Ecological drivers influence the distributions of two cryptic

lineages in an earthworm morphospecies

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ABSTRACT

Substantial genetic diversity exists within earthworm morphotypes, such that traditional species designations may be incomplete. It is, however, currently not known whether these different genetic variants show ubiquity or specialty in their distribution across separated sites subject to different climatic, biotic or soil physicochemical factors. Here we report on the results of a survey in which individuals of the *Lumbricus rubellus* morphotype, a species known to comprise two deeply divergent genetic lineages in England and Wales, were sampled from 26 plots. Sequences from the mitochondrial cytochrome oxidase I gene were used to distinguish lineages for 787 individuals. In conjunction, a range of geographic, climatic, biotic and soil physiochemical variables were also collected for each locality.

Genotyping indicated that Lineage A was more common than Lineage B, comprising 58% of the collected *L. rubellus*. Six site populations comprised only Lineage A, while only a single site comprised entirely Lineage B. The remaining 20 sites containing both lineages. A multivariate ordination of site variables identified major difference between sites were associated with low pH, organic-rich soils in Western wet upland areas and pollutant levels associated with sites in the South. Earthworm genotype (as proportion of Lineage A) was not correlated with either of these major environmental axes. When individual variables of soil pH and the percentage of soil organic matter, which are known to be key driver of soil species distributions, were investigated as single variables significant relationship with lineage frequency were found. Soil organic matter content was significantly negatively correlated with Lineage A proportion, while pH was significantly positively correlated. This lineage preference may be related to lineage metabolism and/or behavioral differences.

Measurement of tissue metal concentrations in worms from 17 sites identified a significant site effect in all cases, but a lineage effect only for arsenic (higher Lineage B). Tissue arsenic concentrations varied between lineages, supporting previous observations that there are differences in the way the two lineages have adapted to manage exposure to this metalloid.

1. INTRODUCTION

58 Soils contain a wealth of invertebrate biodiversity recognised for their important contributions

59 to ecological processes (Bardgett and van der Putten, 2014; Fitter et al., 2005; Giller, 1996).

60 One key group of species are the "ecosystem engineers": those organisms that modify the

61 physical state of the soil and resource availability for other species. Earthworms are known

62 as a key group of ecosystem engineers in many habitats. They perform a range of physical

63 (aeration, bioturbation, litter fragmentation) and biological (microbial interactions, exudate

64 production) roles in soil (Blouin et al., 2013; Lavelle et al., 1997; Sackett et al., 2013;

65 Umarov et al., 2008). Because of their functional importance, earthworms have emerged as

66 a major taxon for biomonitoring and biomarker assessments of human induced pressures on

67 soil communities (Cluzeau et al., 2012; Rutgers et al., 2009).

68

69 As soil invertebrate species, including earthworms, have been shown to be sensitive to a

70 range of land use change and pollution impacts (Bundy et al., 2007; Cluzeau et al., 2012),

71 different soil taxa have become a natural focus for research on the relationships between

72 environmental pressures, biodiversity and soil functioning (Bartlett et al., 2010; Leveque et

73 al., 2015; Rutgers et al., 2016). For community studies, a major constraint relates to current

74 uncertainties in earthworm taxonomy. Traditionally earthworm identification has relied on

75 morphology, but the paucity of suitable local keys and problems with application to juveniles

76 has also recently encouraged the use of molecular methods (Dominguez et al., 2015;

77 Emerson et al., 2011; Klarica et al., 2012). These genotyping studies have begun to

78 challenge current understanding of diversity through the identification of genetically distinct

79 cryptic lineages within previously established morphospecies.

80

81 Earthworm species in which cryptic lineage diversity has to date been identified include

82 *Eisenia fetida/andrei* (Römbke et al., 2016), *Lumbricus terrestris* (James et al., 2010),

83 *Aporrectodea caliginosa* (PerezLosada et al., 2009), *Allolobophora chlorotica* (King et al.,

84 2008), *Amynthas gracilis / Amynthas cortici* (Novo et al., 2015) and *Lumbricus rubellus*. For

85 *L. rubellus*, genotyping studies based on mitochondrial cytochrome oxidase I and II markers

86 have identified as many as 6 cryptic lineages across Europe (Giska et al., 2015), two of

87 which are found in the UK (Andre et al., 2010; Kille et al., 2013). The two UK lineages have

88 10-15% divergence for the mitochondrial COI and COII sequences. While this implies they

89 may actually be cryptic species, recent analysis of multiple nuclear markers using RADseq

90 has not supported this interpretation, instead suggesting that different *L. rubellus* lineages

91 may actually correspond to a single highly polymorphic species (Giska et al., 2015).

