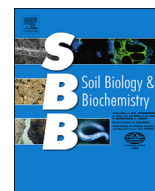


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# The worm has turned: Behavioural drivers of reproductive isolation between cryptic lineages

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## ABSTRACT

Behavioural processes such as species recognition and mate attraction signals enforce and reinforce the reproductive isolation required for speciation. The earthworm *Lumbricus rubellus* in the UK is deeply differentiated into two major genetic lineages, 'A' and 'B'. These are often sympatric at certain sites, but it is not known whether they are to some extent reproductively isolated. Behavioural tests were performed, in which individually genotyped worms were able to choose between soils previously worked either by genetically similar or dissimilar individuals (N = 45). We found that individuals (75%) were significantly (P < 0.05) more likely to orientate towards the soil conditioned by worms of their own lineage. Further testing involved a choice design based on filter papers wetted with water extracts of soils worked by a different genotype on each side (N = 18) or extracts from worked soil vs. un-worked control soil (N = 10). Again, earthworms orientated towards the extract from their kindred genotype (P < 0.05). These findings indicate that genotype-specific water-soluble chemicals are released by *L. rubellus*; furthermore, they are behaviour-modifying, and play a role in reproductive isolation between sympatric earthworm lineages of cryptic sibling species, through pre-copulatory assortative mate choice.

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## 1. Introduction

An important question in the understanding of speciation is: what mechanisms are involved in the origin and maintenance of reproductive isolation between populations? Behavioural processes such as mate choice potentially play an important role in pre-copulatory reproductive isolation, leading to genetic divergence between the isolated lineages and, ultimately, to speciation (Mendelson and Shaw, 2012). Divergence in mate choice preferences can occur between populations that become geographically isolated (allopatric reproductive isolation), leading to the persistence of reproductive isolation following secondary contact between the populations (sympatric reproductive isolation). Examples of signals that have contributed to behavioural isolation involve visual signals observed in butterflies (Wiernasz and Kingsolver, 1992), damselflies (Saetre et al., 1997), fish (Seehausen and Van Alphen, 1998) and frogs (Maan and Cummings, 2008),

acoustic signals observed in insects, frogs (Gerhardt and Huber, 2002) and bats (Barlew and Jones, 1997), and chemical signals observed in moths (Linn and Roelofs, 1995), spiders (Trabalon et al., 1997), and flies (Coyne et al., 1994). Variation in these signals between lineages can result in pre-mating reproductive isolation, providing a behavioural mechanism driving speciation (Smadja and Butlin, 2009).

"Cryptic species" are morphologically indistinguishable, but genetically distinct taxa. The advent of molecular techniques has led to many such cryptic species being identified among many animal taxa in recent years (Bickford et al., 2007), and they are particularly common among soil dwellers, such as earthworms (King et al., 2008; James et al., 2010; Novo et al., 2010; Buckley et al., 2011). Morphological stasis despite genetic divergence is commonly manifest in non-visually guided invertebrates that live in opaque media such as soil or turbid waters, where chemical signalling may play a more important role than morphology in sexual selection (Lee and Frost, 2002). Soil-dwelling cryptic species therefore provide an ideal model for investigating veiled processes of behavioural isolation and mate recognition systems.

*Lumbricus rubellus* Hoffmeister, 1843 is a lumbricid earthworm species that comprises two cryptic lineages (namely A and B) living in sympatry in the UK (King et al., 2008; Andre et al., 2010;

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Donnelly et al., 2014). These lineages are genetically differentiated deeply enough to warrant the status of cryptic species (King et al., 2008). According to Sechi (2013), speciation within the *L. rubellus* taxon may have first arisen through allopatric speciation during the last European glaciation event whereby lineages were geographically isolated into separate refugia during the glacial period, followed by secondary contact between lineages in the post-glacial period. The two lineages have remained genetically distinct despite living in sympatry (Andre et al., 2010), but differ in subtle aspects of phenotypic expression, such as disparate responses to high levels of arsenic exposure (Kille et al., 2013) or minor morphological traits (Donnelly et al., 2014).

