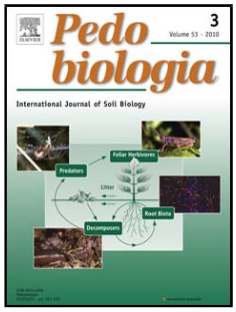

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Mate choice of an endogeic earthworm revealed by microsatellite markers.

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Running title: Mate choice of an endogeic earthworm

Summary

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.

Keywords: Mate choice, endogeic earthworm, sperm, spermathecae, microsatellite markers.

Introduction

Partner preference and mating behaviour have been studied in epigeic (*Eisenia fetida* by Monroy et al., 2005; *E. 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 (Garvin 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, 2001). By sampling different

100 points within a population we tried to infer the dispersal capacity of *H. elisae* and detect its
101 genetic structure at a microgeographical scale.
102
103 **Materials and methods**
104 Sampling and earthworm dissection.
105 A total of 75 sexually mature clitellate individuals of *Hormogaster elisae* were collected by
106 digging and manual sorting at a micro-geographical scale in El Molar, Madrid, Spain (GPS:
107 N40º44´22.9´´ W3º33´53.1´´) in January 2008. The climatic and edaphic characteristics of the
108 site are fully described in Valle et al. (1997) and Gutiérrez et al. (2006). Earthworms were
109 collected from nine different 1m² plots, each separated by 8 m, within a square of 64 m². Six
110 to ten individuals were collected from each plot (see below), which is a relatively high density
111 but assured the possibility of choice among different partners within one square meter.
112
113 After being washed with distilled water, the individuals were preserved in absolute ethanol at
114 –20 ºC. They were dissected, and five different samples were taken from each specimen: a
115 portion of their tegument (0.025 g) was carefully cleaned and their four spermathecae were
116 isolated under the stereomicroscope, resulting in a total of 375 samples.
117
118 Microsatellite analysis
119 DNA from the tegument and spermathecae was isolated using the DNeasy Tissue Kit
120 (QIAGEN) and stored at 4ºC.
121 Four loci (Table 1) with high polymorphism were selected from the microsatellite markers
122 developed for *H. elisae* by Novo et al. (2008). PCR amplifications were carried out in 20 µl
123 reaction volumes with 5 ng of DNA, 1X PCR buffer (Biotools), a locus-specific MgCl2

concentration (Table 1), 1 mM of dNTPs, 0.5 μM of each primer (0.25 μM for Hem 194b) and 1 U *Taq* DNA polymerase (Biotools) on a Perkin Elmer 9700 thermal cycler.

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

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).

Micro-geographical 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 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 spematheca 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 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 kms (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 prep.) 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 Bouwman (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 *Eisenia 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 partner 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*

296 has multiple matings and generally maintains the sperm of two individuals mixed in four
297 spermathecae. Individuals prefer same-sized partners that are found nearby with no need for
298 long-distance dispersion.
299
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304
305 **References**
306 Andre, J., King R.A., Stürzenbaum S.R., Kille P., Hodson M.E., Morgan J. 2010. Molecular
307 genetic differentiation in earthworms inhabiting a heterogeneous Pb-polluted landscape.
308 Environ. Poll. 158, 883-890.
309
310 Birkhead, T.R., Pizzari, T., 2002. Postcopulatory sexual selection. Nat. Rev. Genet. 3, 262-
311 273.
312
313 Butt, K.R., Nuutinen, V., 1998. Reproduction of the earthworm *Lumbricus terrestris* Linné
314 after the first mating. Can. J. Zool. 76, 104-109.
315
316 Díaz-Cosín, D. J., Hernández, P., Trigo, D., Fernández, R., Novo, M., 2009. Algunos aspectos
317 del ciclo biológico del endemismo ibérico, *Hormogaster elisae* Álvarez, 1977 (Oligochaeta,
318 Hormogastridae), en cultivos de laboratorio. Bol. R. Soc. Esp. Hist. Nat. Biol. 103 , 49-57.
319

Edwards, C.A., Bohlen, P.J., 1996. Biology and Ecology of Earthworms, third ed. Chapman and Hall, London.

El Mousadik, A., Petit, R.J., 1996. High level of genetic differentiation for allelic richness among populations of the argan tree [*Argania spinosa* (L.) Skeels] endemic to Morocco. Theor. Appl. Genet. 92, 832-839.