92 Comparative studies of the two lineages in the UK have, nonetheless, identified

93 physiological differences between them, including variation in pheromone production (Jones

94 et al., 2016), maturation time (Anderson et al., 2013), metabolic profiles (Liebeke et al.,

95 2014), mechanism of arsenic adaptation (Kille et al., 2013), trace element metabolism

96 (Andre et al., 2010), and microbiome complement (Pass et al., 2015).

97

98 Despite known biological differences, the extent to which differences in distribution and

99 physiology are related to different geographical, climate and soil physicochemical

100 preferences between the two known UK lineages of *L. rubellus* is not established. The two

101 lineages found co-occur at some, but not all, sites meaning that they have some likely niche

102 divergence that facilitates coexistence (Andre et al., 2010; Giska et al., 2015; Kille et al.,

103 2013). We aim to better understand the nature of the spatial and geochemical drivers of

104 lineage relative abundance, and so here we test the hypothesis that the site distribution of

105 the two cryptic *L. rubellus* lineages is based on one or more geographical, climatic,

106 physiochemical or biotic drivers. We collected and genotyped morphotype *L. rubellus* at

107 multiple well-characterized sites that differed in their properties to investigate the

108 relationships that determine lineage distributions. Tissue metal concentrations were also

109 measured to assess if trace metal levels could also influence distributions, as could be the

110 case if the two lineages had different sensitivity to specific contaminants.

2. METHODS

2.1 Site selection

Twenty six sites located across England and Wales (Fig. 1) were visited between four times (for Devon Great Consols Mine and Control, Shipham Mine and Control, Cwmystwyth Mine and Control) and a single visit (for Porton Down, Parys Mountain, Castell, Clydach, Roman Gravel, Didcot) over four separate sampling events from Spring 2011 to Spring 2014. The chosen sites were selected to capture a range of the habitats and soil conditions under which morphotype *L. rubellus* can be collected. Land-uses covered included arable systems, broadleaf woodland, rough grassland and improved pasture habitats. Sites included both mineral and organic soils, although not true peats.

To allow the role of soil geochemistry and pollution status on lineage distribution to be addressed, sites of different known pollution history were sampled. Sites corresponded to three groups with respect to past land-use and associated expected contamination level. These were: 1) sites with no known pollution source (Unpolluted); 2) sites near to industrial facilities expected to be characterised by moderate pollution (Industrial polluted); and 3) sites at abandoned mining sites that can be expected to have high pollution (Mine polluted). For expected polluted sites from categories 2 and 3, a local control site was also sampled. This reference site was located outside of the area that was expected to be strongly influenced by the main pollution source and so was on soil expected to contain regional background pollutant concentrations.

2.2 Site geographical, biological and soil physiochemical characterisation

To allow the assessment of environmental drivers relating to lineage distribution, we used both publically available resources as well as our own analyses to gather data on each sites. Site geographical locations were collected as Easting and Northings from www.gridreferencefinder.com and site altitudes from www.freemaptools.com/elevation-

138 [finder.htm](). A series of site climate conditions were also assembled from
139 [www.metoffice.gov.uk/](). These were: annual average maximum temperature, annual
140 average minimum temperature, average January minimum temperature, average July
141 minimum temperature, average annual rainfall, average annual rain days and average
142 annual frost days. Initial visits to each site recorded main land-use (arable, broadleaf
143 woodland, rough grassland and improved pasture) and where present the average sward
144 height of vegetation at collection locations. The site was identified according to the level of
145 shade (open, part shaded, shaded) and the presence of livestock was noted.