Chemical communication through pheromones has been described in earthworms, serving as alarm systems (Ressler et al., 1968), or as signals inducing migration (Zirbes et al., 2010) or egg-laying (Oumi et al., 1996). Attractin, Temptin, Enticin and Seductin are small molecules that act as water-borne sex pheromones promoting mate attraction (Painter et al., 1998, 2004; Cummins et al., 2004). These pheromones were first described in the marine mollusc *Aplysia* (sea slug/sea hare), and in terrestrial snails where they are implicated in trail-following (Ng et al., 2013). The expression of Attractin and Temptin has been detected in the transcriptomes of earthworm tissues, including epidermis and digestive tract (Novo et al., 2013), forming physical and functional interfaces with the environment, thus suggesting that the behaviour-modulating molecules could be released through the mucus or faeces (“casts”) to create a trail (Ressler et al., 1968) analogous to those in snails for the attraction of potential mates.

Given the known existence of cryptic, sympatric, earthworm lineages (cryptic species) and the identification of sex pheromones in *L. rubellus* (Novo et al., 2013), our aim was to test the hypothesis that chemical cues play a role in reproductive isolation, through pre-copulatory assortative mate choice between cryptic species of earthworms. This hypothesis was tested using a behavioural assay on genotyped individual *L. rubellus* derived from a site where preliminary observations indicated that the two lineages co-exist in approximately equal numbers. A further experiment tested whether the behaviour-modulating chemical substances released by the two lineages are water-soluble and retain their specific activities on worm behaviour when presented as soil water-extracts.

## 2. Material and methods

### 2.1. Earthworm collection and housing

Earthworms of the species *L. rubellus* were collected by manual digging and hand sorting from a single site in Rudry, South Wales (Fig. 1A; N51°34'19" W3°10'52") and were transported back to the laboratory in their native soil. The site was a lowland dry acid grassland, not polluted by heavy metals (Supplementary Table S1). Only mature worms (i.e. with a clearly visible clitellum) were used in the experiments. On return to the laboratory, the worms were individually weighed, and placed into numbered containers filled with native soil from the extraction site. Throughout the duration of the experiments the worms were maintained in an unlit climate chamber of 13 °C. Posterior segments (‘tail clips’) of approximately  $\leq 1$  cm were amputated from each individual and preserved in Eppendorf tubes of absolute ethanol prior to DNA extraction for genotyping. Experiments were started two weeks after caudal amputation.

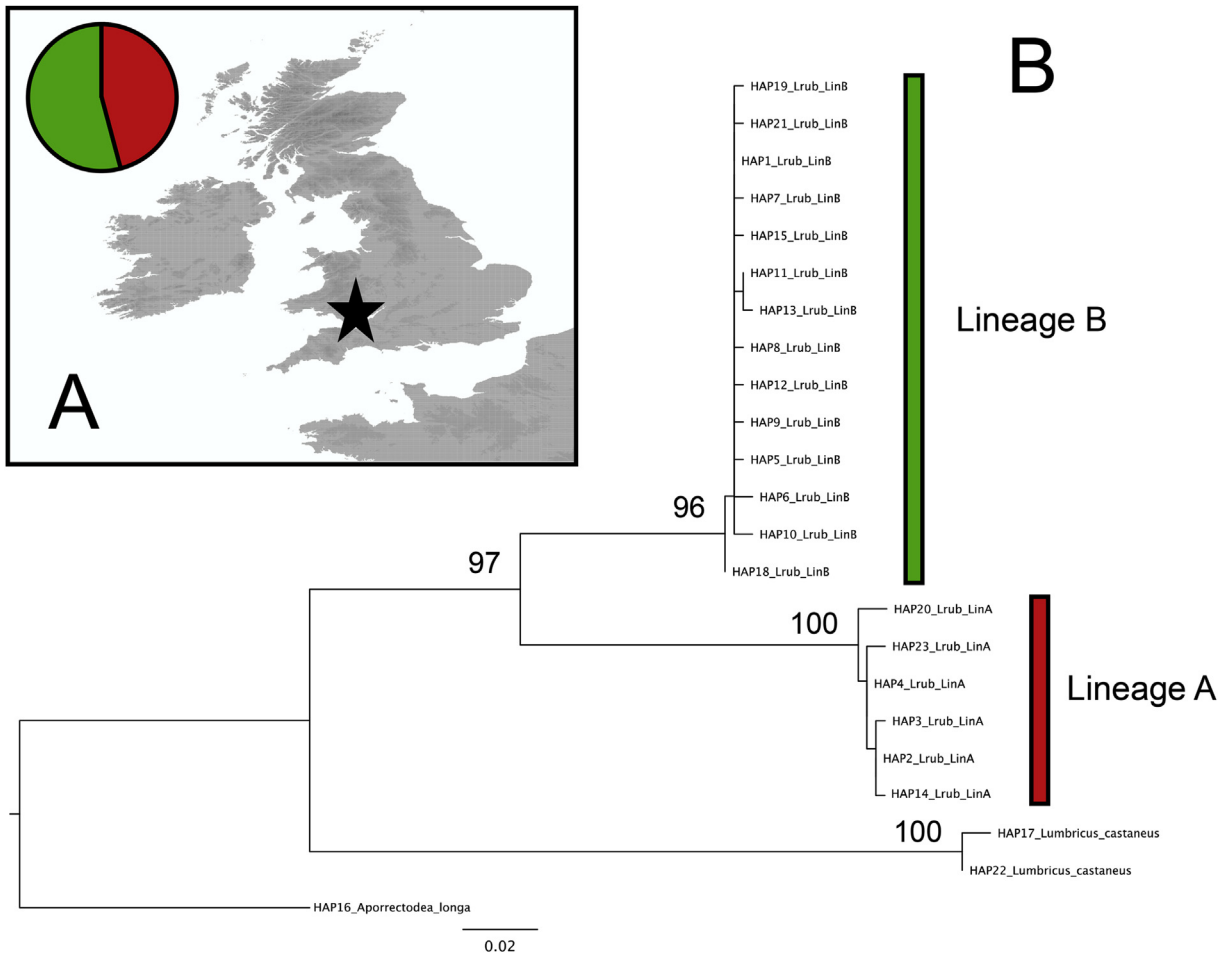
### 2.2. Molecular techniques for lineage identification

