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Molecular genetic differentiation in earthworms inhabiting a heterogeneous Pb-polluted landscape

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Landscapes punctuated by Pb-polluted islands have engendered local genetic differentiation in resident earthworms.

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ABSTRACT

A Pb-mine site situated on acidic soil, but comprising of Ca-enriched islands around derelict buildings was used to study the spatial pattern of genetic diversity in *Lumbricus rubellus*. Two distinct genetic lineages ('A' and 'B'), differentiated at both the mitochondrial (mtDNA COII) and nuclear level (AFLPs) were revealed with a mean inter-lineage mtDNA sequence divergence of approximately 13%, indicative of a cryptic species complex. AFLP analysis indicates that lineage A individuals within one central 'ecological island' site are uniquely clustered, with little genetic overlap with lineage A individuals at the two peripheral sites. FTIR microspectroscopy of Pb-sequestering chloragocytes revealed different phosphate profiles in residents of adjacent acidic and calcareous islands. Bioinformatics found over-representation of Ca pathway genes in EST_{Pb} libraries. Subsequent sequencing of a Ca-transport gene, SERCA, revealed mutations in the protein's cytosolic domain. We recommend the mandatory genotyping of all individuals prior to field-based ecotoxicological assays, particularly those using discriminating genomic technologies.

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1. Introduction

Sites contaminated to different degrees with metals and metalloids are globally widespread, from geogenic deposits such as serpentine soils to anthropogenically modified soils associated with mining, various industries, and agricultural practices. Abandoned mine sites typically display conspicuous spatial heterogeneities, with geological features combining with diverse anthropogenic inputs to produce a mosaic of physicochemically contrasting ecological 'islands' to which constituents of the local biota have evidently, and perhaps variously, adapted. For example, the Cwmystwyth Valley, Wales (UK), is a region of base-poor upland grassland containing a disused Pb-mine whose shallow acidic soil is punctuated by more-or-less discrete calcareous micro-habitats around derelict buildings. Galena (PbS) extraction from the Cwmystwyth Mine stopped in about 1921, and in the intervening period the site has been colonised by two epigeic earthworm species, the subject of the present study, *Lumbricus rubellus*, and *Dendrodrilus rubidus* (Morgan and Morgan, 1991). The ancestors of these resident soil dwellers at Cwmystwyth would have survived the major glaciations and climatic instability of the Devensian period in one or more of the sheltered southern European refugia. With the onset of each stadial period and reformation of ice-sheets, retreating bottlenecked populations would have experienced shrinkage, dissection and extinction, whereas upon post-glacial expansion they would have undergone adaptation and selection to new environments (Hewitt, 2000). Repeated climatic oscillations and changes in habitat range have therefore yielded increased species diversity through several genome reorganisations, manifested on the one hand by the recently described splitting of *L. rubellus* into two deep genetic lineages (King et al., 2008) which may warrant the status of cryptic species, and on the other hand by the capacity of this peregrine taxon (Blakemore, 2002) to successfully colonize diverse soil types and conditions across wide geographical ranges. The physiological versatility of the species is exemplified by the fact that field populations inhabit soils ranging from pH 3.8 to 8.4 (Sims and Gerard, 1985), and soils contaminated with Pb to a degree exceeding by an order of magnitude the exposure level that severely compromises reproduction in spiked laboratory soils (Spurgeon et al., 1994).

Ecotoxicology seeks easily interpretable exposure ('environment') versus response ('phenotype') relationships, analogous to those that underpin classical toxicology. This relationship can however be confounded in field populations by local stress-mediated genetic differentiation (Evenden and Depledge, 1997; Morgan et al., 2007). A body of evidence has accumulated indicating

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significant differences in the responses to certain inorganic and organic environmental toxicants of cryptic (sibling) species belonging to a number of aquatic taxa (Sturmbauer et al., 1999; Rocha-Olivares et al., 2004; Bach et al., 2005; Palmqvist and Forbes, 2008). *L. rubellus* has recently been exploited as a sentinel organism in a number of ecotoxicogenomic studies (Bundy et al., 2008; Owen et al., 2008; LaCourse et al., 2009; Stürzenbaum et al., 2009), but each of these studies was performed before the extent and possible functional implications of the high genetic diversity within the 'species' (King et al., 2008) was fully appreciated. The first aim of the present study, therefore, was to use mitochondrial (mt-DNACOII) and Amplified Fragment Length Polymorphism (AFLP) markers to genotype *L. rubellus* at four discrete and geochemically contrasting sub-sites along a transect across the Cwmystwyth Mine following prior *in-situ* spatial mapping of surface soil Pb concentrations with a combination of portable X-ray fluorescence spectroscopy (XRF) and GPS localization. The objectives were to determine: (i) whether both *L. rubellus* genetic lineages identified by King et al. (2008) are present in the locality; (ii) whether the distribution patterns of the two lineages, if present, could be related to the soil composition of ecological islands across the site; and (iii) whether or not within-lineage genetic diversity has been locally eroded (van Straalen and Timmermans, 2002) by chemical stressors. Although a recent publication (Langdon et al., 2009) based on the acute toxicity testing of laboratory-reared offspring indicated that a population of *L. rubellus* inhabiting a field soil heavily contaminated with As has evolved metalloid resistance, it is important to emphasise that the present phylogenetic study was motivated by the need to establish the extent and distribution of genetic diversity in a highly heterogeneous landscape. An earlier attempt to use laboratory-bred offspring from the Cwmystwyth Mine to reveal evidence of heritable Pb resistance proved inconclusive (Aziz et al., 1999) but this is not to say that *L. rubellus* at the site is not spatially differentiated into more-or-less distinct genotype clusters. Whilst evolution is considered to be an almost inevitable consequence of stress, the means by which adaptation is achieved may, in general, be deemed to be either facultative or constitutive (Bradshaw and Hardwick, 1989). For instance, the phenotypic plasticity that is usually favoured in heterogeneous or variable environments is itself genetically determined (Windig et al., 2004). On the other hand, when selection pressure is heavy and relatively constant over several generations, it is probable that an organism will evolve a fixed resistance mechanism (Bradshaw and Hardwick, 1989). In this study we did not explicitly seek to resolve whether Cwmystwyth Mine worms are facultatively or constitutively adapted to local stress challenges; rather, we were engaged in determining the range of genetic variability or amplitude upon which stressors have impinged.