146

147 An initial site survey identified points on the site where morphospecies *L. rubellus* could be
148 found. Thereafter all collections were focussed on these locations. For any one sampling
149 event at each site, between 6 and 25 fully clitellate *L. rubellus* were collected by digging and
150 hand-sorting from the soil to 20 cm depth. Generally the required number of worms could be
151 collected within a reasonable search period (approximately 2 h duration). There were,
152 however, some locations where this was not possible for particular sampling events. Climate
153 factors (notably dry soils), low frequency of adults in the population or the requirement to
154 limit site damage caused by digging were the major constraints. During collection, the
155 presence of other earthworm morphospecies was noted. Only common species were
156 recorded (>5 individuals observed). In total 10 other species were found: *Aporrectodea*
157 *caliginosa*, *Aporrectodea rosea*, *Aporrectodea longa*, *Allolobophora chlorotica*, *Lumbricus*
158 *castaneus*, *Dendrobaena rubida*, *Lumbricus terrestris*, *Lumbricus festivus*, *Octolasion*
159 *cyaneum*, and *Octolasion tyrtaeum tyrtaeum*. At the end of sampling, the *L. rubellus*
160 collected were washed and blotted dry on-site and then snap frozen in liquid nitrogen before
161 being transfered to the laboratory under dry ice storage.

162

163 Triplicate soil samples from surface to 5 cm depth were collected from each site collection
164 location. All soil samples were oven dried at 80°C to constant weight and then sieved

165 through a 2 mm mesh to remove large roots and stones. Total concentrations of aluminium,

166 arsenic, barium, cadmium, cobalt, chromium, copper, iron, lead, manganese, mercury,

167 molybdenum, nickel, selenium, titanium, vanadium, zinc, calcium and total phosphorous

168 were determined in a 1 g sample of this processed soil following an aqua regia digestion

169 protocol (Arnold et al., 2008; Emmett et al., 2010; Spurgeon et al., 2008). Digests were

170 subsequently analysed on a Perkin Elmer Optima 7300 DV inductively coupled plasma

171 optical emission spectrometry instrument. For quality control, an in house reference

172 traceable to BCR-143R (Commission of the European Communities, Community Bureau of

173 Reference) was included with each batch of digestions. Measured concentrations were

174 within 10% of certified values for all measured elements with the exception of Al where the

175 value was 55%. Organic matter content of each soil sample was measured by proxy using

176 loss on ignition following combustion at 500°C (Rowell, 1994) and soil pH was quantified by

177 electrode from a 1:2.5 volume soil:water mix (i.e. 1 volume soil with 2.5 volumes water

178 added)(International Organisation for Standards, 2005).

179

180 *2.3 Lineage assignment by mitochondrial cytochrome oxidase I (COI) sequencing*

181 DNA was extracted from ~10 mg of frozen tissue (taken from the tail of each individual using

182 a scalpel) by automated DNA extraction using a Nucleoplex Plants Tissues DNA Extraction

183 Kit (Nucleoplex, Manchester, UK). After DNA quantification using Nanodrop (Thermo

184 Scientific, Willmington, DE), polymerase chain reaction amplification of the COI gene was

185 conducted using a set of established forward (GGTCAACAAATCATAAAGATATTGG) and

186 reverse (TAAACTTCAGGGTGACCAAAAAATCA) primers (Folmer et al., 1994) amplified

187 after 5 minutes at 95°C over 40 cycles of 30 sec 95°C, 30 seconds 48°C and 60 seconds

188 48°C. A sub-set of all PCR products were checked by gel electrophoresis to ensure

189 successful amplification and purified for sequencing using 0.25 U each of Exonuclease I and

190 Shrimp Alkaline Phosphatase (NEB, Hitchin, UK), incubated at 37°C for 45 minutes and 80

191 ^{o}C for 15 minutes. Purified PCR products were then sequenced as in Andre et al. (2010),

192 using ABI PRISM® BigDye v3.1 Terminator Sequencing technology (Applied Biosystems,

193 USA).

194

195 Sequences were aligned and trimmed for tree construction using the Maximum Likelihood

196 method and General Time Reversible substitution model with a gamma distribution in Mega

197 v5.01. Sequences for *L. rubellus* associated with specific mitochondrial lineages already

198 documented in the UK were incorporated into the analysis as anchor sequences (Anderson

199 et al., 2013), with sequences for *L. terrestris*, *L, festivus* and *L. castaneus* included as an

200 out-group. Tree topology was supported by bootstrap analyses over 1000 iterations.