In total, 134 earthworms were genotyped; including 5 individuals of the species *Lumbricus castaneus* and a single individual

of *Aporrectodea longa*, in order to be used as outgroups for the phylogenetic tree. The remaining 128 earthworms were of *L. rubellus* lineages A or B. Genomic DNA was extracted from 25 mg of tissue with the Qiagen DNeasy Blood and Tissue kit (Qiagen LTD, UK) following the manufacturer’s instructions. A 407 bp fragment of the mitochondrial gene cytochrome oxidase subunit II (COII) was amplified using specific primers for *L. rubellus* (Andre et al., 2010). PCR reactions had a final volume of 20  $\mu$ l, with 1  $\mu$ l of DNA template, 0.5  $\mu$ M of each primer, 0.25 mM dNTPs and 1.25 units of GoTaq® DNA polymerase (PROMEGA) buffered with 1.3 $\times$  GoTaq® reaction buffer and supplemented with 2.5 mM MgCl<sub>2</sub>. The PCR reaction included a denaturation step of 95 °C for 5 min and then cycled 35 times, at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. This was followed by a 10 min final extension at 70 °C. PCR products were purified and sequenced by Eurofins ([www.eurofinsgenomics.eu](http://www.eurofinsgenomics.eu)). Sequences were aligned and cut using Mega 6.06 (Tamura et al., 2013) and the ClustalW option. Genetic variability was measured in DNAsp v 5.10.1 (Librado and Rozas, 2009) and a parsimony network with a 95% connection limit was built in TCS v1.21 (Clement et al., 2000). Unique haplotypes were retrieved with DNACollapser in FaBox (Villesen, 2007) and were then used for maximum likelihood tree construction in Mega with 1000 bootstrap repetitions under the model GTR + I + G.

