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Annual Reproductive Performance of *Eisenia andrei* and *E. fetida* 2 in Intra- and Inter-Specific Pairs and Lack of Reproduction of Isolated Virgin Earthworms

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Original article

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Eisenia andrei/fetida complex of lumbricid earthworms contains *E. andrei* (Ea) and two mitochondrial lineages of *E. fetida* (Ef), referred to as Ef1 and Ef2. These earthworms are hermaphrodites capable of self-fertilization and hybridization as evidenced in laboratory mated earthworms from Ea and Ef1 lineage of Ef. The aim of the present investigations was to compare reproductive performance of Ea and Ef2 lineage from French laboratory stocks reared for a decade in Polish laboratories. These were cultured either in isolation and/or in intra-specific or inter-specific pairs for up to 57 weeks from hatching. Parental specimens and offspring were identified by species/lineage-specific sequences of the haploid mitochondrial COI gene, either 'a' or 'f2', and species-specific sequences of the nuclear 28s rRNA gene, either 'AA' or 'FF', thus delimitated as aAA or f2FF for Ea or Ef2, respectively, or aAF for hybrids. Isolated virgin earthworms produced a few sterile cocoons only, more frequently in Ef2 than in Ea, but no hatchlings. Analysis of cocoon production and reproduction of laboratory-mated intra-specific Ea+Ea and Ef2+Ef2 pairs revealed higher fecundity of Ea than Ef2 measured by numbers of cocoons and hatchlings, while inter-specific Ea+Ef2 pairs gave plenty cocoons but low numbers of aAF hybrids developed from Ea ova fertilized by Ef2 spermatozoa.

Key words: lumbricid earthworms, cocoons, hatchlings, hybridization, self-fertilization.

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Lumbricid earthworms *Eisenia andrei* (Ea) and *Eisenia fetida* (Ef) are important model species in various disciplines such as comparative immunology (e.g. DVORAK *et al.* 2013), ecotoxicology (e.g. COELHO *et al.* 2018), biomedicine (e.g. LI *et al.* 2011) and vermicomposting (e.g. SULEIMAN *et al.* 2017; DOMINGUEZ & EDWARDS 2011), thus their proper identification is crucial for scientific purposes. It turned out, however, that precise delimitation of these species is not simple on the basis of morphological criteria and requires modern molecular tools. A phylogenetic tree based on mitochondrial *COI* genes distinguishes among them the Ea branch and Ef branch, the latter with two distinct lineages, Ef1 and Ef2, considered as hypothetical cryptic species (ROMBKE *et al.* 2016) but such supposition did not find support by other studies (MARTINSSON & ERSEUS 2018). The Ea and Ef branches with two clusters within the latter were consistently formed on *COI*-based phylograms of lumbricid species genotyped for proper identification of earthworms investigated during our previous studies (RORAT *et al.* 2014; SANTOCKI *et al.* 2016; SWIDERSKA *et al.* 2017; PLYTYCZ *et al.* 2016, 2018a).

Most recently, inter-specific hybrids were detected in natural populations of Ea and Ef from Scandinavia (MARTINSSON & ERSEUS 2018) and the fertile hybrids were found during controlled laboratory mating between Ea and the Ef1 mitochondrial lineage of Ef from French/Polish laboratory stocks. The latter were genotyped by both mitochondrial COI genes ('a' or 'f/f2') and diploid nuclear genes ('A' or 'F') as aAA (Ea) or fFF/f2FF (Ef1/Ef2), or as interspecific hybrids derived from Ea cocoons (aAF) or Ef1 cocoons (fFA) (PLYTYCZ *et al*. 2018a,b). In contrast, interspecific hybrids were absent in Ea and Ef earthworms from Spain and Brazil (DOMINGUEZ *et al*. 2005). On the other hand, Spanish specimens of Ea and Ef were capable of uniparental reproduction due to self-fertilization, since isolated virgin earthworms produced cocoons and hatchlings (DOMINGUEZ *et al.* 2003), while such phenomenon was absent in *Eisenia* sp. cultured in Polish laboratories (unpublished observations). Therefore, further studies on reproduction of these hermaphroditic species capable of self-fertilization were worthwhile.