201 Individuals that showed a close relationship with one of the two previously identified UK *L.*

202 *rubellus* lineages were identified from the analysis. Any individuals showing intermediate

203 status resulting from probable sequencing errors were excluded from further analysis.

204

205 *2.4 Earthworm tissue trace element concentrations*

206 Earthworm tissues from 494 individuals taken from a sub-set of 17 sites (Alice Holt ECN

207 Control, Avonmouth Control, Avonmouth Incinerator, Avonmouth Savalco, Cwmystwyth

208 control, Cwmystwyth mine, Devon Great Consols Control, Devon Great Consols Mine,

209 Drayton ECN Control, Port Talbot Control, Port Talbot blast furnace, Porton Down ECN,

210 Scunthorpe blast furnace, Scunthorpe Control, Shipham control, Shipham mine, Snowdown

211 ECN control) were prepared for analysis (nb samples from remaining sites were lost due to

212 storage issues). These samples were analysed for tissue Al, As, Cd, Co, Cr, Cu, Fe, Mn, Mo,

213 Ni, Pb, Se, Sr and Zn concentrations. Whole earthworms, after tail removal for DNA

214 extraction, were initially ground to powder under liquid nitrogen in a cryogenic mill. The

215 powder was freeze-dried and a 100 mg sample digested with 10 ml of 70% HNO_3 (Ultrapure)

216 at $200^{o}C$ for 15 minutes within a microwave vessel. Samples were run as two batches on a

217 Perkin Elmer DRCII ICP-MS. Each batch included multiple certified reference material

218 samples for TORT-2 and DOLT-4 (National Research Council, Canada). Certified values for

219 reference materials corroborated well with measured values. Average recovery was 91%

9

220 (range 85% for Se to 110% for Pb) in the first batch of samples and 94.8% (range 53.9 for Al

221 to 129% for Se) in the second batch. Recoveries of only two metals, Al and Se, were outside

222 80% of certified values for any run, with 19 of 27 determinations within 10%. With systematic

223 bias absent, acquired data can be used for statistical processing without requirement for

224 recovery correction.

225

226 *2.5 Data handling and statistical analysis*

227 The number of *L. rubellus* returning COI sequences that were closely related to reference

228 sequences from previously collected lineage A and Lineage B individuals were counted for

229 each study site. These were calculated as proportions before being logit transformed as the

230 most appropriate transformation for biological proportion data (Warton and Hui, 2011), with

231 value of zero and one modified by addition and subtraction of half of the lowest proportion

232 respectively. Environmental drivers were established as either categorical (e.g. site type, site

233 shading, livestock presence/absence, earthworm species presence/absence) or as

234 continuous measured variables. The values for soil metal concentrations were log

235 transformed to obtain a Gaussian distribution in accordance with established practice

236 (Davies, 1989).

237

238 Relationships amongst site geographical, climate and soil variables (after appropriate

239 transformation) were initially investigated using principal component analysis in Minitab 14

240 (Minitab, PA, USA). This reduces the dimensionality of the original complex dataset in an

241 unsupervised fashion, by successively generating new axes (principal components), that are

242 the linear combinations of the original data that explain greatest overall variance. The

243 principal components (PCs) arising can be interpreted as ordinations representing a

244 summary of environmental factors. Pearson correlations between (logit transformed)

245 proportions of Lineage A individuals at each site and the individual principal component

246 scores were then calculated to investigate the relationships between ordinated site

247 characteristics and genotype. Based on this analysis and prior knowledge, possible

248 individual primary driver variables were identified that were in turn assessed for Pearson

249 correlation with genotype. Because of this key role, percentage organic matter content and

250 soil pH were selected as focus variables. Site and lineage effects on earthworm tissue log

251 transformed trace element concentrations were analysed using a mixed model based

252 general linear model in Minitab 14. Within the model, site and lineage were included as fixed

253 variables, with sampling campaign (1-4) as a random factor.