### 2.3. Experiment 1: preferences of worms for lineage-specific secretants in soil

The primary experiment was carried out in the form of a classical animal behaviour side-choice experiment, similar to that carried out on earthworms by Lukkari and Haimi (2005). Experiments were conducted in food-quality plastic container mesocosms (length: 27 cm, width: 10 cm, height: 5.5 cm). A vertical PVC divider was placed in the middle of each container. A well-mixed ‘standard soil’ was made in a separate container for each mesocosm in turn, totalling 871 g, and was made up of 732 g Boughton Kettering Loam (composition shown in Supplementary Table S2), 39 g of Organic Farmyard Manure Gro-sureManure (product found at: <http://www.gardenhealth.com/>), and 100 g of water. From this mixture, 400 g was placed in each compartment of the mesocosm, in order to ensure homogeneity within each replicate. In each mesocosm (n = 51), one worm from lineage A was placed in one compartment, and a worm from lineage B placed in the other compartment (Fig. 2A). Pairs of worms from the two lineages were chosen according to similarity in weight in order to account for the possibilities of larger earthworms secreting more chemical signals, and thus mate selection decisions being made on the basis of size (Michiels et al., 2001). The worms were then left in the mesocosms for a period of 31 days, in an unlit climate chamber at 13 °C, in order for them to secrete potential chemical signals into the surrounding soil. After the elapsed conditioning period both worms were carefully removed from the mesocosms, the central divider was removed so that the soils in both halves of the mesocosm were in contact, and a third worm (of known lineage, either A or B) was placed on the soil surface in the middle of the mesocosm (i.e. aligned with the boundary between the two contrasting lineage-worked soils). Due to stock limitations, earthworms used for working the soils were used afterwards for the test. A small number of the mesocosms were discarded from the subsequent behavioural tests because of the death of one of the pre-test worms (final n = 45 mesocosms). The containers were left in a climate chamber at 13 °C and after 48 h the side choice of the worm was recorded, either as that of the same lineage (deemed the ‘correct side choice’), or that of the opposite lineage (‘incorrect’). If there was a part of the worm still in the central area, the side chosen was considered to be the side where the anterior part of the worm lay. For a subset of worms



**Fig. 1.** Phylogenetic analysis of *Lumbricus rubellus* cryptic lineages. Lineage B is represented in green; lineage A in red. A: Map of UK showing the position of the locality sampled and the proportion of lineages of *Lumbricus rubellus* found. B: Maximum likelihood tree based on the mitochondrial COII showing the two cryptic lineages whose mean uncorrected pairwise distance is 11.52% Bootstrap values higher than 70 are shown on the branches. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

( $n = 27$ ), the distance that the worm had burrowed into the soil was also recorded, as a measure of the extent to which a worm had exhibited a preferred direction of movement.

#### 2.4. Experiment 2: preferences of worms for lineage-specific soil water extracts

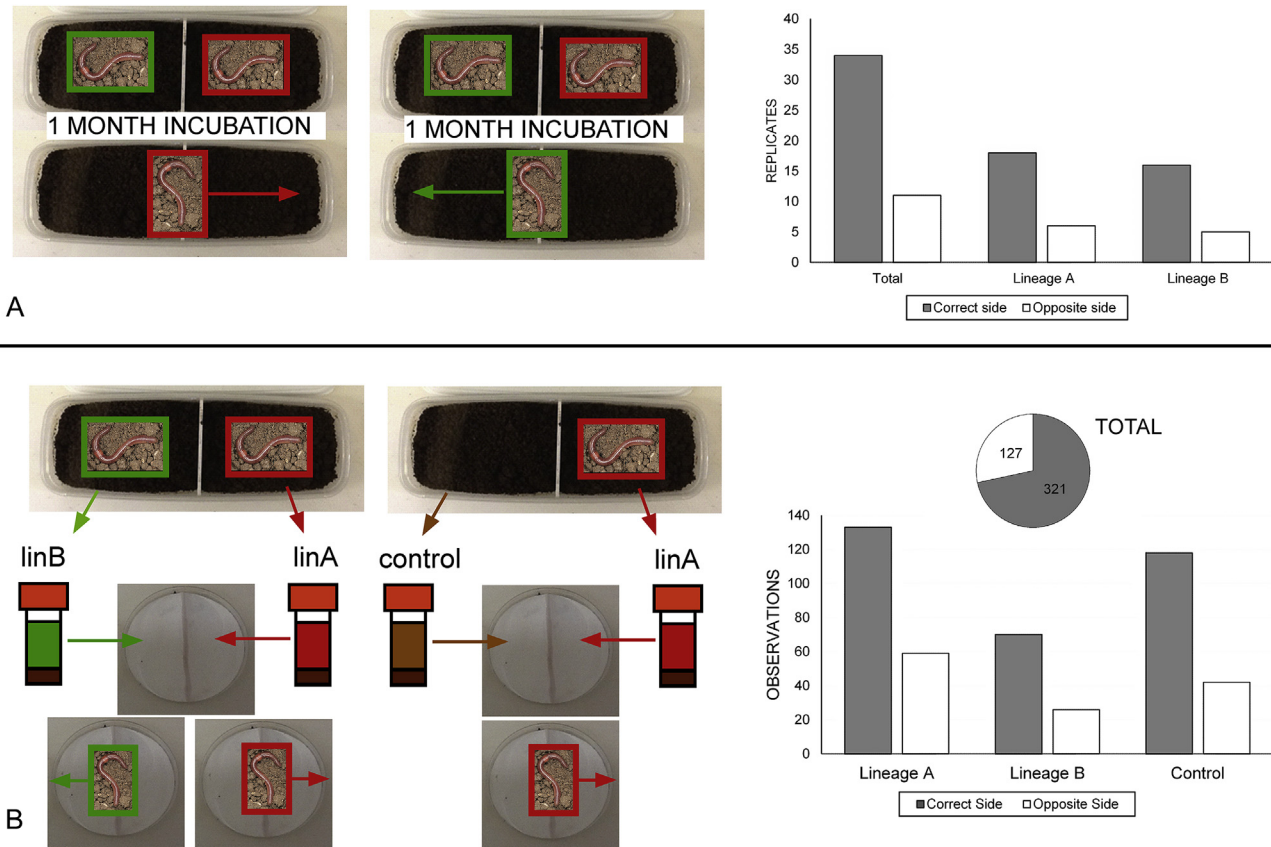
Genotyped earthworms were placed into plastic mesocosms with 400 g of soil (same size and composition as described in Experiment 1) and left for one month at 13 °C. Due to earthworm stock limitations, 22 lineage A worms and only 6 lineage B worms were available for the second experiment. In order to observe whether the pheromones involved in mate attraction are water-soluble and if they have the same effect on the worms as in the primary experiment, 4 g of soil from each container was mixed with 10 ml of de-ionized water and then vigorously shaken overnight. The solutions were then centrifuged at 6000 rpm for 4 min. After centrifugation, 10 ml of the supernatant was transferred to new test tubes. Control samples (i.e. aliquots of the standard soil mix with no earthworms maintained in it) were also created, for comparison with the lineage-specific samples (Fig. 2B).