Present investigations conducted on Ea/Ef2 earthworms from French/Polish laboratories focused on (1) reproductive capabilities of virgin specimens living over one year in isolation; (2) comparisons of cocoon production and hatchability between intra-specific (Ea+Ea and Ef2+Ef2) and inter-specific (Ea+Ef2) pairs of earthworms investigated throughout whole year under the same laboratory conditions.

Material and Methods

Earthworms

Adult composting *E. fetida* and *E. andrei* earthworms deriving from laboratory stocks at the University in Lille (France) were cultured for a decade in the laboratory of the Institute of Zoology and Biomedical Research of the Jagiellonian University (Krakow, Poland). The present investigations were performed in the laboratories of Rzeszow University (Poland) and Jagiellonian University (Krakow, Poland) on earthworms cultured in boxes with commercial soil (Kronen Universalerde: pH ($CaCl_2$) 5.5-6.5; N – 200-450 mg/l; P_2O_5 – 200-400 mg/l; K_2O – 300-500 mg/l) at room temperature and fed *ad libitum* on boiled/dried tea, nettle and dandelion leaves.

Experimental scheme

E. andrei (Ea) and *E. fetida* (Ef) were identified by specific sequences of mitochondrial COI gene and nuclear 28S gene as Ea, Ef1, and Ef2 (PLYTYCZ *et al.* 2018a) and progeny of these three groups were cultured separately.

Reproduction of isolated and paired Ea and Ef2 earthworms

Freshly hatched specimens of Ea and Ef2 (0.18±0.03 g body mass) were put into boxes (150 ml) with soil (100 g) either individually, 6 Ea, and 5 Ef2, or in pairs, either intra-specific, 6 (Ea+Ea) and 5 (Ef2+Ef2) or inter-specific, 7 (Ea+Ef2). Cultures were maintained throughout a year (from 23 March 2017 till 26 April 2018). During this period, weighed earthworms were eight times transferred to boxes with new soil either individually (Ea and Ef2) or, in the case of pairs, either with original partners (Ea+Ea; Ef2+Ef2, Ea+Ef2) or temporarily separated (Ea-Ea; Ef2-Ef2, Ea-Ef2) to two boxes, and then rejoined with the same partners. The 'old' soil was kept in the 'old' boxes for manual counting of cocoons and hatchlings, and the latter were cultured further, each specimen in the individual box with fresh soil, and their tail tips were cut for genotyping.

Earthworm genotyping

Amputated posterior segments were ethanol fixed and used for genotyping in respect of mitochondrial *COI* sequences as 'a' (for Ea) or 'f2' (for Ef2), and the nuclear 28S sequences as AA (Ea) or FF (Ef) pure species or AF/FA hybrids, thus earthworms may be identified as aAA, f2FF, or aAF/fFA specimens. DNA extraction, PCR amplification and sequencing were performed as described previously (PLYTYCZ *et al.* 2018a).

Statistical analysis

The results are presented as means and standard deviations ($\bar{x}$±SD). STATISTICA v. 10 (StatSoft) was used for statistical analyses. Before using the proper tests, it was checked whether the data distributions were consistent with the normal distribution (Shapiro-Wilk test) and whether the variances in the groups were homogeneous (Brown-Forsyth test). For comparisons two groups t-test was used, for more than two groups ANOVA with LSD test were used. Differences were considered statistically significant at $\alpha<0.05$.

Results

Earthworm viability

Parental specimens were vital throughout a whole year of investigations except two Ef2 specimens from the Ef2+Ef2 group; their growth was inhibited versus their partners and they died before reaching maturity. Therefore, two pairs had to be excluded, and only three pairs Ef2+Ef2 were considered during final analyses.