254 *3. RESULTS*

255 High quality *L. rubellus* COI sequences were obtained from DNA samples taken from 787

256 earthworms for assignment as either Lineage A or Lineage B individuals. The maximum

257 number of sequences from any one site was 73, from Cwmystwyth Mine, and the minimum

258 3, from Avonmouth Control (Fig. 1). In total, 457 individuals were assigned as Lineage A,

259 58% of the number collected. The remaining 330 (42%) were assigned as Lineage B. Eight

260 sites (Avonmouth Savalco, Avonmouth Incinerator, Clydach Smelter, Didcot Power Station,

261 Dinas Powys, Parys Mountain, Scunthorpe Control and Scunthorpe) had populations

262 comprising only Lineage A individuals. Seven sites (Avonmouth Control, Castell Mine,

263 Cwmystwyth Control, Cwmystwyth Mine, Drayton ECN Control, Shipham Control and

264 Shipham Mine) contained ==populations comprised largely Lineage B, although only Castell==

265 ==Mine was exclusively B.== All remaining sites had mixed lineage populations, although with

266 more Lineage A than B individuals.

267

268 The site geographical, physical and soil characteristics were analysed using principal

269 component analysis. The first PC explained 21.4% of total variance. A number of

270 parameters were positively correlated with this axis, including soil % loss on ignition (LOI);

271 soil log Fe, log Co and log Al concentrations; some earthworm species; and site altitude and

272 climate variables including average rainfall and number of rain days. Negatively correlated

273 variables included soil pH; average July max temperature and average temperature; log Ca

274 and log P concentrations; and Easting (Fig 2). This first PC axis could therefore be

275 interpreted as representing a set of variables characterised by the presence of high organic

276 matter, low pH soils, associated with wetter and colder upland regions located mainly in the

277 West of England and Wales. The second PC axis explained a further 17% of variation, and

278 was positively associated with Northing and the weather variable of average frost days and

279 average rain days. Variables negatively associated with this axis included pollutant metal

280 concentrations such as log Pb, log Zn and log Cd concentrations (Fig 2). This axis can be

281 interpreted as representing a gradient of metal contamination of sites located primarily in the

282 South of England and Wales.

283

284 To assess if the site characteristics summarised by the two first PCs potentially act as

285 drivers of lineage distribution, the PC1 and PC2 scores were correlated with the (logit

286 transformed) Lineage A proportion at each location. This did not identify significantly

287 relationships between Lineage A proportion with site PC1 or PC2 score (p=0.25 and

288 p=0.074 respectively). As sites PC scores were not significant, we next went on to

289 investigate if individual variables measured are related to the relative frequency of *L.*

290 *rubellus* lineages. Specifically we selected soil pH and LOI for initial assessment, as these

291 are established as drivers of patterns of diversity (Griffiths et al., 2011; Raty and Huhta,

292 2003). Both variables were significantly correlated with logit Lineage A proportion (soil % OM

293 -0.529, p=0.005; pH -0.392, p=0.048). The nature of these two relationships were

294 summarised by locally weighted scatterplot smoother model fits. These indicate a decline in

295 proportion of Lineage A (higher logit transformed values) as site soil % LOI increases from

296 0-20%, thereafter remaining constant. The model fits for pH indicated an initial decline in the

297 proportion of Lineage A individuals (higher logit transformed values) as pH increases from

298 4.5 to 5, with, thereafter, an increase in frequency (lower logit transformed values) where

299 site pH increases from 5 to 7.5 (Fig. 4 a,b). Amongst other measured variables, only log soil

300 Ca concentrations (-0.487, P=0.012) and the average annual number of rain days (0.433,

301 p=0.027) were also significantly correlated with Linage A proportion. Both of these variables

302 are, however, also significantly correlated with soil pH (Annual rain days: -0.584, p=0.002;

303 log soil Ca concentration:-0.751, p<0.001) making precise attribution of cause challenging.

