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Mate choice of an endogeic earthworm revealed by microsatellite markers

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ABSTRACT

Endogeic earthworms are difficult study subjects due to the cryptic medium in which they live; thus, only the behaviour of epigeic and anecic earthworms has been studied before. We used microsatellite markers as a tool to elucidate the mate choice processes of *Hormogaster elisae*, an endogeic earthworm. It was shown to normally mate with two partners, preferably of the same size that are found in close proximity thereby eliminating the need for long-distance dispersion, which could explain the previously observed high genetic differentiation between populations. The genetic analyses of the sperm within each of its four spermathecae showed a uniform distribution with no signs of differential storage of sperm from different partners.

Introduction

Partner preference and mating behaviour have been studied in epigeic (*Eisenia fetida* by Monroy et al. 2005; *Eisenia andrei* by Tato et al. 2006) and anecic (*Lumbricus terrestris* by Nuutinen and Butt 1997; Butt and Nuutinen 1998; Michiels et al. 2001; Sahm et al. 2009) earthworms, but no attention has been given to endogeic earthworms, whose biological cycles and vital strategies are substantially different. Endogeic earthworms inhabit the sub-surface layers of the soil, rarely reaching the surface (Valle et al. 1999), which makes observation and consequently, behavioural studies extremely difficult. Nevertheless, endogeic earthworms probably have their own reproductive strategies because their life cycles are much longer and their rates of cocoon production are lower than those of epigeic or anecic earthworms (Edwards and Bohlen 1996; Díaz-Cosín et al. 2009); hence, it is interesting to investigate their unknown mating behaviour and partner preference.

Hormogaster elisae is a typical endogeic earthworm. It is an obligate out-crossing hermaphrodite, endemic to the central Iberian Peninsula, and may represent a complex of cryptic species, according to Novo et al. (2009). *H. elisae* needs, on average, 484 days for clitellum development from hatching, and its cocoon production rate ranges from 0.9 to 2.29 cocoons earthworm^{-1} year^{-1} (Díaz-Cosín et al. 2009).

Because endogeic earthworms live in a very cryptic medium, analysis of molecular markers, such as microsatellites, is a good way to unravel some of their behavioural and reproductive characteristics. These markers have been used recently by Sahm et al. (2009) to study mating preferences in *L. terrestris*, and specific primers are available for the species *H. elisae* (Novo et al. 2008).

We address some questions on pre-copulatory sexual selection, such as: (1) What is the average number of partners? (2) Do worms choose partners according to the size of the mate? and (3) Does the mating success of the studied earthworms depend on their size or their genetic variability?

A different strategy, known as sperm competition (Parker 1970) or cryptic female choice (Thornhill 1983), is possible after copulation (Birkhead and Pizzari 2002) when multiple copulation partners exist. Two possible mechanisms of sperm competition in earthworms are old sperm digestion (Richards and Fleming 1982) or a differential storage pattern within the spermathecae. We tested the second hypothesis in *H. elisae*, which has two pairs of large tubular spermathecae. The posterior pair of spermathecae has been found to contain more allosperm than the anterior pair (Garvín et al. 2003). Other Hormogastrid earthworms exhibit much smaller spermathecae, and, in some cases, different chambers are found within each spermatheca (Qiu and Bouché 1998). Given that *H. elisae* has no tissue subdivision inside the spermathecae, we tested whether they use the different spermathecae to achieve the function of different chambers and store sperm from different partners.

There is no information about the movements of *H. elisae* individuals under the soil, and it remains unknown whether they relocate to seek a partner or whether they stay within a certain home range. The latter behaviour was observed in the anecic *L. terrestris*, which is known to be anchored in its burrows (Michiels et al. 2001). Nevertheless, the burrow system that *H. elisae* uses is possibly more complex, as endogeic earthworms normally build semi-permanent or temporary gallery systems (Lavelle and Spain

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Table 1
Details of the microsatellite markers used in the study of *Hormogaster elisae* and overall genetic diversity for each locus.