Reproduction of single and paired Ea and Ef2 earthworms

a. Single earthworms

During the whole experimental period, i.e. by week 57 after hatching, only three cocoons appeared in soil from one of six Ea earthworms kept in isolation, but as many as 193 in two out of five isolated Ef2 earthworms. No hatchlings appeared in the whole experimental period (Table 1, 2). The differences of mean numbers of cocoons per earthworms were statistically insignificant between Ea and Ef2 earthworms (Table 1).

b. Pairs of earthworms

During the whole experimental period, i.e. till week 57 (Table 1, 2), the Ea+Ea, Ef2+Ef2, and Ea+Ef2 pairs of earthworms produced numerous cocoons (1664, 608, 1852, respectively) and hatchlings (1791, 425, 150, respectively). In a case of temporary separated pure species, Ea-Ea and Ef2-Ef2, cocoons and hatchlings appeared in soils from both separated partners. In a case of inter-specific pairs, cocoons were present in soils from both partners, Ea and Ef2, while all but one hatchlings appeared in soil from the Ea partner (Table 1).

Numbers of cocoons per pair were highest in Ea+Ea, intermediate in Ea+Ef2, and lowest in Ef2+Ef2 groups

Table 1

Cocoon production and hatchability of *E. andrei* (Ea) and *E. fetida* 2 (Ef2) earthworms kept from hatching to week 57 either individually (Ea or Ef2) or in pairs, either intra-specific (Ea+Ea) or (Ef2+Ef2), or inter-specific (Ea+Ef2). Within columns, different superscripts in capital letters show statistically significantly differences according to t-test (single specimens) and ANOVA and LSD test (pairs) ($p<0.05$)

Numbers of cocoons and hatchlings counted during 57 weeks after hatching ($\bar{x}$±SD)						
Groups (genotypes)	cocoons			hatchlings		
single	total	per earthworm		total	per earthworm	
6 Ea (aAA)	3*	$(0.5±1.2)^{A}$	p=0.131	0	0	
5 Ef2 (f2FF)	193**	$(38.6±56.8)^{A}$		0	0	
	cocoons			hatchlings		
pairs	total	per pair		total	per pair	
6 (Ea+Ea) (aAA+aAA)	1664	$(277.3±49.0)^{A}$	p=0.044	1791	$(298.5±145.0)^{A}$	p=0.001
3 (Ef2+Ef2) (f2FF+f2FF)	608	$(202.6±23.8)^{B}$		425	$(141.6±27.0)^{B}$	
7 (Ea+Ef2) (aAA+f2FF)	1852	$(264.6±59.0)^{AB}$		150***#	$(21.4±26.8)^{B}$	

* – all cocoons from one earthworm; ** – all cocoons from two earthworms; *** – one pair without hatchlings; # – almost all hatchlings from Ea cocoons

Table 2

Cocoon production and hatchability of particular *E. andrei* (Ea) and *E. fetida 2* (Ef2) earthworms kept from hatching to week 57 either individually (Ea or Ef2) or in pairs, either intra-specific (Ea+Ea) or (Ef2+Ef2), or inter-specific (Ea+Ef2). '–' not done

		Numbers of cocoons and hatchlings							
Earthworm/pair No.		1	2	3	4	5	6	7	total
Ea	cocoons	0	3	0	0	0	0	–	3
	hatchlings	0	0	0	0	0	0	–	0
Ef2	cocoons	0	0	126	67	0	–	–	193
	hatchlings	0	0	0	0	0	–	–	0
Ea+Ea	cocoons	305	192	292	300	300	275	–	1664
	hatchlings	311	49	476	267	261	427	–	1791
Ef2+Ef2	cocoons	230	188	190	–	–	–	–	608
	hatchlings	152	160	113	–	–	–	–	425
Ea+Ef2	cocoons	313	322	253	283	218	214	249	1852
	hatchlings	77	19	26	0	20	1	7	150

Table 3

List of species and GenBank accession numbers used in the present study. Accession numbers marked in bold correspond to the sequences obtained in the present study. In case of the *Eisenia* hybrids, all COI sequences belong to *andrei* haplotype; the 28S sequences were deposited as two haplotypes – *andrei* and *fetida*

Taxa	COI	28S	References
Eisenia andrei	**MN711555-MN711579**	**MN719731-MN719755**	present study
Eisenia fetida 2	**MN711580-MN711626**	**MN719850-MN719867**	present study
Eisenia hybrids	**MN711627-MN711644**	**MN719756-MN719849**	present study
Eisenia fetida 1	MG031096-MG031098	MG030880-MG030882	PLYTYCZ *et al.* 2018a
Eisenia eiseni	AY874488	–	PEREZ-LOSADA *et al.* 2005

