluorophore considered to be molecular marker of Ea [16].

Out of 22 Ef specimens, only two f2FA hybrids and two fFF specimens were M-positive. Chromatogram of earthworm fFF484/495/511 (three times genotyped, arrow at Ef clade of Fig. 2A) combined with high M-derived fluorescence intensity (intensity 389) (S2C) suggested that this fFF earthworm possesses unique for this species two dominant alleles MM, thus its putative genotype might be fFFMM.

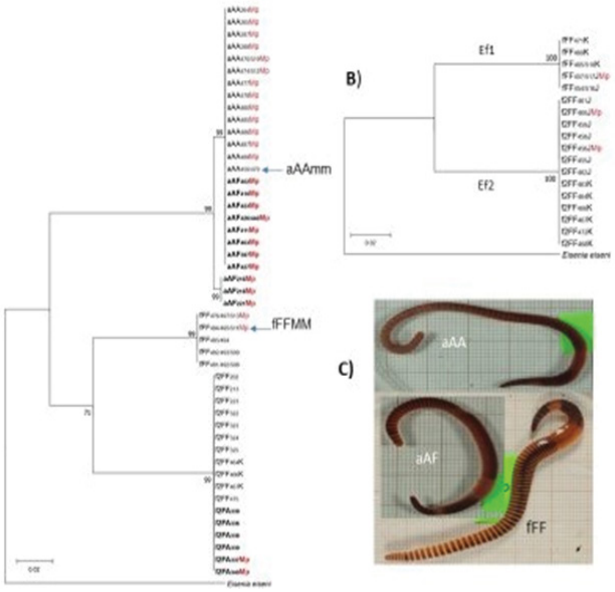

Figure 2: Phylograms and photos of representative earthworms *Eisenia andrei* (aAA), *E. fetida* (fFF and f2FF) and interspecific hybrids (aAF and f2FA), some of them with quinazoline M-fluorophore (Mp), investigated during present experiments. A) specimens tested by spectrofluorometry and HPLC; B) specimens tested by spectrofluorometry only. C) pigmentation patterns of representative specimens.

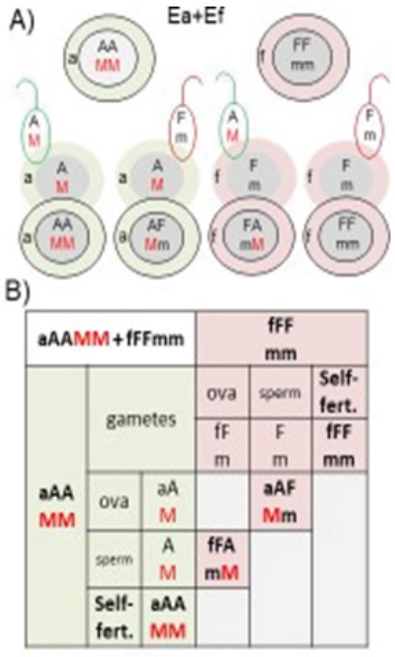

Figure 3: Hypothetical genotypes of Ea (aAAMM), Ef (fFFmm) pair and their offspring. A) Scheme parental cells, their gametes (ova, spermatozoa) and zygotes. B) Punnett squares adapted to pair of hermaphroditic earthworms capable to self-fertilization with assumption that quinazoline M-fluorescence is encoded/controlled by dominant 'M' allele and recessive 'm' allele segregating independently from the nuclear A/F sequences of 28S rRNA gene.

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During present investigations, 18 earthworms derived from semi-natural stocks in wastewater treatment plant in Kluczbork (K) and manure and compost cheap close to moist forest in Jasienczyk (J) were genotyped as fFF or f2FF, i.e. belonged to two distinct mitochondrial sublines of Ef genealogical tree while spectrofluorometric analysis revealed among them three M-positive specimens (Figure 2B). Existence of hybrids is connected with appearance of earthworms with pigmentation patterns different from that characteristic for totally pigmented Ea and Ef with pigmentless bands extending from the furrows between segments (Figure 2C). Such atypical specimens were described earlier [12] and inclined also other investigators to consider them as Ea x Ef hybrids [19-20].

Genealogy of investigated specimens

Eisenia sp. belongs to hermaphrodites with cross fertilization, i.e. simultaneously transferring and receiving sperm during copulation. Spanish populations can reproduce uniparentally by self-fertilization of isolated virgin specimens [21] while isolated specimens from Polish and French populations have started reproduction only after copulation with the earthworm of the same species or closely related partner; offspring of such pairs originated either by cross-fertilization and/or 'facilitated self- fertilization' connected with a mixture of the own sperm with the partner's one [14].