The detrimental effects of Pb exposure arise from its ability to mimic the trafficking and metabolism of essential cations, notably Ca (Clarkson, 1993; Warren et al., 1998). Intracellular interactions between Pb and Ca are well documented, with non-sequestered Pb metal ions shown to interact and associate with proteins active in the calcium signalling pathway. This shared chemical affinity between Pb and Ca lead us to hypothesise that the network of mechanisms evolved by all living cells to regulate potentially lethal free Ca^{2+} levels are somehow implicated in the handling of its non-essential cationic analogue. The main molecular mechanisms underlying metal tolerance in invertebrates entails either metal efflux pumps (Callaghan and Denny, 2002) or sequestration by one of three classes of thiol-rich peptides, namely glutathione, phytochelatin and metallothionein (Vatamaniuk et al., 2005; Janssens et al., 2007). However, neither of these generic mechanisms has been found to underpin Pb adaptations in earthworms. Indeed, Pb is sequestered by earthworms within the calcium phosphate-rich matrix of chloragosomes, unique organelles with certain lysosome-like properties that are located in the chloragogenous tissue (Morgan and Morgan, 1989). It is plausible that specific transport molecules reside in the limiting membranes of earthworm chloragosomes that promote both the uptake of O_2-seeking metals such as Pb and Ca and also provide the negatively-charged counter-ion (i.e. phosphate) required for mineralization. Consequently, the second major aim of the present study was to explore provisionally certain candidate molecular mechanisms of Pb management and adaptation in chronically exposed earthworm populations. This was achieved through global transcriptomic analyses, *in-situ* biochemical fingerprinting by FTIR microspectroscopy of cryo-sectioned chloragogenous tissue from earthworms quench frozen in the field to maintain compositional fidelity, and targeted single locus experiments focused on an important intracellular transmembrane Ca-transporter, SERCA (Bolotina and Csutora, 2005). Thus, the study employed an unprecedented combination of geochemical and molecular-genetic tools to obtain information about population-level genetic differentiation in an ecosystem engineering sentinel organism, and about predicted functionally important structural modifications in a potentially key molecular component underlying Pb/Ca tolerance traits.

2. Materials and methods

2.1. Portable X-ray fluorescence (XRF), pH mapping of the Cwmystwyth site and ICP-OES determination of total soil and body metal concentrations

A portable XRF (NITON XLiand, Thermo Scientific Inc, Germany) and GPS system (Garmin, Etrex Venture, UK) were used in order to create a Pb profile of the Cwmystwyth valley, with a total of 97 random measurements taken across the site. At 70 of these sites a soil sample (~ 50 g, taken from the soil litter and upper layers of the soil) was also collected and the pH recorded. The mapping software SURFER© was used to convert both the metal and pH data sets into a series of 3D rendered surface maps, stacked alongside a base-map of the valley. The Pb concentration of earthworms and soil from each site, C1 (OS grid reference, SN 809749), C2 (SN 801746), C3 (SN 804746), C4 (SN 797743) and R1 (ST 149723), was determined. Several soil samples were randomly collected from each site and pooled. Soil was dried, sieved to <2 mm through a stainless steel mesh, digested in boiling aqua regia and analysed for Pb by ICP-OES (Arnold et al., 2008). Earthworms ($n = 3$) were transported back to the laboratory in their native soil and depurated as described in (Arnold et al., 2007). Following this depuration period the earthworms were placed individually into Sterilin tubes, stored at -18 ± 1 °C until digestion, and analysed for Pb by ICP-OES (Langdon et al., 2005).

2.2. Mitochondrial (cytochrome oxidase II) and amplified fragment length polymorphism (AFLP) genotyping

L. rubellus earthworms were collected by digging and hand-sorting. The animals were transported back to the laboratory in their native soil and depurated as described in Arnold and Hodson (2007). Four populations, $C1^{pH5}_{Pb^*}$ ($n = 27$),$C2^{pH4}_{Pb^*}$ ($n = 33$),$C3^{pH7}_{Pb_{**}*}$ ($n = 32$) and $C4^{pH6}_{Pb^*}$ ($n = 30$), were sampled from four locations across the study site. C1 and C4 were located at the periphery of the site, thereby representing on-site references. C2 and C3 are highly contaminated sites with contrasting pH and soil chemistry; C2 is acidic whereas C3 is circumneutral in pH and calcareous in nature. The interaction of soil metal load and pH is important when considering bioavailability, and is reflected in accumulated metal body loads. The number of asterisks denotes the level of contamination as classified by the Kelly index (ICRCL 59/83) (KellyIndices, 1980): * contaminated (1000–2000 mg kg^{-1}), ** heavily contaminated (2000–10 000 mg kg^{-1}) and *** unusually heavily contaminated (>10 000 mg kg^{-1}). Genomic DNA was extracted from all four populations using DNAzol reagent (Invitrogen Ltd., Paisley, UK.). DNA was also isolated from *Lumbricus castaneus* and *Lumbricus eiseni*. Forward (5′-TAGCTCACTTAGATGCCA) and reverse (5′-GTATGCGGATTTCTAATTGT) *L. rubellus* specific cytochrome oxidase II (COII) primers were designed from mitochondrial sequences deposited in LumbriBASE (www.earthworms.org). For each PCR reaction ~ 100 ng DNA template was amplified using 10 pmol/μl forward and reverse primer, 10 mM dNTP mix and 5U/μl Taq DNA polymerase buffered with 5 X Mg-free Taq PCR amplification buffer and supplemented with $MgCl_2$ (1.5 mM). The reaction was denatured at 95 °C for 10 min and then cycled 35 times at 95 °C for 30 s, 30 s at the required primer annealing temperature and 72° for 1 min. This was followed by a 10 min final extension at 70 °C. The amplicon (469 bp) was resolved by electrophoresis in 1 X TAE buffer at 120 V for approximately 30 min in a Pharmacia GNA-100 tank. Nucleic acid bands were then visualised on a UV gel documentation system. Prior to sequencing PCR clean-ups were performed using Exo-SAP-IT (Amersham Pharmacia, UK) reagents. Exonuclease 1 (0.25 μl) and Shrimp Alkaline Phosphatase (0.5 μl) were mixed with