Locus	Repeat motif	Primer sequence (5′–3′)	Mg^{2+}	Tag[a]	GenBank accession	Size range (bp)[b]	*N*	NA	Ho	He	HWE *P*-value	PIC
Hem07	(CT)26[c]	F:CTGTTCTCCGTGACTTCGAG R:CAGGGAGTCAGACAGGCAGT	2.5	FAM	AM902182	134–202 (156, 164)	75	24	0.89	0.94	0.0000	0.93
Hem194b	(GTCTCT)4	F:GCCCCATCCCCGCTTCTTTGTAT R:GCGCACCAAAATAAAGCCACACTAGTA	2.5	VIC	AM902190	154–244 (154)	75	12	0.91	0.83	0.0022	0.80
Hem188	(CT)24	F:CCGGGAGCCTCATGCAACAG R:CCGATAAACTCAGAAAAAACGCATAAACT	1.5	VIC	AM902188	222–310 (256)	75	20	0.41	0.91	0.0000	0.90
Hem193	(GT)43[c]	F:CAGTTATGTATGTGTTTTGCGTGGGTGTA R:CAAAGAGAGCTCCGCCCAGTTACGTAGAC	3	FAM	AM902189	124–178 (148)	75	17	0.67	0.88	0.0000	0.86

Mg^{2+}: Magnesium ion concentration in mM used for PCR amplifications.
N: number of individuals analysed.
NA: number of alleles.
Ho: observed heterozygosity.
He: expected heterozygosity.
HWE: Hardy–Weinberg equilibrium.
PIC: polymorphism information content.
[a] Fluorescence label at 5′-end of primer.
[b] Numbers in parentheses are the most frequent allele(s) sizes.
[c] Microsatellites contain interruptions among repeats.

2001). By sampling different points within a population we tried to infer the dispersal capacity of *H. elisae* and detect its genetic structure at a microgeographical scale.

Materials and methods

Sampling and earthworm dissection

A total of 75 sexually mature clitellate individuals of *H. elisae* were collected by digging and manual sorting at a microgeographical scale in El Molar, Madrid, Spain (GPS: 40°44′22.9″N, 3°33′53.1″W) in January 2008. The climatic and edaphic characteristics of the site are fully described in Valle et al. (1997) and Gutiérrez et al. (2006). Earthworms were collected from nine different 1 m^2 plots, each separated by 8 m, within a square of 64 m^2. Six to ten individuals were collected from each plot (see below), which is a relatively high density but assured the possibility of choice among different partners within 1 m^2.

After being washed with distilled water, the individuals were preserved in absolute ethanol at −20 °C. They were dissected, and five different samples were taken from each specimen: a portion of their tegument (0.025 g) was carefully cleaned and their four spermathecae were isolated under the stereomicroscope, resulting in a total of 375 samples.

Microsatellite analysis

DNA from the tegument and spermathecae was isolated using the DNeasy Tissue Kit (QIAGEN) and stored at 4 °C.

Four loci (Table 1) with high polymorphism were selected from the microsatellite markers developed for *H. elisae* by Novo et al. (2008). PCR amplifications were carried out in 20 μl reaction volumes with 5 ng of DNA, 1× PCR buffer (Biotools), a locus-specific $MgCl_2$ concentration (Table 1), 1 mM of dNTPs, 0.5 μM of each primer (0.25 μM for Hem194b) and 1 U *Taq* DNA polymerase (Biotools) on a Perkin Elmer 9700 thermal cycler.

The PCR profile was 94 °C (1 min), 30 cycles of [94 °C (30 s), 60 °C (15 s), 72 °C (15 s)] and a final extension of 3 min at 72 °C. For the locus Hem193, the PCR profile was 94 °C (5 min), 35 cycles of [94 °C (1 min), 60 °C (1 min), 72 °C (1 min)] and a final extension of 7 min at 72 °C.

The amplified products were first checked on a 1% agarose gel and then analysed on a 3730 DNA Analyzer (Applied Biosystems) following the manufacturer's instructions. The alleles were sized using the GS-500 LIZ size standard and Peak Scanner Software v. 1.0 (Applied Biosystems).