Filter paper (calculated to be able to absorb approximately 640  $\mu$ l of liquid) was placed inside Petri dishes (8.5 cm in diameter) and were used as mesocosms for Experiment 2. A line was drawn

down the centre of the filter paper using a wax DakoPen, creating a non-permeable barrier between the two solutions and preventing any spill over or absorption between the two halves of the filter paper. Then, 320  $\mu$ l of each solution (A or B; A or control) was pipetted onto each side of the filter paper. Next, a worm of known lineage was placed in the middle of the filter paper, aligned with the wax barrier, and the side choice of the worm was recorded every 3 min for the first 15 min and then noted every 15 min, for a total of 3 h. In total, 12 lineage A worms and 6 lineage B worms were used for the A/B selection, and a further 10 lineage A worms were used for the A/control selection. Extracts came from different worms and separate control samples for each replicate and no worms used to create the soil solutions were used in the same choice trials.

#### 2.5. Statistical analyses

Statistical analysis were conducted using the software R (R Development Core Team, 2012). Chi-squared tests were used to examine deviation in worm head orientation from expected (i.e. random) orientation. The data for Experiment 1 were also analysed using a Generalised Linear Model (GLM) with a binomial error structure and logit link function (starting model) and cauchit link function (minimal model). The dependent variable was whether



**Fig. 2. Experiments settings and main results.** A. Setting of the primary experiment and results showing that earthworms tended to go to the side where their same lineage had left their chemical substances. B. Setting of the secondary experiment and results showing that those chemicals may be water-borne substances since the earthworms had the same behaviour as in the primary experiment when left to choose between sides with water incubated with the soils. Control refers to an extra treatment where water for one side was incubated with soil with no worm on it. *L. rubellus* image: Copyright Malcolm Storey/[www.discoverlife.org](http://www.discoverlife.org).

the worm turned towards the side of the mesocosm that had previously housed a worm of the same lineage, or towards the side that had previously housed a worm of the other lineage. Independent variables were: lineage (same/different), body mass, experimental round block, and the distance that the worm had burrowed into the soil. Model selection was based on AIC comparisons, to identify a minimal adequate model. Biologically relevant two-way interactions were examined, and retained in the minimal model on the basis of AIC comparisons and significance tests. Model validation was based on examination of residual plots, following Thomas et al. (2015).