the PCR product (10 μl) and incubated at 37 °C for 45 min followed by 80 °C for 15 min. DNA was sequenced using ABI PRISM® BigDye v3.1 Terminator Sequencing technology (Applied Biosystems, USA) on the ABI PRISM® 3100 DNA Sequencer run by the Cardiff University Molecular Biology Support Unit. Raw sequence traces were confirmed using Finch TV before being imported into Mega v3.1 (Kumar et al., 2004) for alignment and tree construction. The distance-based neighbour joining (NJ) algorithm (Saitou and Nei, 1987), using p-distances, was used to estimate tree topology and calculate branch lengths. Relationships between phylogenetic haplotypes were determined by maximum parsimony (MP), maximum likelihood (ML) and Bayesian methods using PAUP v3.1 and MRBAYES respectively (Huelsenbeck and Crandall, 1997; Huelsenbeck and Ronquist, 2001), MRMODELTEST v2.2 (Nylander, 2004) and the Akaike Information Criterion (AIC) were used to select the optimum model (HKY + G) of sequence evolution that best fitted the data (base frequencies of $A = 0.3638$, $C = 0.2244$, $G = 0.1277$, $T = 0.2842$ and T-ratio = 3.5050 and Gamma distribution parameter = 0.2005). Node support for MP and ML analyses was determined using a non-parametric bootstrap, with 500 and 1000 replicates respectively (Holmes, 2003). For the analysis 3 × 106 generations were run, with one tree retained every 300th generation and the first 2500 trees discarded as burn-in. Genetic distances were calculated using p-distance in Mega and median-joining networks were drawn using NETWORK and dnasp4.

AFLP analysis was adapted from Ajmone-Marsan et al. (1997) with approximately 200 ng of genomic DNA extracted from $C1^{pH5}_{Pb*}$ ($n = 24$),$C2^{pH4}_{Pb*}$ ($n = 30$),$C3^{pH7}_{Pb***}$ ($n = 23$) and $C4^{pH6}_{Pb*}$ ($n = 18$) individuals. Pre-selective EcoR1 (GACTGCGTACCAATTCA) and Taq1 (GACTGCGTACCAATTCC) primers were used and for the selective amplifications two primer combinations E32 (5′-GACTGCGTACCAATTCAAC-3′)/T32 (5′-GATGAGTCCTGACCGAAAC-3′) and E32/T38 (5′-GATGAGTCCTGAC CGAACT-3′) were employed. Reactions were run by the Cardiff University Molecular Biology Support Unit and analysed on an Applied Biosystems 3130 × 1 fragment analyser. Bands between 70 and 325 base pairs (bp) and with a minimum peak height of 70 units were scored using GeneMapper analysis software. Microsoft Access, Excel and the Excel macro GenAlEx6 were used to create a cumulative table of all loci from each individual and transform the data into a binary form. Principal coordinates (PCO) analysis and phylogenetic tree construction, supported by bootstrap analysis (1000 replicates), was performed using the neighbour joining algorithm (based upon the Nei's distance) to in PAUP v4.0b10 to estimate tree topology and calculate branch length. The software Structure v2.2 was used to delineate clusters of individuals on a multi-locus, genotype basis using a Bayesian algorithm. The number of inferred populations ran from 1 to 5, with 8 replicate runs, a burn-in of 75 000 cycles followed by 300 000 for data collection. L(K), the modal choice criterion, is calculated in Structure and the true number of populations (K) can be deferred from its maximal value. ΔK, the rate of change in the log probability of data between successive K-values, provides a visual means to easily identify the number of clusters in a sample of individuals (Evanno et al., 2005).

2.3. EST libraries and informatics

Upon sampling $C3^{pH7}_{Pb***}$ ($n = 5$) earthworms were immediately immersed and maintained in liquid nitrogen and stored at −80 °C until required. Earthworms were also collected from a South Wales reference site, R1 ($n = 5$), and transported back to the laboratory in their native soil. These individuals were exposed to 500, 750, 1250, 1750 and 2250 mg kg^{-1} Pb respectively, in the form of $Pb(NO_3)_2$ spiked Kettering loam (Barrycroft Stores Limited, Kettering, UK) soil (Davies et al., 2003) and maintained at a WHC of 75% for three weeks at 15 °C. Spiked soil was left to equilibrate for 3 days prior to earthworm addition. Following exposure, earthworms were snap-frozen in liquid nitrogen and stored at −80 °C. Earthworm total RNA (~1.25 mg) was extracted using the TRI-reagent method (Sigma–Aldrich, UK) and mRNA isolated using an mRNA Purification Kit (Amersham, UK). $C3^{pH7}_{Pb***}$ and R1 cDNA-libraries were constructed using the pBluescript® II XR cDNA Library Construction Kit (Stratagene Europe, The Netherlands). PCR was used to screen the libraries and quantify insert size. Each PCR contained 5 μl neat culture, 10 X Triton free PCR Buffer (10 μl), MgCl2 (0.25 μl, 1 mM), universal M13F and M13R primers (0.2 μl, 10 mM), dATP, dCTP, dTTP, dGTP (0.2 μl, 100 mM) and Taq polymerase (0.16 μl, 50U/ml) in a 95 μl reaction. The reaction was denatured at 95 °C for 10 min and then cycled 35 times at 95 °C for 30 s, 30 s at the primer annealing temperature of 56 °C and 72 °C for 1 min. This was followed by a 10 min final extension at 70 °C. Products were resolved by electrophoresis using E-Gel® technology (Invitrogen Ltd., UK) and associated editing software. High quality clones were cherry-picked using the MultiPROBE® II HT EX liquid handling system (PerkinElmer, Bucks., UK) and associated WinPrep® software. The composite plate products were purified using Montage® Multiscreen PCRμ96 cleanup plates by vacuum filtration and the DNA was resuspended in sterile water (30 μl). Sequencing reactions were completed by the SBSSS facility at Edinburgh University and sequences named according to the NERC Environmental Genomics scheme to allow for bioinformatics analysis. The raw trace chromatograms from the sequencing reaction were processed using trace2dbEST (Sturzenbaum et al., 2003) which contains a base calling component (phred) and a sequence trimming component (cross_match). This software produces good quality EST sequences, formatted for submitting to NCBI dbEST (http://www.ncbi.nlm.nih.gov/dbEST). The EST sequences were clustered using CLOBB (Sturzenbaum et al., 2003) to derive a consensus putative gene sequence contig and then processed by the software package PartiGene (Sturzenbaum et al., 2003) (http://www.nematodes.org/PartiGene). Cluster information can be retrieved by LumbriBASE (http://www.earthworms.org) through simple text queries, identification of sequence similarity and library specific searches. The biological process and molecular function of gene sequences were described by defining their Gene Ontology (GO) classification using blast2go (http://www.blast2GO.de).