Microgeographical genetic analysis

The program Arlequin v. 3.1 (Excoffier et al. 2005) was used to obtain results on overall genetic diversity from tegument samples. The allele number (NA), and the observed (Ho) and expected heterozygosity (He) were calculated for each locus. Hardy–Weinberg equilibrium (HWE) was tested following the procedure described in Guo and Thompson (1992), and tests for linkage disequilibrium were conducted following Slatkin (1994). Polymorphism information content (PIC) was calculated using the Excel Microsatellite Toolkit (Park 2001).

The genetic variation in samples, including mean unbiased expected heterozygosity (He) and allelic richness (A), were estimated using Fstat v. 2.9.3 (Goudet 2001). For A, Fstat estimates the number of alleles in a sample corrected to the smallest sample size, as recommended by El Mousadik and Petit (1996).

We examined the existence of a genetic structure within the studied area using a hierarchical analysis of molecular variance (AMOVA) framework (Excoffier et al. 1992), as implemented in

Table 2
Mean genetic variability expressed as mean allelic richness (A), mean expected heterozygosity (He), and inbreeding coefficients (Fis) within each sample plot (Pn) of *Hormogaster elisae* at four microsatellite loci.

Sampling point	*N*	*A*	He	Fis
P1	7	6.55	0.87	0.189*
P2	6	6.50	0.85	0.234*
P3	9	7.13	0.89	0.161*
P4	9	5.76	0.78	0.121
P5	8	6.68	0.88	0.195*
P6	10	6.05	0.84	0.174*
P7	10	6.43	0.87	0.173*
P8	8	7.16	0.87	0.185*
P9	8	6.63	0.86	0.096
Total	75	7.32	0.89	0.191

N: Number of individuals analysed.
* Asterisks in Fis represent significant coefficients at $P < 0.05$.

Arlequin 3.1 (Excoffier et al. 2005). Weir and Cockerham's (1984) *F*-statistics were calculated: pairwise Fst values (as estimates of genetic differentiation between sample plots) and Fis values (inbreeding coefficients within sample plots).

A pattern of isolation by distance was tested with a Mantel (1967) test, which correlates the matrix of genetic distances between sample plots (Fst) with geographical distance (the length of a straight line between sample plots). The significance of correlation between the matrices was evaluated with 10,000 random permutations in Arlequin v.3.1 (Excoffier et al. 2005).

Sexual selection analysis

The alleles of each spermatheca were scored separately in order to observe whether there was a differential sperm storage pattern in the four spermathecae. Alleles from the tissue samples of the focal individual were removed from the analysis because the spermathecae walls had been included in the DNA extraction. Then, all the alleles from the four spermathecae were combined to calculate the minimum number of partners for each individual. The locus that exhibited the maximum number of alleles was considered for the estimate, and this number was divided by two, as *H. elisae* is diploid.

Mating probabilities were calculated following Sahm et al. (2009). For each individual of a given sample plot, the probability of having received sperm from all the other individuals from that plot was calculated. For that purpose, the presence (1) or absence (0) of the tegument alleles from each of the potential donors in the DNA from the recipient's spermathecae was scored. We estimated the probability that an allele observed in the spermatheca of the focal individual arrived there due to sperm donation from another individual. This probability is high if the pertinent allele frequency in the rest of the population is low. So the probability of any particular spermatheca allele being donated was estimated by subtracting its frequency in the population from unity (probability = 1 − allele frequency). Finally, the average of the probabilities that the two alleles were donated was calculated for each locus, and then the average of the probabilities for all four loci was determined. The mating probability between two individuals was calculated as the average of the probabilities of each individual receiving sperm from the other.

The mating success of each individual was estimated and expressed as the global probability of receiving and giving sperm, and the global probability of copulation (the average value of the previous two probabilities).

Correlation analyses were implemented in STATISTICA v. 6.1 (StatSoft, Inc; www.statsoft.com). The mating probabilities between individuals were tested for a correlation with their differences in size (expressed as weight). A correlation between global probabilities of mating success (donation, reception and copulation) and weight of individuals was explored. An ANOVA was also performed to explore the possibility that the mating probabilities of individuals depended on their degrees of heterozygosity (categories: number of heterozygous loci). This analysis was controlled for their weights with an ANCOVA.