In the cycle of experiments [12-13] we were dealing with first, second and third generations offspring of M-positive Ea and M-negative Ef parental specimens. In the Ea+Ef pair of earthworm, aAAMM specimen produces 'aAM' ova and 'AM' spermatozoa, whereas fFFmm partner produces 'fFm' ova and 'Fm' spermatozoa. The 'aAM' ova may be self-fertilized by 'AM' spermatozoa or cross-fertilized by 'Fm' spermatozoa giving new aAAMM earthworms and the first generation aAFMm hybrids. The 'fFm' ova self-fertilized by 'Fm' spermatozoa give new fFFmm specimens, while cross-fertilized by 'AM' spermatozoa should give fFAmM hybrids. In other words, among offspring of isolated Ea+Ef pair might be expected some self-fertilized aAAMM and fFFmm specimens and cross-fertilized aAFMm and fFAmM hybrids (Figures 3A, B) but the latter were so far absent among first generation offspring [7, 13-14].

Punnett squares from Figures 3B, 4 and 5 are adapted to pairs of hermaphroditic earthworms capable to self-fertilization with assumption that M-fluorescence is encoded/ controlled by dominant 'M' allele and recessive 'm' allele segregating independently from the nuclear A/F sequences of 28S rRNA gene.

The aAF hybrids produce four typed of ova ('aAM', 'aAm', 'aFM', 'aFm') and four types of spermatozoa ('AM', 'Am', 'FM', 'Fm'). They are fertile and gave progeny when back-crossed with Ea (Figure 4A) or Ef specimens (Figure 4B) accompanied by their siblings derived by self-fertilization (Figure 4C). So far only sterile cocoons were laid by hybrid-hybrid pairs [13-14] thus such possibility is excluded here.

Partner-induced facilitated self-fertilization of aAFMm hybrids (Figure 4C) leads to development of aAA, aAF/aFA (impossible to be distinguished by present methods), and aFF specimens, the latter with cyto-nuclear incompatibility [13, 22], each of them being either M-positive (MM or Mm) or M-negative (mm). Among them have appeared the "aberrant" M-negative aAAmm specimen (encircled on Figure 4C and marked by arrow at the Ea clade of Figure 2A) developed by self- fertilization of 'aAm' ova of aAFMm hybrid by its own 'Am' spermatozoon carrying the recessive 'm' allele (Figure 4C). Theoretically possible self-fertilized M-negative hybrids aAFmm/aFAmm were absent among specimens tested during our investigations in respect of M-fluorescence.

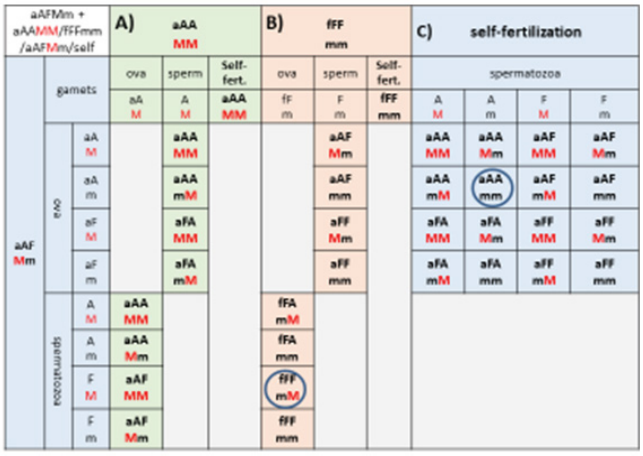

Figure 4: Hypothetical genotypes of the offspring of test-crosses of aAFMm hybrid with parental specimens: A) aAAMM, B) fFFmm, C) self-fertilized. Symbols are the same as on Figure 3.

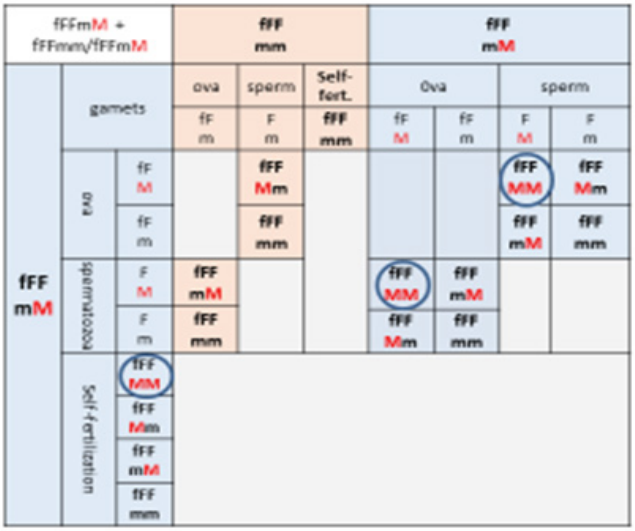

Figure 5: Hypothetical genotypes of the offspring of fFFMm specimen with fFFmm or fFFMm or self-fertilized. Symbols are the same as on Figure 3.