2.4. Fourier-transform infrared spectroscopy

Soil and adult (fully clitellate) *L. rubellus* earthworms were collected from $C2^{pH4}_{Pb*}$ and $C3^{pH7}_{Pb***}$ and the posterior segments immediately excised and quench-frozen in liquid nitrogen. The frozen tissue was transported to the laboratory under liquid nitrogen and stored at −20 °C until required. Tissues were mounted in CryoEmbed and sectioned longitudinally at a nominal thickness of 50 μm in a Bright cryostat. Sections were mounted on Kevley slides and air-dried overnight in the cold chamber of the cryostat. Infra-red spectra were collected in transmission mode from station 11.1 at the CLRC Daresbury Synchrotron Radiation Source. The chloragogenous tissue was visually identified and each section imaged and analysed, with five spectra from five different regions of the tissue (i.e. x25 spectra per individual earthworm) collected. Following FTIR, genomic DNA was extracted directly from tissue sections using the QIAamp DNA Micro Kit according to the manufacturer's instructions (Qiagen Ltd., UK). Buffer ATL (180 μl) was pipetted directly onto the Kevely slide to remove the section, prior to lysis in a microcentrifuge tube at 56 °C overnight.

2.5. SERCA

Plasmid preparations of individual LumbriBASE clones (Genbank accession numbers CF416761 and CO048347) were prepared using a Wizard® Plus SV Miniprep kit (Promega Ltd., UK). Preparations were sequenced in their entirety by "walking" along the gene, after each step re-designing a specific reverse primer to complement the universal M13 forward. Primers were designed using the software Primer3 (Rozen and Skaletsky, 2000) and Oligo® (MBI Inc, USA) and sequencing performed as described above. These full-length library sequences were used to design *L. rubellus* specific SERCA primers in order to amplify the gene transcribed in individuals of each genealogical lineage. Reactions were denatured at 95 °C for 10 min and then cycled 35 times at 95 °C for 30 s, 30 s at the required primer annealing temperature and 72° for 1 min. This was followed by a 10 min final extension at 72 °C. DNA was sequenced as described above by the Cardiff University Molecular Biology Support Unit.

Total RNA was extracted from tail-clips of an adult individual sampled from $C3^{pH7}_{Pb***}$ and $C2^{pH4}_{Pb*}$ using the Tri-reagent method (Sigma–Aldrich, UK). Complementary DNA (cDNA) was synthesised from messenger RNA (mRNA) using reverse transcriptase. Total RNA (7–20 μg) was heated at 65 °C for 3 min and combined with anchored oligo d(T) (1 μl, 100 mM) and random hexamers (2 μl, 100 mM) and incubated at 70 °C for 10 min. A reaction mix of 5 X RT buffer (6 μl), DTT (3 μl, 0.1 M) and dNTP mix (1.2 μl, 10 mM) was prepared and added to the RNA mix and incubated at 25 °C for 2 min. Superscript (1 μl, Invitrogen Ltd., Paisley, UK.) was added and the reaction incubated at 42 °C for 3 h. A series of three PCR reactions were optimised and performed in order to obtain the full-length SERCA sequence of each individual; between sequenced sections of the gene there was a large overlap to ensure the same SERCA isoform was being amplified in each instance. PCRs were performed as described above. Reactions that yielded products >2000 bp were modified. For these reactions the DNA (~100 ng) template was amplified using 10 μM forward and reverse primer, 25 mM dNTP mix and 1 μl Herculase® II Fusion DNA Polymerase buffered with 5 X Herculase® II PCR reaction buffer (Stratagene Europe, The Netherlands). Each reaction was supplemented with an optimised quantity of $MgCl_2$ (25 mM). The reaction was denatured at 95 °C for 10 min and then cycled 35 times at 95 °C for 20 s, 20 s at the required primer annealing temperature and 68 °C for 4 min. This was followed by a 4 min final extension at 68 °C. Protein sequences were aligned using bioinformatic software tool Mega v3.1 (Kumar et al., 2004) and modelled using SWISS-MODEL (http://swissmodel.expasy.org//SWISSMODEL.html), Swiss-PdbViewer and Pymol.

Total RNA was extracted from nine, previously genotyped (mtDNA) adult individuals sampled from, $C2^{pH4}_{Pb*}$ and $C3^{pH7}_{Pb***}$, four from $C4^{pH6}_{Pb*}$ and three from $C1^{pH5}_{Pb*}$. This was followed by cDNA synthesis, as described above. A PCR was designed to enable easy identification by gel electrophoresis of the expressed isoform, with a combination of three primers used in each reaction (F1 5′-CTGGCCGGAATTCGTGTTATC-3′, F2 5′-ATACTCTTCGCTGTCTTGCGT-3′, R1 5′-CCGCTGGCTCTTCTTCCG-3′). The two forward primers were designed so that each one isolated one of the two isoforms. The resulting products were of different sizes to enable simple identification on an agarose gel following resolution by electrophoresis.

3. Results and discussion

3.1. The 'field laboratory': the metalliferous site and its resident earthworms

In the decades since its abandonment, the spatially chequered and hostile Cwmystwyth Pb-mine site has developed micro-habitats

colonised by a limited variety of naturally occurring plants and invertebrates. Thus, it serves as an ideal evolutionary field laboratory. *L. rubellus* was sampled from four sites, effectively 'ecological islands', across the mine: $C1^{pH5}_{Pb*}$, $C2^{pH4}_{Pb*}$, $C3^{pH7}_{Pb***}$, and $C4^{pH6}_{Pb*}$ (Fig. 1). In addition to measuring soil Pb concentrations at these locations, whole-earthworm Pb contents were also measured in order to account for the integrated effects of local edaphic factors on Pb availability to earthworms (Peijnenburg, 2002). The relationship between pH and bioavailability is evident; earthworms inhabiting the soil with low ambient pH ($C2^{pH4}_{Pb*}$) have accumulated a significantly higher Pb burden than their counterparts inhabiting a circumneutral soil containing a much higher 'total' Pb content ($C3^{pH7}_{Pb***}$) (Table 1), where the Pb concentration factors (worm: soil Pb ratio) are 4.99 and 0.32, respectively.

Population divergence was measured using amplified fragment length polymorphism (AFLP) analysis and mitochondrial cytochrome oxidase II (mtDNA COII) gene sequence data of individuals sampled from the four sites. Two distinct genetic lineages, differentiated at both the mitochondrial and nuclear level, were revealed with a mean inter-lineage mtDNA sequence divergence of approximately 13%, indicative of a cryptic species complex (Fig. 2A and B). Such cryptic complexes are typical in taxa that thrive in specialised environments and have been noted for other earthworm species (King et al., 2008; Shepeleva et al., 2008; Pérez-Losada et al., 2009).