Excoffier, L., Smouse, P.E., Quattro, J.M., 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131, 479–491.

Excoffier, L., Laval, G., Schneider, S., 2005. Arlequin ver. 3.0: an integrated software package for population genetics data analysis. Evol. Bioinform. Online 1, 47–50.

Garvín, M.H., Trigo, D., Hernández, P., Díaz Cosín, D.J., 2003 Gametogenesis and reproduction in *Hormogaster elisae* (Oligochaeta, Hormogastridae). Inv. Biol. 122,152-157.

Goudet, J., 2001. FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Available from http://www.unil.ch/izea/softwares/fstat.html.

Grove, A.J., Cowley, L.F., 1926. On the reproductive processes of the brandling worm, *Eisenia foetida* (Sav.). Quart. J. Microsc. Sci. 70, 559-581.

Guo, S., Thompson, E., 1992. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. Biometrics 48, 361-372.

Gutiérrez, M., Jesús, J.B., Trigo, D., Díaz-Cosín, D.J., 2006. Is *Hormogaster elisae* (Oligochaeta, Hormogastridae) a predator of mite and springtails? Eur. J.Soil Biol. 42, S186–S190.

Hale, C.M., Frelich, L.E., Reich, P.B., 2005. Exotic European earthworm invasion dynamics in northern hardwood forest of Minnesota, USA. Ecol. Appl. 15, 848–860.

Lavelle, P., Spain, A.V., 2001. Soil Ecology, Kluwer Academic Publishers, London, UK.

Ligthart, T.N., Peek, G.J.C.W., 1997. Evolution of earthworm burrow systems after inoculation of lumbricid earthworms in a pasture in the Netherlands. Soil Biol. Biochem. 29, 453–462.

Mantel, N., 1967. The detection of disease clustering and generalized regression approach. Cancer Res. 27, 209–220.

Meyer, W.J., Bowman, H., 1994. Mating period and cocoon production in *Eisenia fetida*. In Abstracts of the 5th International Symposium on Earthworm Ecology, Columbus, Ohio, 5-9 July 1994. p. 128 (Abstr.).

Meyer, W.J., Bowman, H., 1995. Suitable characters for selective breeding in *Eisenia fetida* (Oligochaeta). Biol. Fertil. Soils 20, 53-56.

Meyer, W.J., Bowman, H., 1997. Anisopary in compost earthworm reproductive strategies (Oligochaeta). Soil Biol. Biochem. 29, 731-735.

Michiels, N.K., Hohner, A., Vorndran, I.C., 2001. Precopulatory mate assessment in relation to body size in the earthworm *Lumbricus terrestris:* avoidance of dangerous liaisons? Behav. Ecol. 12(5), 612–618.

Monroy, F., Aira, M., Velando, A., 2005. Size-assortative mating in the earthworm *Eisenia fetida* (Oligochaeta, Lumbricidae). J. Ethol. 23, 69-70.

Novo, M., Velavan, T.P., Almodóvar, A., Schulenburg, H., Díaz Cosín, D.J., Michiels, N.K., 2008. Microsatellite markers for the drought-resistant earthworm *Hormogaster elisae*. Mol. Ecol. Res. 8, 901–903.

Novo, M, Almodóvar, A., Díaz-Cosín, D.J., 2009. High genetic divergence of hormogastrid earthworms (Annelida, Oligochaeta) in the central Iberian Peninsula: evolutionary and demographic implications. Zool. Script. 38, 537-552.

Nuutinen, V., Butt, K.R., 1997. The mating behaviour of the earthworm *Lumbricus terrestris* (Oligochaeta: Lumbricidae). J. Zool. Lond. 242, 783-798.

Park, S.D.E., 2001. Trypanotolerance in West African Cattle and the Population Genetic Effects of Selection [Ph.D. thesis] University of Dublin.