The data for Experiment 2 were analysed using a Generalised Linear Mixed Model (GLMM) with a binomial error structure and complimentary log–log (“cloglog”) link function. The dependent variable was whether the worm turned towards the side of the Petri dish containing extract from soil that had previously housed the same lineage. The repeated measurements of the same individuals over time were represented in the model, by including individual identity as a random variable. The fixed terms were; time, body mass of the focal worm, and treatment (a factor with three levels, namely “treatment A” comprising lineage A worms choosing between lineage A extracts and lineage B extracts; “treatment B” comprising lineage B worms choosing between lineage B extracts and lineage A extracts; and a control group, comprising lineage A worms choosing between lineage A extracts and a control solution containing no worm extracts). Model selection and validation procedures were as for Experiment 1, above.

The null hypothesis for both of these experiments was that there would be no significant attraction to a specific side, i.e. the number

of worms showing a preference for soil previously housing the same lineage would be the same as the number of worms showing a preference for soil previously housing the opposite lineage, or no worm (control). Conversely, our expectation was that worms would be significantly more likely to turn towards the side of the mesocosm (Experiment 1) or Petri dish (Experiment 2) containing soil or soil water-extract, respectively, derived from its own lineage.

### 3. Results

#### 3.1. Lineage identification

Generated sequences have been deposited in GenBank (Supplementary Table S3). The 128 genotyped individuals of *L. rubellus* belonged to two distinct lineages (named A and B as in Andre et al., 2010); 56 individuals belonging to lineage A and 72 individuals belonging to lineage B were found. The total number of haplotypes was 20 (haplotype diversity  $h$ : 0.724; nucleotide diversity  $\pi$ : 0.058), 6 of those haplotypes being unique to lineage A ( $h$ : 0.51;  $\pi$ : 0.0023) and 14 unique to lineage B ( $h$ : 0.417;  $\pi$ : 0.0014). Sixty segregating sites were found within the 407 bp sequence. Networks from both species were separated according to the parsimony limit and each showed a more abundant haplotype, considered as the ancestral by the program (indicated by a square network shape), and less abundant derived ones, showing a star-like network shape in case of lineage B (see Supplementary Fig. S1). Mean uncorrected p-distance between both lineages was 11.52%, whereas within-lineage the distances ranged from 0.25% to 1.47% for lineage A and from 0.25% to 0.98% for lineage B. The



phylogenetic tree presented in Fig. 1B clearly shows the phylogenetic divergence between the two cryptic lineages. Earthworms from lineage B were substantially heavier (mean = 0.98 g; s.d. = 0.31) than earthworms from lineage A (mean = 0.63 g; s.d. = 0.24). A GLM (Gaussian error, log-link) explaining body mass demonstrated a significant difference in mass between the two lineages ( $F_{1,100} = 43.264$ ,  $p < 0.0001$ ).

### 3.2. Experiment 1: earthworms are attracted to soil previously inhabited by worms of the same lineage

Earthworms showed a tendency to move into the side of the mesocosms containing soil that had previously occupied by worms from the same lineage (Fig. 2A). This was true for both lineages separately, with 75% of worms from lineage A choosing the side of their same lineage (significantly different from random;  $\chi^2 = 6$ ,  $DF = 1$ ,  $p = 0.0143$ ) and 76% of the worms from lineage B ( $\chi^2 = 5.762$ ,  $DF = 1$ ,  $p = 0.0164$ ) moving into the side previously occupied by worms of the same lineage. A  $2 \times 2$  contingency table test showed that there was no significant difference between lineages in this regard ( $\chi^2 = 0$ ,  $DF = 1$ ,  $p > 0.999$ ) and that, therefore, they could be analysed together in order to improve statistical power. The combined results showed that worms exhibited a significant preference to move into soils previously occupied by worms of the same lineage as themselves ( $\chi^2 = 11.756$ ,  $DF = 1$ ,  $p = 0.0006$ ). A GLM to explain whether worms moved into soils occupied by worms of either the same or different lineage (binomial error GLM, logit-link) showed that there was no significant effect of lineage, body mass or experimental round on the outcome (all LRT values  $< 0.06$ ,  $DF = 1$  in each case, all  $p > 0.800$ ). The minimal adequate GLM also revealed a significant association between outcome and the distance that the worm had burrowed into the soil (binomial error GLM, cauchit-link;  $LRT = 7.696$ ,  $DF = 1$ ,  $p = 0.006$ ), with worms that had burrowed further into the soil being more likely to have moved towards soil that had previously contained the same genotype (Supplementary Fig. S2).