Results

Microgeographical genetic analysis

Total genetic diversity values for each microsatellite locus are shown in Table 1. The loci exhibited high polymorphism, and the number of alleles detected ranged from 12 to 24. The heterozygosity ranged from 0.41 to 0.91. The polymorphism information content was from 0.80 to 0.93. The loci showed differences between observed and expected heterozygosities.

Analysis of linkage disequilibrium yielded two significant cases (Hem07 vs. Hem188, $P = 0.031$; Hem188 vs. Hem193, $P = 0.017$) out of 6 pairwise comparisons. None of these remained significant after Bonferroni correction (critical significance level of $P = 0.008$), indicating that the loci used are unlinked.

The genetic variability within each sample point and the Fis values are shown in Table 2. The inbreeding coefficients were all positive and ranged from 0.096 to 0.234. There was no significant

Table 3
Results of analysis of molecular variance (AMOVA) in *Hormogaster elisae* at four microsatellite loci. Partitioning of genetic variance at three hierarchical levels is shown. Corresponding fixation indices (Weir and Cockerham 1984) and their *P*-values are given.

Source of variationSource of variation	d.f.	Sum of squares	Variance components	Fixation indices	*P*-values	Percentages of variation
Among sites	8	23.61	0.056	0.031 (Fst)	0.76	3.14
Among individuals within sites	66	133.31	0.290	0.168 (Fis)	0.00	16.23
Within individuals	75	108.00	1.440	0.193 (Fit)	0.00	80.63

d.f.: degrees of freedom.

Table 4
Average pairwise Fst values between sample plots (Pn) of *Hormogaster elisae* based on four microsatellite loci.

	P1	P2	P3	P4	P5	P6	P7	P8
P1	–							
P2	0.05532	–						
P3	0.07034*	0.02812	–					
P4	0.02113	0.10343*	0.09370	–				
P5	0.02027	0.02211	0.02609	0.04499*	–			
P6	0.06941*	0.05501*	0.02140	0.09478*	0.03096	–		
P7	0.02947	0.03361	0.04114*	0.04915*	0.02298	0.03029	–	
P8	0.05222*	0.02526	0.03913	0.08298*	0.00657	−0.01049	0.02871	–
P9	0.06820*	0.00164	0.02461	0.09595*	0.01037	0.02564	0.04721*	0.01520

* Fst represent significance at $P < 0.05$.

genetic structure (Table 3) within the studied area, and the differentiation between plots explained only 3.14% of the genetic variation found, which was explained mostly by differences between and within individuals.

Pairwise Fst values between samples ranged from −0.01049 to 0.10343, exhibiting low genetic differentiation (Table 4).

The Mantel test indicated that the pairwise genetic distances between sample sites (Fst) and the distance between sites in metres were significantly correlated ($P = 0.03$, $r = 0.34$).

Mate choice analysis

Most of the individuals (62.66%) stored sperm from a minimum of two partners, whereas 18.67% of the earthworms copulated with a minimum of one partner, and the remainder (18.67%) stored sperm from at least three different individuals.

There were no differences in the pattern of sperm storage between the four spermathecae, and the same alleles were generally found in all the spermathecae from one individual.

A significant negative correlation was found between the mating probability and the difference in weight of the specimens ($r = -0.22$, $P < 0.05$), thus indicating that individuals prefer to mate with partners of similar weights to themselves.

No significant results were found in the remaining statistical analyses.