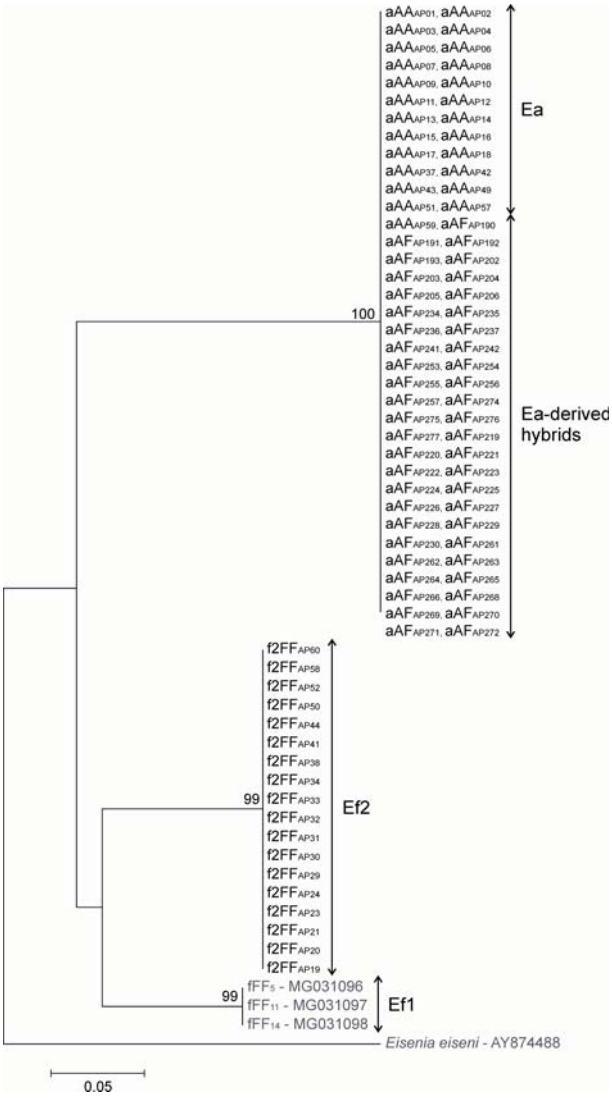

Fig. 1. Phylogram of genotyped parental *E. andrei* and *E. fetida* specimens and some of their offspring from present experiments. The maximum-likelihood phylogram for the 'a' or 'f2' COI gene combined with the 'A' or 'F' nuclear 28S rRNA genes of the same individuals with the same code. The reference sequences were marked in grey font. The *Eisenia eiseni* sequence was used as outgroups. All sequences included in Table 3 are deposited in GenBank.

(277.3±49.0; 264.6±59.0; and 202.6±23.8, respectively), while numbers of hatchling per pair during the whole experimental period was higher in Ea+Ea (298.5±145.0) than in the both Ef2+Ef2 (141.6±27.0) and Ea+Ef2 (21.4±26.8) groups (Table 1).

Hatchlings were absent in one of seven Ea+Ef2 pairs (Table 2). As evidenced by counting hatchlings from temporary separated Ea+Ef2 pairs, all but one early dying hatchlings derived from cocoons produced by the Ea partner. Among 150 offspring of the AF2 pairs, 47 specimens have been genotyped so far, and all of them were identified as Ea-derived hybrids aAF (Fig. 1).

c. Earthworm genetic identification

On the phylogram of earthworms used in present experiments, the Ea cluster contained both the pure aAA specimens and Ea-derived aAF hybrids, the latter being the first generation offspring of Ea+Ef2 pairs, while the Ef2 cluster contained pure f2FF (Ef2) specimens. Some reference fFF (Ef1) specimens were added from France-derived stocks (Fig. 1).