### 3.3. Experiment 2: secreted attractants are water-soluble molecules

The results of Experiment 2 mirrored those from Experiment 1, with the difference being that each earthworm was presented with a choice between two halves of a Petri dish, containing filter paper wetted on one half with water-soluble chemicals extracted from soil previously occupied by worms of the same lineage as themselves and on the other half with water extracted from soil worked by individual worms belonging to the other lineage (treatments A and B), or a control solution (control group). Overall, there was a high proportion of 'correct side' choice (72% of worms;  $\chi^2 = 84.01$ ,  $DF = 1$ ,  $p < 0.0001$ ) exhibited by worms of both lineages across the 3-h test period (Fig. 2B).

A GLM (binomial error, clog-log link) revealed that there was no significant variation in the orientation behaviour of the earthworms across the observation period (covariate = Time,  $LRT = 2.476$ ,  $DF = 1$ ,  $p = 0.116$ ), (Supplementary Fig. S3). There was also no difference in the ability of the two lineages to orientate towards the side containing extract of the same lineage (pairwise comparison of treatment A vs. treatment B;  $Z = 0.750$ ,  $DF = 1$ ,  $p = 0.451$ ). Nevertheless the behaviour of the control group was significantly different from the earthworms in treatments A and B, with worms in the control group showing a stronger preference for the 'correct side' (factor = Treatment;  $LRT = 6.454$ ,  $DF = 2,443$ ,  $p = 0.040$ . Pairwise comparison of control vs. treatment A;  $Z = 2.48$ ,  $DF = 1$ ,  $p = 0.013$ . Pairwise comparison of control vs. treatment B;  $Z = 2.28$ ,  $df = 1$ ,  $p = 0.022$ ). The same GLM revealed a significant positive association between the body mass of the worm and its

preference with heavier worms showing a stronger preference for the extract from the same lineage (covariate = body mass;  $LRT = 7.768$ ,  $DF = 1$ ,  $p = 0.005$ ) (Supplementary Fig. S4).

## 4. Discussion

Our results provide evidence that water-soluble molecules mediate the attraction of individual *L. rubellus* to individuals of the same genetic lineage. This behavioural response to chemical signals released by con(cryptic)specifics into the soil is a candidate mechanism to explain the maintenance of pre-copulatory reproductive isolation between cryptic lineages of earthworms. Our study examined the direction of movement towards or away from soil- and water-borne extracts from different lineages as a measure of attraction; future studies could examine whether these behavioural responses do indeed lead to assortative mating between the two lineages.

### 4.1. Cryptic speciation in *Lumbricus rubellus*

Genetic characterization through the mitochondrial gene COII and phylogenetic analyses, confirmed the presence of two cryptic lineages within *L. rubellus* collected from the field site. The subtle intraspecific genetic variation shown within this species has been previously documented by King et al. (2008) and Andre et al. (2010) and our sequences clustered together with the described lineages A and B in these previous studies. Donnelly et al. (2013) confirmed the lack of gene flow between these two lineages using microsatellite markers. Although RAD-Seq analyses revealed that certain European *L. rubellus* lineages may not be reproductively isolated (Giska et al., 2015), a similar genetic analysis observed that no hybridization occurs between A and B lineages in a number of UK locations where the lineages co-existed (Anderson pers. comm.). According to Sechi (2013) the two sympatric lineages appear to have evolved in allopatry during the last glaciation, thus suggesting that pre-copulatory isolation mechanisms may have developed during allopatry (see below) before their secondary contact. However, as stated above, post-copulatory isolation mechanisms (such as the production of unviable cocoons) cannot be dismissed and experiments addressing this point are worth considering. This is an example of cryptic speciation, which appears to be relatively common within soil invertebrate taxa and perhaps especially in earthworms (James et al., 2010; Novo et al., 2010; Buckley et al., 2011). The named *L. rubellus* cryptic lineages were found in a 44/56 A:B abundance, and their mean genetic uncorrected inter-lineage divergence was 11.5%, at the field site chosen for the present study. In contrast, the intraspecific divergence for lineage A (0.25–1.47%) and lineage B (0.25–0.98%) are much lower and may reflect reproductive isolation between lineages. Constructed haplotype networks suggest that Haplotypes 1 and 2 are ancestral in lineages B and A, respectively, and may therefore represent the genotype of the founders of the lineages in the studied population.