Discussion

Microgeographical genetic analysis

Overall genetic diversity values were high, similar to the ones found by Velavan et al. (2009) for *L. terrestris* and even higher with respect to average allelic richness. Departure from the HWE was detected, as in a previous study by Novo et al. (2008). Their hypothesis for explaining these significant departures was a possible population sub-structuring (i.e. Wahlund effect) that could lead to inbreeding. Indeed, positive Fis values indicated a moderate amount of inbreeding within the sampled plots, but the inbreeding was not very pronounced, permitting high genetic diversity. The AMOVA indicated no genetic sub-structuring, but the significant result of the Mantel test indicated isolation by distance. Therefore, it seems that individuals of *H. elisae* are rather sedentary and do not relocate over long distances to find mating partners. Sahm et al. (2009) found that distance was the most important factor for mate choice in *L. terrestris*, but because endogeic *H. elisae* is not anchored to vertical burrows and does not copulate at the surface, it was not expected to have this pattern. *H. elisae* individuals are not exposed to predators during copulation, and so they can move underground to seek appropriate partners. Nevertheless, these results could lead us to two hypotheses, namely that *H. elisae* earthworms could be somehow linked to a permanent or semi-permanent burrow system or that, due to a high density of individuals in the studied area (Table 2), they do not need to move long distances to find partners.

This reduced capacity for mobility has been also found in other earthworm species that have observed natural dispersal rates of only 1.4–9 m/year (Ligthart and Peek 1997; Hale et al. 2005) and could explain the deep genetic isolation between populations found by Novo et al. (2009) that led to cryptic speciation. Deep genetic splits between populations of earthworms are also observed in *Lumbricus rubellus* (which has a surface-active activity), (see Andre et al. 2010), and is in contrast with other soil invertebrates such as Collembola, in which populations are structured on a scale extending to several kilometres (Van der Wurff et al. 2003).

Sexual selection

It seems that, on average, individuals of *H. elisae* copulate with two partners. Nevertheless, this finding could represent only their very recent mating history if only the sperm of the last mating partners are maintained for future use. In fact, a histological study of spermathecae from *H. elisae* (in preparation) has shown that there were degrading sperm loads in the central areas of the spermathecae. Richards and Fleming (1982) found spermatozoal phagocytosis by the spermathecae of *Dendrobaena subrubicunda* and other lumbricids that was probably related to ageing or aberrant sperm removal during the months when cocoon production was minimal. Butt and Nuutinen (1998) observed that *L. terrestris* was capable of successfully maintaining received sperm for up to 6 months, and Meyer and Bowman (1994) reported that *E. fetida* continued cocoon production for up to 12 months after the earthworms were isolated from their partners, although the viability of the cocoons was not measured.

No differences in the pattern of sperm storage between the four spermathecae were found, which indicates that if sperm competition is present in these animals it should be orchestrated by mechanisms inside each spermatheca. Grove and Cowley (1926) observed that transmission of sperm in *E. fetida* normally occurs on both sides of the individual, whereas in *L. terrestris* some individuals were found with spermatophores in only one side of their bodies (Butt and Nuutinen 1998). Future histochemical studies are necessary to unravel such mechanisms.

A negative correlation was found between weight differences and mating probabilities, which means that *H. elisae* individuals select partners with similar weights. This selection was already discussed by Michiels et al. (2001) who found that pairs of similarly sized anecic *L. terrestris* earthworms copulated earlier than pairs with different sizes in laboratory experiments. It was also found by Monroy et al. (2005) that the epigeic *E. fetida* showed size-assortative mating in the field with individuals choosing similarly sized partners. This selection could be explained for epigeic and anecic earthworms as a trade-off between choosing a larger partner, given that female fecundity (as measured by cocoon production, cocoon size and hatchling size) is related to body size (Meyer and Bowman 1995, 1997), and choosing a smaller part-

ner that would decrease predation risk. However, as endogeic *H. elisae* copulates underground, larger partners could be expected to be selected. The proposal that assortative mating could be constrained by physical incompatibility of the copula among partners of different sizes (Michiels et al. 2001) could explain the similar-sized selection in *H. elisae*, although this incompatibility would be caused only by excessive size differences. Therefore, it is more plausible that this pattern is caused by the conflict of every earthworm seeking a larger partner that finally led to an equilibrium of similar weight partners, thus balancing expectations of both mates on female and male functions.

Conclusions

This is the first time that sexual behaviour has been studied in an endogeic earthworm. Different questions arise from the findings of this study, but in general, it seems that *H. elisae* has multiple matings and generally maintains the sperm of two individuals mixed in four spermathecae. Individuals prefer same-sized partners that are found nearby with no need for long-distance dispersion.

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