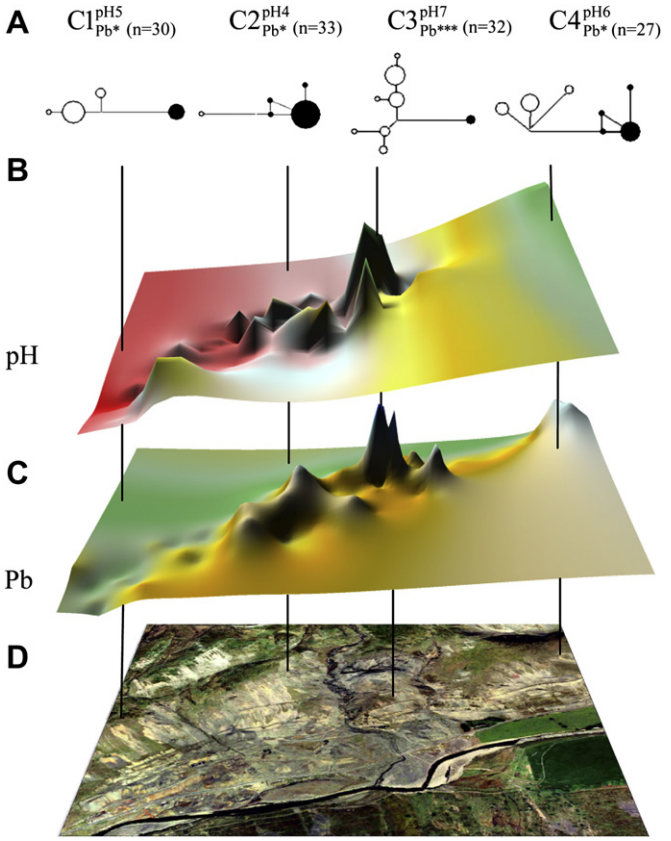


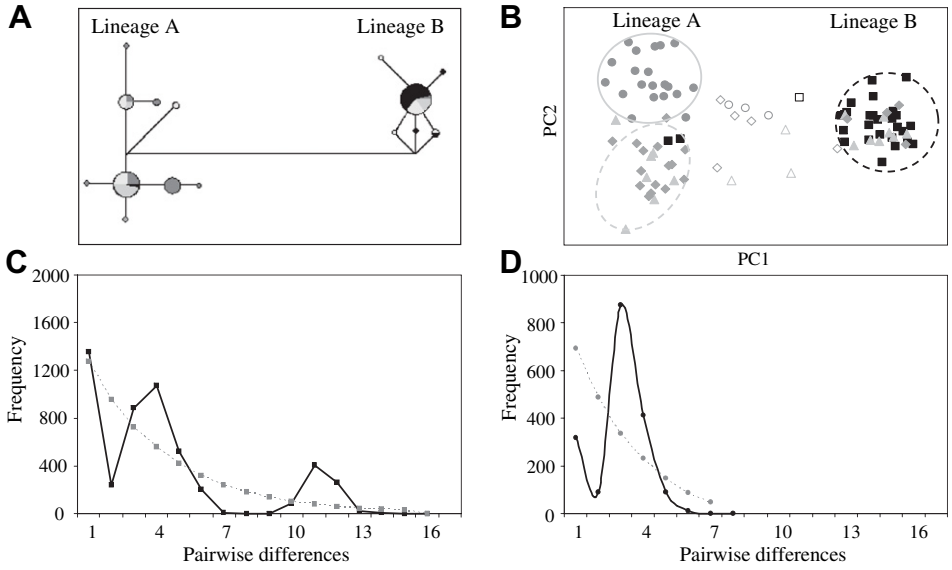
Fig. 1. Earthworm population structure superimposed on a geochemical map of the Cwmystwyth Pb mine. Surface maps depicting the pH (B) and Pb (C) levels are overlaid on a topographical map of the Cwmystwyth valley (D), derived from 71 to 97 independent measurements respectively, and generated using SURFER©. The four earthworm sampling sites are indicated by vertical black guide lines together with the median-joining networks depicting the phylogenetic structure, based upon cytochrome oxidase II sequence data, of each population studied (A). The size of each haplotype group within the network is proportional to the total number of individuals attributed to the genotype. Mitochondrial lineage A individuals are shown as open circles whilst lineage B are filled circles.

Table 1
The total Pb body burden of earthworms collected from each Cwmystwyth valley site, ($C1^{pH5}_{Pb*}$, $C2^{pH4}_{Pb*}$, $C3^{pH7}_{Pb***}$, $C4^{pH6}_{Pb*}$) and reference site (R1) alongside the total Pb concentration of all the soils and their pH. Values are expressed as the mean ± the standard error.

Site	pH	Soil Pb ($mgkg^{-1}$)	Earthworm Pb ($mgkg^{-1}$)
C1	5.9	2851 ± 68	415 ± 71
C2	4.4	1217 ± 51	6077 ± 1706
C3	6.5	11928 ± 659	3850 ± 390
C4	5.1	615 ± 8	153 ± 67
R1	6.9	103	50 ± 14

It is interesting that Lineage A predominates at the intensely Pb-polluted calcareous site ($C3^{pH7}_{Pb***}$) but Lineage B predominates and the almost adjacent moderately Pb-polluted site ($C2^{pH4}_{Pb*}$), whereas the less contaminated flanking sites ($C1^{pH5}_{Pb*}$, $C4^{pH6}_{Pb*}$) have representatives of both lineages (Fig. 2A and B). If the two lineages can be assigned the status of cryptic species, then the two central but geochemically contrasting micro-habitats appear to have experienced differential losses of earthworm biodiversity, analogous to events described by Rocha-Olivares et al. (2004) in species complexes of aquatic copepods. On the other hand, if the lineages are manifestations of extremely high intra-species genetic diversity, then the *L. rubellus* populations inhabiting $C2^{pH4}_{Pb*}$ and $C3^{pH7}_{Pb***}$ are almost certainly the evolved products of differential genetic erosion (van Straalen and Timmermans, 2002). It is noteworthy that AFLP analysis indicates that lineage A individuals at site $C3^{pH7}_{Pb***}$ are uniquely clustered, with little genetic overlap with lineage A individuals at the two peripheral sites (Fig. 2B), implying strong within-lineage selection at this location. In comparison, AFLP analysis indicates that there is considerable overlap in the genetic constitutions of lineage B individuals at site $C2^{pH4}_{Pb*}$ with those of lineage B individuals at both peripheral sites $C1^{pH5}_{Pb*}$ and $C4^{pH6}_{Pb*}$ (Fig. 2B). These observations indicate that there is a high degree of spatially localised genetic differentiation, and possibly genetic erosion, in earthworm populations inhabiting contrasting microhabitat islands across this complex mine site. Since the phenotypic characteristics of site-specific genotypes has not yet been established, it is premature to conclude whether the two deeply differentiated *L. rubellus* genetic lineages display fitness advantages under different edaphic conditions even though the genotype spatial patterns at the Cwmystwyth mine are not inconsistent with the notion.