Among offspring of aAFMm+aAAMM pair may be present exclusively M-positive (MM and Mm) specimens, both self- and cross-fertilized aAAMM/aAAMm of the "pure" Ea species and cross-fertilized aAF/aFA hybrids (impossible to distinguish), either MM or mM/Mm (Figure 4A). Among offspring of aAFMm+fFFmm pair may be present M-positive or M-negative aAF/fFA hybrids and aFF specimens with cytonuclear incompatibility [13, 22], and also new generation of self-or cross-fertilized fFFmm and some M-positive fFFmM earthworms (Figure 4B). The M allele once introduced to Ef earthworm by cross-fertilization of 'fFm' ovum by hybrid- derived 'FM' spermatozoon (encircled on Figure 4B) may be fixed in Ef population by facilitated self- fertilization or cross-fertilization of fFFmM earthworm paired with fFFmm or another fFFmM specimen (Figure 5). Among their progeny there are Ef earthworms with two MM alleles, fFFMM, encircled on Figure 5, and one of them marked by arrow on the Ef branch of Figure 2A.

During our cycle of experiments, ancestors of earthworms from Jasienczyk and Kluczbork were sampled in 2013 from their dense semi-natural stocks and hand-sorted on a basis of external appearance into Ea and Ef. This was initially verified by mitochondrial 'a' and 'f' sequences of COI gene, as we were then unaware on possibility of hybridization of these species, e.g. [4, 8]. The M-derived fluorescence in some descendants of these field-derived earthworms genotyped only by the 'f' sequence of mitochondrial COI gene might be signatures of past hybridization in nature (Figure 2C). Signs of the past hybridization of Ea and Ef in indoor and outdoor compost populations in Sweden and Norway were reported by [11].

Evolutionary considerations

Ea and Ef inhabit organic matter rich location at high population densities [14, 23], thus formation of interspecific Ea+Ef pairs may be quite common. Hermaphroditic earthworm species exhibit multiple mating to increase reproductive success [24-25] and sperm from multiple males with the admixture of the own sperm may be stored in spermatheca.

As a result the one cocoon may contain ova fertilized by spermatozoa of various partners. From this reason hybridization may be more common in nature that that recorded in our laboratory experiments focused on the offspring of permanent partners selected by investigator. During our laboratory studies reproductive activity of Ea-derived hybrids was impaired in the second and third generation [13], but in a dense colony the earthworms have opportunity of partners' selection and exchange, thus gene transfer and introgression may be continued. The theoretical Punnett's squares (Figures 3B, 4, 5) show much more possibilities of progeny of the initial Ea+Ef pairs than those recorded in our experiments, and hypothetical progeny of hybrid+hybrid pair would further increase these possibilities. Metabolic origin and physiological function of the M-fluorophores is still an open question. It is known that quinazolines have potent antimicrobial and cytotoxic activities [26-29] that might be at least partly responsible for better viability and higher fecundity of *E. andrei* than those of *E. fetida* [7, 14, 20, 23, 30-31]. Hypothetical participation of microbiome-derived factors from bacterial symbionts of *E. andrei* [32-34] in production of M-fluorophore cannot be neglected [12].

Conclusions

Both spectrofluorometry and HPLC are suitable for the detection of quinazoline M-fluorophores in the earthworm coelomic fluid. The results of investigations from the present paper fully confirmed that the hypothetical gene/s encoding/ controlling the presence of M-fluorophore in coelomic fluid of Ea might be transferred via Ea-derived hybrids mated with Ef to some pure Ef specimens and then be fixed in Ef population. The present results might inspire the further research on 1) metabolic pathway/s leading to M-positive phenotype; 2) studies on possible biological function/s of the M-fluorophore itself or factors connected with its metabolism; 3) application of M-fluorophore in screening of laboratory/field populations of *Eisenia* sp. in respect of hybridization; 4) studies on the ongoing evolution of *Eisenia* sp. 5) Ea and/or Ef are used as a source of biologically active substances for scientific and/or paramedical applications thus a possibility of "contaminations" from another species due to their hybridization shall be always seriously considered and controlled.