304

305 Separate univariate models were generated to analyse tissue metal concentrations in

306 relation to collection site and lineage. The collection site had a highly significant (p<0.001)

307 influence on tissue concentrations for all analysed trace elements. This is only to be

308 expected, given that the sites include locations with no history of local pollution, to highly

309   contaminated industrial and mine sites. Lineage was also a significant factor in the model for

310   As ($p<0.02$). This difference is, however, based on only a relatively small difference in

311   average tissue concentrations between lineages across all sites. Thus, average

312   concentrations in Linage A of 10.69 (n=300) was slightly lower than the average tissue

313   arsenic concentrations of 11.7 mg/kg (n=195) for Lineage B, Hence although statistically

314   significant, the absolute magnitude of difference in tissue As concentrations between

315   lineages is small. For all other analysed metals, there was no significant effect of lineage on

316   tissue concentration ($p>0.05$).

4. DISCUSSION

Species distributions can be affected by a range of environmental drivers, including physiological tolerances, dispersal constraints, biotic interactions and anthropogenic influences (Dennis and Hellberg, 2010; Gaston, 2003). Among earthworms, species show preference for certain habitats, for example common compost earthworm species such as *Eisenia fetida*, *Perionyx excavatus* and *Eudrilus eugeniae* preferentially occupy organic matter rich habitats associated with animal manure or composting vegetation (Edwards, 2004). Further, some species also have preference for different soil physiochemical properties. For example, Jaensch et al (2013) found differences in morphospecies preference across different soil pH classes, with species such as *Allolobophora chlorotica*, *Aporrectodea rosea*, *Aporrectodea longa* and *Lumbricus terrestris* preferring soils with pH >5.6, and *Dendrodrilus rubida*, *Dendrobaena octaedra* and *L. rubellus* soils with pH <5.6.

The UK earthworm fauna is notably denuded, comprising only around 20-25 native species, compared to about 180 species that are found in neighboring France (Bouché, 1972; Sims and Gerard, 1985). The reduced earthworm fauna of the UK can be linked to its recent history of glaciation and the severing of the land bridge to Europe that restricted earthworm colonization after glacial retreat. This influence of quaternary glaciation is consistent with what is known about the current distribution and genetic structure of a range of species across Europe and the UK (Hewitt, 2000). Among UK earthworm species, the majority show a widespread and cosmopolitan distribution (Boag et al., 1997; Carpenter et al., 2012; Rutgers et al., 2016; Sims and Gerard, 1985). Habitat preferences are known, such as those for pH and for organic rich habitats as discussed previously, however the spatial heterogeneity of terrestrial habitats means that at coarse recording scales (e.g. 10 km^2 or even 1 km^2), a significant proportion of UK earthworm species may be present in any given sampling area (e.g. a mixed land-use area subject to comprehensive earthworm sampling within different vegetation stands and habitats).

15

Genetic marker studies have identified deeply divergent cryptic lineages within many common UK earthworm morphospecies based on mitochondrial or nuclear genetic marker analysis. An active debate currently surrounds the question of whether these cryptic lineages correspond to cryptic species or highly polymorphic species variants (Blakemore et al., 2010; Giska et al., 2015; King et al., 2008). In the specific case of *L. rubellus*, the presence of cryptic lineages is established from studies conducted from measurement of highly divergent (13-15%) sequences for both of the cytochrome oxidase I and II mitochondrial genes (Andre et al., 2010; Donnelly et al., 2014; Kille et al., 2013). Pan-European studies have shown that at continental scale, morphotype *L. rubellus* may comprise of 5 or more such deeply divergent lineages (Giska et al., 2015), two of which were here found across sites in England and Wales (Fig 1). Recently RADseq analysis suggests that cryptic *L. rubellus* lineages may represent a case of a highly polymorphic single species rather than true cryptic species (Giska et al., 2015). Nonetheless, previous studies of the lineage physiology have identified a number of differential responses between lineages (as previously outlined notably for the two UK lineages). For example, Jones et al. (2016) found that the two lineage were favorably attracted to soils that had previously been worked by earthworm of their own rather than the alternative lineage. These results suggests that pheromone attractants may allow mate selection in mix populations,. such as those that are found at the majority of our sampled site. Such selection has the potential to underpin lineage differences in habitat preference and, as a consequence, different spatial distributions at local scale.