As phylogenetic population structure is shaped by ongoing processes of genetic drift and gene flow, combined with past historical events, unravelling the *L. rubellus* species complex requires inferences on both the structure of the phylogeny and demographic tendencies. The timeline of divergence leading to sustained differentiation is neither rapid nor definable and, due to the combined effects of gene flow and selection of adaptively important genes, the genomes of incompletely isolated populations will contain an assortment of variable and undifferentiated regions (Supporting data). Fluctuations in the global climate have led to major ice ages during the Quaternary period, with the Pleistocene epoch (1 808 000–11 500 before present (BP)) covering the most recent period of repeated glaciations. Glaciation evidence can be related to the profile of mtDNA haplotypes in both lineage A and B, the shape of the corresponding mismatch distributions (Fig. 2C and D), and estimated time since population expansion. Lineage A comprises nine haplotypes that contain two or more individuals. This, combined with a ragged multimodal mismatch distribution, is suggestive of a stationary population that has undergone multiple introductions and bottleneck episodes (Harpending, 1994). Additionally, from the parameters Tau and date of growth in mutational units, expansion is estimated to have occurred approximately 250 000 years BP (assuming one generation per year) and may have corresponded



E

	SSD	*P*SSD	Rg	*P*RG	τ	*t*
Lineage A	0.023	0.848	0.053	0.639	62	244479
Lineage B	0.073	0.181	0.202	0.196	4.3	16956

Fig. 2. Mitochondrial and nuclear analysis of the earthworm, *L. rubellus*, population structure and corresponding mitochondrial mismatch distributions. Earthworms collected at four equally specially distributed sites with contrasting geochemical properties were analysed for their mitochondrial (panel A) and nuclear (panel B) genotype. Sites included $C4^{pH6}_{Pb*}$ (light grey triangles) and $C1^{pH5}_{Pb*}$ (grey diamonds), at the boundary of mine, together with $C3^{pH7}_{Pb***}$ (dark grey circles) and $C2^{pH4}_{Pb*}$ (black squares). (A) Median-joining network analysis based upon 440 bp sequence of the cytochrome oxidase II mitochondrial gene of 122 *L. rubellus* individuals. The size of each haplotype group within the network is proportion to the total number of individuals attributed to the genotype whilst the earthworm source is indicated by fill color. The left and right hand branches of the network are denoted lineage A & B respectively. (B) AFLP multi-locus profiling principal component analysis showing individuals from the four sample stations. Those individuals exhibiting mitochondrial lineage B genotype are circled in a dotted black circle whilst those attributed to lineage A are circled in a dotted grey circle. The clustering of $C3^{pH7}_{Pb***}$ earthworms distinct from other lineage A earthworms is indicated with a solid grey circle. Hybrid individuals are shown by a lack of fill color. (C & D) Arlequin simulated mitochondrial mismatch distributions, using the model of demographic expansion, of lineage A (C) and lineage B (D) haplotypes respectively. The solid lines are the observed mismatch distribution and the dotted line shows the distribution simulated under the expansion model. (E) Associated sum of squared deviation (SSD), Raggedness (Rg) and *p*-value statistics, based on 1000 data bootstraps.

with a non-glacial environment such as the Hoxnian interstadial (~250 000 BP) (Brown, 1979) (Fig. 2E). In contrast, lineage B consists of three haplotypes that contain two or more individuals, and displays an unimodal mismatch distribution, and a post-glacial population expansion time of approximately 17 000 years BP was calculated. This combined evidence suggests that the population experienced a single burst of growth and expanded after the height of the last glaciation period (~25 000 BP) with adaptation or selection occurring in response to the warmer climate experienced towards the end of the Devensian glaciation and onset of the Windermere interstadial (Brown, 1979; Harpending, 1994).

3.2. 'In-situ' FTIR microspectroscopical biochemical fingerprinting

These field earthworm populations prodigiously accumulate up to 1.5% of total body dry mass Pb (Morgan et al., 2001), with Ca/PO4-rich earthworm chloragocyte cells constituting the main metal sequestering organ (Cotter-Howells et al., 2005). Fourier transform infra-red (FT-IR) microspectroscopy on a high energy synchrotron source was used to determine the chemical composition of cryo-sectioned chloragocytes in earthworms belonging to each lineage at the two heavily polluted, albeit one acidic ($C2^{pH4}_{Pb*}$) and one calcareous ($C3^{pH7}_{Pb***}$), mine sites. The chlorogogenous tissue was found to have a distinctive FTIR spectrum (Supporting data) and site-specific disparities in the composition of chlorogogenous tissue (a second peak is observed in the 1100cm−1 region of the $C2^{pH4}_{Pb*}$ spectrum) were apparent, which correlated with phosphorous-containing functional groups (Fig. 3A and B) (Coates, 2000). The earthworm chloragocyte is thought to be involved in haem biosynthesis, a conserved pathway that is inhibited by Pb at several junctures (Jamieson and Molyneux, 1981). As such, Pb trafficking into and across earthworm chloragocytes must be tightly regulated in these animals that are continuously exposed to high concentrations of metal in their native environments and whose strategy for dealing with it involves intracellular accumulative immobilization. Indeed, inferences on the mechanisms of adaptive evolution to environmental heterogeneity require not only abstract genotype- to phenotype associations but more meaningful molecular genetic interpretations regarding the nature of induced phenotypic variation.