### 4.2. Chemical attraction of cryptic lineages

The data gathered in Experiment 1 support the hypothesis that chemicals released into their surrounding environment by *L. rubellus* have properties involved in mate attraction. Worms showed a significant preference to move towards the mesocosm side that had previously been occupied by a worm of the same lineage. This suggests that the worms are able to detect the specific chemical signals secreted by con(cryptic)specifics into soil in the relatively confined space in our laboratory-based mesocosms. Whether this phenomenon is operative under field conditions remains an open question, as is the persistence of the molecules

involved. The fact that the choice tests were conducted in mesocosms using single worms (i.e. in the absence of the worms that had provided the chemical signals) eliminates non-chemical modes of driving directional movement, for example direct tactile contact or locomotion-mediated vibrations. *L. rubellus* has already been shown to be able to actively avoid soils laced with Cu and Zn (Lukkari and Haimi, 2005), supporting the conclusion that chemoreception is an effective means of detecting abiotic chemical stimuli within their environment. The behaviour-modifying role of biogenic compounds secreted and released by conspecific earthworms adds considerably to the present knowledge of the behavioural- and population-ecology of this taxon of soil-dwelling ecosystem engineers. The relationship between distance moved from the centre of the mesocosm and the apparent preference recorded, suggests a methodological improvement for such behavioural choice assays; the further the worms moved into the mesocosm, the more likely the choice was to be “correct”. (i.e. worms that were near the middle of the mesocosms were more prone to be “wrong” about their choice). Therefore, subsequent analyses of this type should record the preferences of worms once they have moved more than 2 cm in either direction from the centre line.

#### 4.3. Chemical attraction is driven by water-borne molecules

With mate-attraction pheromones (Attractin and Temptin) having previously been identified in *L. rubellus* (Novo et al., 2013), we hypothesize that the release of lineage-specific pheromones acts as a means of lineage-specific mate choice. These pheromones are water-borne molecules, analogous to those originally identified in the marine mollusc *Aplysia*, that function as mate attractants (Cummins et al., 2007). The data generated over the 3 h in Experiment 2 indicates that more worms of lineage A and B are attracted to a filter paper side that was soaked with a soil solution derived from soils occupied by worms of the same lineage. The same was true for the trials comparing lineage A solutes versus controls, which showed even a stronger effect since there was no confounder in the other side of the test arena. The results demonstrate that the worms of both lineages of *L. rubellus* detected specifically water-borne chemicals and altered their movements accordingly. Further studies are clearly needed to identify the molecular attractants secreted by the two *L. rubellus* lineages, to characterise their individual and combined effects, and to evaluate their persistence under a range of realistic environmental conditions.

#### 4.4. Weight differences and niche partitioning

The mean body mass of lineage B worms was shown to be significantly greater than that of lineage A worms in the study site, indicating possible size dimorphism between the two cryptic lineages, something previously reported for other cryptic species of *Lumbricus* (James et al., 2010). This may be a contributing factor in their reproductive isolation, as size assortative mating is known to occur in other earthworm species such as *Eisenia fetida* (Monroy et al., 2005) and has been documented to occur to a degree within *Lumbricus terrestris* (Michiels et al., 2001). The difference in average weights could also suggest niche partitioning, whereby the two lineages use different strategies for the exploitation of available resources. Kille et al. (2013) reported distinct adaptive responses to soil contamination of the two lineages, which also exhibit differing environmental preferences (Spurgeon pers. comm.). These studies could suggest commencement of niche specialization. Klok et al. (2006) found that frequent floods made the reproductively mature *L. rubellus* to be half weight of those from non-flooded sites.

Further work on several mixed lineage A + B populations is warranted to determine if mature lineage B individuals are consistently larger than their lineage A counterparts, and to establish whether the size difference if it exists plays a role in assortative mating.

## 5. Conclusions

The results show that two cryptic species of the earthworm *L. rubellus*, which evolved initially as allopatric lineages, live in sympatry and are found in a similar proportion within the studied area, where they maintain their genetic diversity and differentiation by means of reproductive isolation. This study has provided evidence for pre-copulatory sexual selection mechanisms driven by the release of lineage-specific chemical signals, which act as a recognition flag for worms of the same lineage to aggregate towards. An experiment involving soil extracts indicated that this attraction is mediated by water-borne molecules. Further studies would shed light on the exact nature and blend of molecules exerting this effect and their genetic basis.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.soilbio.2016.03.015>.

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