393 Parker, G.A., 1970. Sperm competition and its evolutionary consequences in insects. Biol.
394 Rev. 45, 525-567.
395
396 Qiu, J.P., Bouché, M.B., 1998. Contribution to the taxonomy of Hormogastridae (Annelida:
397 Oligochaeta) with description of new species from Spain. Doc. Pedozool. Integrol. 4, 164–
398 177.
399
400 Richards, K.S., Fleming, T.P., 1982. Spermatozoal phagocytosis by the spermathecae of
401 *Dendrobaena subrubicunda* and other lumbricids (Oligochaeta, Annelida). Int. J. Invert. Rep.
402 5, 233-241.
403
404 Sahm, S., Velavan, T.P., Schulenburg, H., Michiels, N.K., 2009. Reconstruction of mating
405 history - a retrospective analysis of *Lumbricus terrestris* mate choice criteria in natural
406 populations. In: Velavan T.P. 2009. Population genetics of Host - Parasite interactions in
407 *Lumbricus terrestris* and *Monocystis sp*. (Apicomplexa: Gregarinea). Phd Thesis, pp. 43-57.
408 Tübingen University.
409
410 Slatkin, M., 1994. Linkage disequilibrium in growing and stable populations. Genetics 137,
411 331-336.
412
413 Tato, A., Velando, A., Domínguez, J., 2006. Influence of size and partner preference on the
414 female function of the earthworm *Eisenia andrei* (Oligochaeta, Lumbricidae). Eur. J. Soil
415 Biol. 42, S331-S333.
416

Thornhill, R., 1983. Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. Am. Nat. 122, 765-788.

Valle, J.V., Moro, R.P., Garvín, M.H., Trigo, D., Díaz Cosín, D.J., 1997. Annual dynamics of the earthworm *Hormogaster elisae* (Oligochaeta, Hormogastridae) in Central Spain. Soil Biol. Biochem. 29, 309–312.

Valle, J.V., Garvín, M.H., Trigo, D., Martínez, F., Belinchón, C., Díaz Cosín, D.J., 1999. Vertical distribution of *Hormogaster elisae* (Oligochaeta, Hormogastridae) in soil at El Molar (Central Spain). Pedobiologia 43, 859-865.

Van der Wurff, A.W.G., Isaaks J.A., Einsting, G, Van Straalen N.M. 2003. Population substructures in the soil invertebrate Orchesella cincta, as revealed by microsatellite and TE-AFLP markers. Mol. Ecol. 12, 1349-1359.

Velavan, T.P., Sahm, S., Schulenburg, H., Michiels, N.K., 2009. High genetic diversity and heterogeneous parasite load in the earthworm *Lumbricus terrestris* on a German meadow. Soil Biol. Biochem. 41, 1591-1595.

Weir, B.S., Cockerham, C.C., 1984. Estimating F-statistics for the analysis of population structure. Evolution 38, 1358–1370.

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

	Repeat motif	Primer sequence(5' - 3')	Mg++	Tag*	GenBank Accession	Size range (bp)**	N	NA	Ho	He	HWE P-valu
	(CT)26†	F:CTGTTCTCCGTGACTTCGAG R:CAGGGAGTCAGACAGGCAGT	2.5	FAM	AM902182	134-202 (156,164)	75	24	0.89	0.94	0.000
b	(GTCTCT)4	F:GCCCCATCCCCGCTTCTTTGTAT R:GCGCACCAAAATAAAGCCACACTAGTA	2.5	VIC	AM902190	154-244 (154)	75	12	0.91	0.83	0.002
	(CT)24	F:CCGGGAGCCTCATGCAACAG R:CCGATAAACTCAGAAAAACGCATAAACT	1.5	VIC	AM902188	222-310 (256)	75	20	0.41	0.91	0.000
	(GT)43†	F:CAGTTATGTATGTGTTTTGCGTGGGTGTA R:CAAAGAGAGCTCCGCCAGTTACGTAGAC	3	FAM	AM902189	124-178 (148)	75	17	0.67	0.88	0.000

†: Microsatellites contain interruptions among repeats
Mg++: Magnesium ion concentration in mM used for PCR amplifications
*: Fluorescence label at 5´- end of primer
**: Numbers in parentheses are the most frequent allele(s) sizes.
N: Number of individuals analysed
NA: Number of alleles
Ho: Observed heterozygosity
He: Expected heterozygosity
HWE: Hardy–Weinberg equilibrium
PIC: Polymorphism Information Content

Table 2. Mean genetic variability espressed 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

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 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

Asterisks in Fst represent significance at $P<0.05$