3.3. EST libraries from Pb-mine and laboratory exposed naïve worms

The transcriptomic profile of an organism provides a snapshot of gene expression to provide information regarding developmental stage, life-history or responses in relation to particular environmental stressors. EST libraries are also the substrate for comparative genomic studies, through investigating differential expression between cDNA populations. Two libraries were constructed from earthworm populations with contrasting histories of Pb exposure; $C3^{pH7}_{Pb***}$ earthworms as representatives of a chronically Pb-exposed field population, and earthworms sampled from a clean reference site, R1, acutely exposed to lead in the form of $Pb(NO_3)_2$ under laboratory conditions. In combination with the plethora of EST cluster information already available in LumbriBASE (www.earthworms.org), a metal tolerant genotype may be related to phenotype and

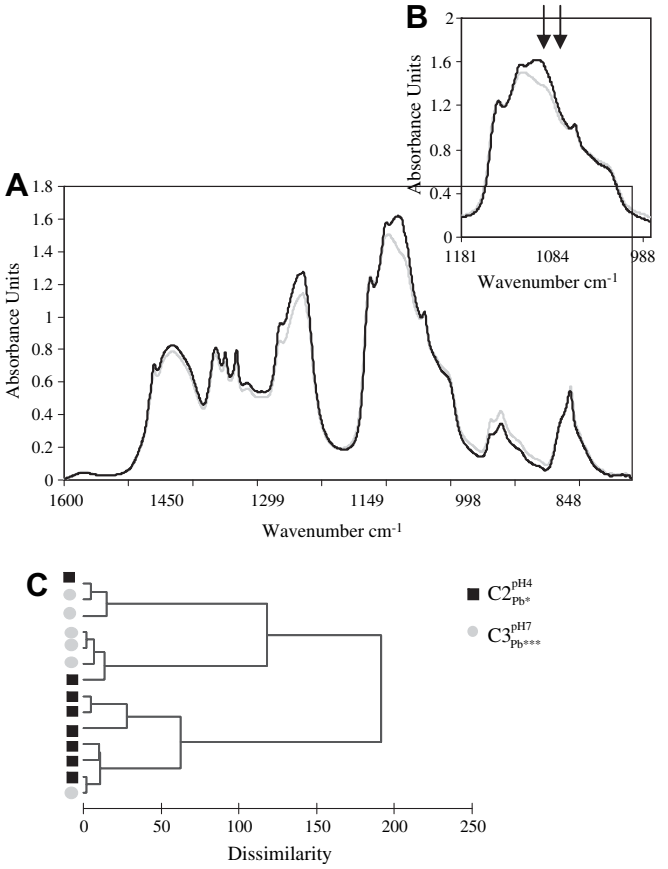


Fig. 3. Metabolomic fingerprinting of earthworm chloragogenous tissue using Fourier Transform Infrared spectroscopy. (A) The fingerprint region of averaged infra-red spectra of earthworm chloragogenous tissue collected from $C3^{pH7}_{Pb***}$ (grey) and $C2^{pH4}_{Pb*}$ (black). Individual spectra were processed by the software package OPUS©. (B) The main difference in $C3^{pH7}_{Pb***}$ and $C2^{pH4}_{Pb*}$ averaged spectra (~1080 cm^{-1}), corresponded to phosphorus-containing functional groups (Coates, 2000). (C) XLSTAT simulated dendrogram illustrating the clustering of $C3^{pH7}_{Pb***}$ and $C2^{pH4}_{Pb*}$ earthworms according to their infra-red spectral patterns (1096–1123 cm^{-1}).

the functional systems that underlie lead handling within these earthworm populations defined. Both libraries comprised high quality sequences with an average length of between 500 and 600 base pairs. This ensured the maximum numbers of sequences were annotated to enable accurate downstream analysis and interpretation using the software LumbriBASE, Blast2GO and associated KEGG resource, which generates pathway maps that highlight gene ontology relationships between annotated sequences. Of interest was the significant number of $C3^{pH7}_{Pb***}$ gene products (when compared to R1) associated with intracellular Ca^{2+} sensing and buffering. These included Calmodulin, with ten (per thousand library sequences and with an alignment score of $<10^{-5}$) $C3^{pH7}_{Pb***}$ hits compared to two R1 matches and Troponin C and Sarcoplasmic calcium binding protein (SCP) that had six and seven $C3^{pH7}_{Pb***}$ matches respectively with zero in R1. All these proteins belong to the EF-hand super-family of proteins implicated in calcium binding and central to the Ca-signalling pathway (Gao et al., 2006; Ishida and Vogel, 2006). These observations may indicate that components of the Ca-signalling pathway are central to Pb sequestration within chloragocytes which, in turn, may be associated with adjustments in the metabolism of their common complexing PO_4^- anion (Fig. 3B). This yields a number of candidate loci that may contribute to a Pb-tolerance phenotype by modifying molecules involved in the cellular physiology of an essential cation (Ca^{2+}) to accommodate its non-essential cationic mimic (Pb^{2+}).