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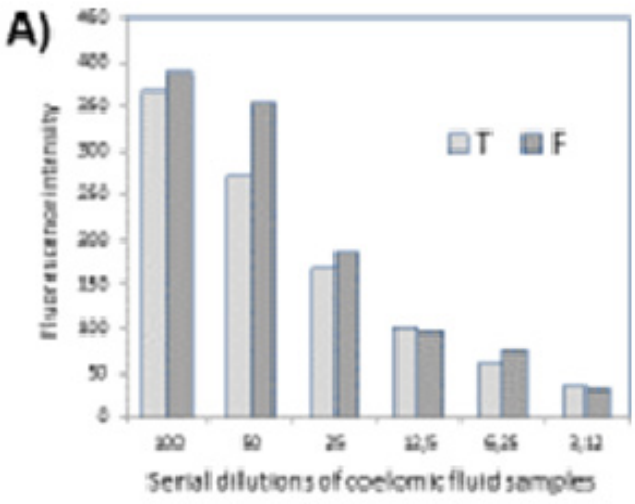

Figure S1: Comparison of the results of intensity of M-fluoresence in samples prepared by Triton solubilization (T) and Freezing-Thawing (F).

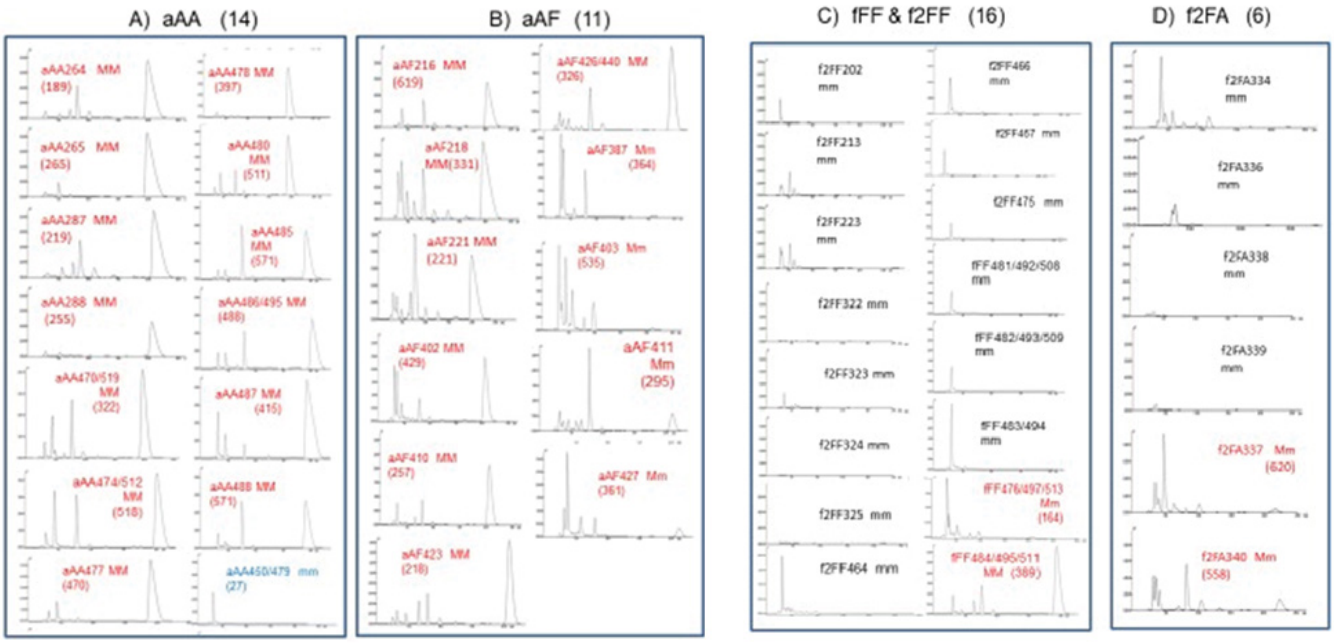

Figure S2: HPLC chromatograms of fluorescent compounds of coelomic fluid from A) aAA; B) aAF; C) fFF/f2FF; D) f2FF.

All sequences are deposited in GenBank under the following accession numbers: MT646844-MT646845, MT649311-MT649312 (Jaskulak et al. 2021); MT090084-MT090085, MT090092, MT090097-MT090098, MT090102-MT090105, MT090118-MT090119, MT126829-MT126830, MT126849, MT126867, MT126902-MT126903, MT126906-MT126909, MT126914-MT126916, MT126927, MT126955-MT126956, MT126959-MT126962, MT126964, MT126976-MT126979, MT126989, MT133075, MT133095-MT133098, MT133108, MT133119, MT133121, MT133123, MT133125, MT133195,

MT133201, MT133203, MT133205, MT133207, MT133209 (Plytycz et al. 2020); and for new sequences (numbered from BP450): OK268027-OK268039, OK268052-OK268073, OK268081-OK268094, OK275549-OK275561, OK275586-OK275607, OK275617-OK275628.