Discussion

Contrary to data concerning uniparental reproduction of hermaphroditic *E. andrei* and *E. fetida* earthworms from Spanish populations (DOMINGUEZ *et al.* 2003), French-derived specimens of Ea and Ef2 from the present experiments did not reproduce throughout a whole year when kept from hatching in isolation. During such a long time virgin Ea and Ef2 produced only a few sterile cocoons, more frequent in Ef than Ea, but this difference was statistically insignificant. Other earthworms were kept in isolation for about three months from hatching giving a few sterile cocoons only, but started cocoon production and reproduction soon after joining them with closely related partner (in preparation). Hypothetically, reproduction might be stimulated by copulatory behavior that is governed by a neuroendocrine network well devel-

oped in lumbricid species (e.g. OUMI *et al.* 1996; FUJINO *et al.* 1999; KAWADA *et al.* 2004; WILHELM *et al.* 2006; HERBERT *et al.* 2009; OKRZESIK *et al.* 2013; PLYTYCZ *et al*. 2016).

Higher fecundity of Ea than Ef were previously described in several populations (ELVIRA e*t al.* 1997; DOMINGUEZ *et al*. 2005), and its seasonal changes were recorded (PULIKESHI *et al.* 2003; BIRADAR & AMOJI 2003; MONROY *et al.* 2006) but without distinction of two lineages of the Ef complex. In present investigations, the numbers of cocoons were similar in Ea and Ef2, while their hatchability was higher in the former.

Interspecific pairs of Ea with either Ef1 (PLYTYCZ *et al.* 2018a) or Ef2 (present paper) produced many cocoons but relatively low numbers of hatchlings, among them fertile Ea-derived aAF hybrids resulted from fertilization of Ea ova by Ef spermatozoa. Among first generation offspring of Ea+Ef1 pairs (PLYTYCZ *et al.* 2018a), besides of Ea-derived hybrids, appeared also relatively common Ea specimens and a few Ef earthworms, both of them hypothetically resulted from partner-induced self-fertilization of cocoon-producing earthworms. Such self-fertilized specimens, either Ea or Ef2, were not identified so far among first generation of genotyped progeny of Ea+Ef2 pairs from the present studies, but appeared among progeny of first generation hybrids, that is worthy of further elucidation (in preparation). Fertility of aAF hybrids between Ea and Ef1 was evidenced in previous studies (PLYTYCZ *et al*. 2018a,b), as first generation hybrids backcrossed with Ea gave second generation of Ea-derived hybrids and pure Ea specimens, while aAF hybrids backcrossed with Ef1 gave new generation aAF hybrids plus rare and sterile Ef1 derived hybrids fFA developed from Ef1 ova fertilized by Ea spermatozoa, and a few Ef1 specimens (PLYTYCZ *et al.* 2018a, b).

Interspecific hybrids between Ea and Ef also exist in nature. The majority of Ea and Ef from natural populations in Norway and Sweden were separated by both mitochondrial (COI) and nuclear (28S and H3) markers, while four of 69 investigated earthworms were interspecific hybrids but they should rather be seen as evidence for historical hybridization between the two species (MARTINSSON & ERSEUS 2018). The presence of hybrids was previously expected on the basis of 'mixed' esterase patterns among offspring of laboratory-mated *Eisenia* sp. (OIEN & STENERSEN (1984).

Conclusions

Neither Ea nor Ef2 lineage from French/Polish laboratory stocks of hermaphroditic *Eisenia* sp. earthworms were capable of uniparental reproduction when cultured from hatching in isolation but reproduced when joined with *Eisenia* sp. partners. Fecundity was higher in Ea+Ea than in Ef2+Ef2 intra-specific intra-lineage pairs. Inter-specific Ea+Ef2 pairs produced relatively low numbers of Ea-derived hybrids. Some of these results obtained on French/Polish laboratory lines of *Eisenia* sp. are different from those on *Eisenia* sp. earthworms from other natural or laboratory sources, that implies the existence of differential mechanisms of speciation within the Ea/Ef complex. In conclusion, laboratory investigations offer some new opportunities for studies on mechanisms of earthworm speciation.

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Author Contributions

Research concept and design: A.P., B.P.; Collection and/or assembly of data: A.P., S.H., A.O., J.B.; Data analysis and interpretation: A.P., S.H., J.B., B.P.; Writing the article: A.P., B.P.; Critical revision of the article: J.K., B.P.; Final approval of article: A.P, J.K., B.P.

Conflict of Interest

The authors declare no conflict of interest.

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