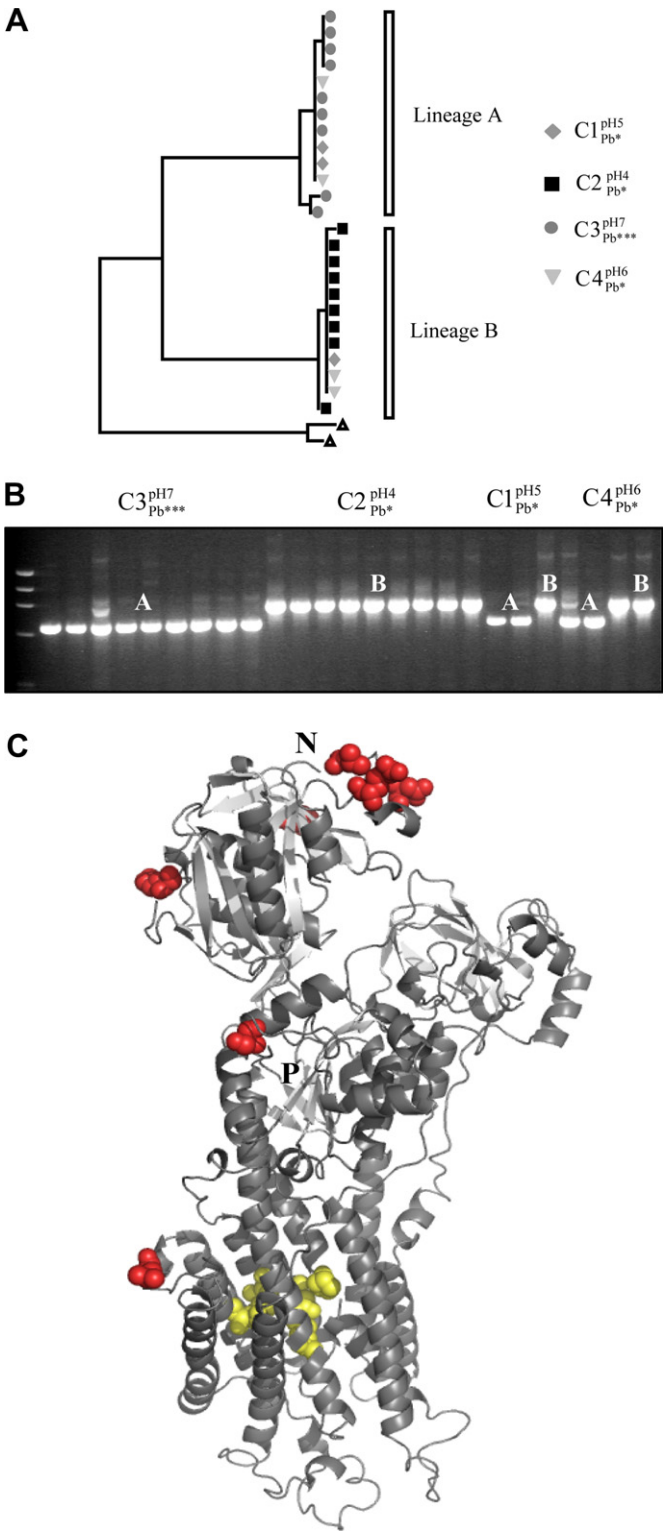


Fig. 4. Analysis of earthworm SERCA variants. (A) Phylogenetic analysis of genotyped individuals, based upon the cytochrome oxidase II gene, from $C4^{pH6}_{Pb*}$ (light grey triangles) and $C1^{pH5}_{Pb*}$ (grey diamonds) at the boundary of the mine, together with $C3^{pH7}_{Pb***}$ (dark grey circles) and $C2^{pH4}_{Pb*}$ (black squares) and a *L. castaneus* and *L. eiseni* individual (white triangles). (B) Discriminatory PCR illustrating the lineage-specific expression of the SERCA variants and (C) PyMol simulated model of SERCA. The conserved calcium binding sites are indicated in yellow and amino acid differences in the two *L. rubellus* isoforms in red. The phosphorylation (P) and nucleotide binding (N) domains are also shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA)

Sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) is a central transport carrier protein of the Ca-signalling pathway that resides in membranes of intracellular storage sites for the uptake of excess Ca and, conceivably, Pb (Tsien et al., 1987). As a consequence this protein was targeted for further analysis. Three isoforms have been described in vertebrates (MacLennan et al., 1985; Campbell et al., 1991; Vilsen and Andersen, 1992) and one in invertebrates (Palmero and Sastre, 1989; Escalante and Sastre, 1994; Shi et al., 1998) fungi (Ghislain et al., 1990) and plants (Wimmers et al., 1992). All isoforms are similar in structure and have a 75–85% identical amino acid sequence (Toyofuku et al., 1992). Despite the identification of several vertebrate isoforms, *L. rubellus* is the first invertebrate found to harbour multiple SERCA variants (GenBank accession numbers GQ911151 and GQ911152). Two structurally different forms were identified in the two populations inhabiting the central geochemically contrasting ecological islands, $C2^{pH4}_{Pb*}$ and $C3^{pH7}_{Pb***}$, and expression was found to be co-incident with the mitochondrial lineage marker (COII), even where nuclear hybridisation was observed (Fig. 4A and B). Their structure differed in amino acids (highlighted in red, Fig. 4C) (For interpretation of the references to color, the reader is referred to the web version of this article.) located in the cytosolic nucleotide-binding domain, or flap, of the protein, a region thought to have a critical role in determining calcium affinity and turnover. This observation may indicate that not only are the intracellular trans-membrane Ca and Pb pathways confluent at the molecular (SERCA) level and are associated with adjustments in the metabolism of their common complexing PO_4^- anion, but the entire machinery is demonstrably genotype-specific. It is important to point out, however, that whist the SERCA molecule is an important connector between the vulnerable cytosol and the intra-vesicular depository of immobilized Pb, other components of the Ca pathway warrant study to determine whether they are structurally or functionally modified.

4. Conclusion

Our observations on field populations of *L. rubellus* with multigenerational histories of exposure to soils with elevated levels of Pb contamination and/or Pb bioavailability lead us to infer that Pb-adaptation traits may be inextricably linked to regulators of Ca physiology. The hypothesis illustrates the contingent face of evolution in that it often innovates by modifying existing structures or pathways. Whilst adaptive changes in enzyme structure are, for good reason, less probable than changes in the promoters that regulate enzyme expression (Crawford et al., 1999), they are clearly not molecular modifications that can be ignored. The ionic radii of Ca^{2+} (1.00 Å) and Pb^{2+} (1.19 Å) (Bridges and Zalups, 2005) appear to be sufficiently similar to allow adaptive structural modifications in the Ca-transporter SERCA to occur in order to accommodate the transport of the non-essential Ca analogue, Pb.

The firm conclusion of the present study is that *L. rubellus* displays a very high degree of genetic diversity, and that the distribution of the various genotypes is not uniform across a heterogeneous metalliferous landscape. This finding raises serious conceptual and practical questions of general importance regarding the use of this (and other) sentinel organisms for field-based ecotoxicology. It cannot yet be assumed that the different genotypes display differential responses or susceptibilities to environmental contaminants, but it is as well to be alert to the possibility. Kautenburger (2006) found a very limited degree of genetic variation in the earthworm *Lumbricus terrestris* sampled from a series of sites in western Germany, and concluded that the genetic uniformity in this species over a limited geographical range meets an essential prerequisite for biomonitoring environmental quality. By direct inference, a lack of genetic uniformity within a species confounds if not invalidates biomonitoring. This, in fact, is one of the key objections promulgated by Forbes et al. (2006) against the use of biomarkers in ecotoxicology. Kautenberger's (2006) recommendation was that the use of earthworms for biomonitoring over wide geographical ranges should be supported by genetic characterisation of the sampled populations. We concur with this principle but, certainly in the case of *L. rubellus*, recommend that exceptionally high genetic differentiation is more a function of highly localised edaphic properties than of geographical distance. This realisation has important implications for how this and other earthworm species are in future exploited as a sentinel, particularly in highly discriminating genomic assays, and argues in favour of the mandatory genotyping of all individuals prior to testing.

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Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envpol.2009.09.021.

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