Earthworms are key ecosystem engineers for the role that play an important role in the creating of the spatial structure and chemistry of the soil habitat through bioturbation, litter degradation and nutrient cycling (Edwards, 2004; Lavelle et al., 1997; Liebeke et al., 2015). The extent to which the divergent lineages of common earthworm species overlap in respect of habitat preference will be an important determinant of morphospecies contributions to different ecosystem processes across space and time. The analysis here suggests that, in

373   the case of the two UK lineages of *L. rubellus*, there are ecological drivers of distribution.

374   Individually, soil pH and % OM were both significant correlated with the proportions of *L.*

375   *rubellus* Lineage A (and conversely Lineage B) collected across the 26 sample sites. These

376   two measurement parameters were selected for particular focus because they are

377   recognized as important environmental drivers of the distribution of a number of soil taxa

378   (Cassagne et al., 2003; Griffiths et al., 2011; Raty and Huhta, 2003). Additionally, there are

379   also correlations with other climate and soil variable that are themselves know to influence

380   soil pH through soil geochemistry and leaching.

381

382   Different soil pH preferences have direct effects on earthworm traits including reproduction,

383   growth and survival (Baker and Whitby, 2003; Spurgeon et al., 2006; Van Gestel et al.,

384   1992). *L. rubellus* is tolerant of relatively low pH, being commonly (and even preferentially)

385   found in moderately acidic soils (Jaensch et al., 2013). Results here suggest that this

386   cosmopolitan nature could partly arise from different lineage pH preferences, with Lineage B

387   found in more acid habitats from pH 4.5 to 5.5 and Lineage A preferentially in nearer neutral

388   pHs of 5.5 and above. Thus, within the current study, Lineage B was absent from 6 of 26

389   sampled sites, while Lineage A was found at all except one of the sampled sites. The

390   detailed genetics of the two cryptic lineages may provide some clues to the basis of such

391   differences. Studies of mitochondrial and genetic marker genes have established that

392   Lineage B has lower genetic diversity of measured traits than Lineage A (Donnelly et al.,

393   2014; Kille et al., 2013). This suggests that Lineage B may have undergone a population

394   bottleneck that restricted the genetic diversity, and possibly, the colonization capacity of this

395   lineage.

396

397   The strongest correlate of lineage frequencies was soil organic matter (% loss on ignition).

398   The fresh and partially degraded soil organic component provides earthworms with food. It

399   is, therefore, possible that this association is driven by different dietary requirements of the

400   two lineages, as has been recognized for different earthworm species (Piearce, 1978).

401   However, in addition to acting as food, soil organic matter also contributes to soil structure

402   and moisture retention. Earthworms are known to be sensitive to soil texture, with regional

403   studies linking species distributions to soil sand, clay and organic matter content (Joschko et

404   al., 2006; Salome et al., 2011). Soils lacking in organic matter are also vulnerable to

405   prolonged periods of high soil moisture deficit. This can be challenging for earthworms given

406   their critical need to retain water balance. The significant correlation with site average rain

407   days also points to a possible influence of soil hydrology on distributions. Metabolomic

408   analyses have identified that many earthworm species contain a high number of betaines

409   which likely act as osmolytes that help to retain soil water balance (Liebeke and Bundy,

410   2013). Any differences in the extent of such protection between lineages may influence

411   colonization ability for more drought susceptible soils.

412

413   Although there is correlation of lineage frequency with both soil pH and soil organic matter,

414   the fact that these two soil variables are co-correlated to other environmental variables

415   makes it hard to unequivocally assign them as the major drivers of lineage distribution. For

416   example, high organic matter/low pH soils are more common in the West of England and

417   Wales than in the East. This geographic relationship could potentially be associated with

418   different recolonization histories for the two lineages, e.g. perhaps recolonization from

419   different glacial refugia (Hewitt, 2000). However, as there is no significant correlation of

420   Easting to lineage proportion, this seems less likely than direct effects of soil organic

421   matter/pH. Ultimately, to tease apart the drivers of lineage preference, higher resolution

422   collection and mapping and experimental manipulation of habitats would be required.

423

424   Differences in physiology that separate species in relation to habitat preference could also

425   affect the way that the two lineages handle and accumulate different trace elements. For the

426   site-level analysis, the soil concentrations of major pollutant metals were correlated with

427   PC2, which was not associated with lineage. For the individual analysis of tissue metals,

428   arsenic was the only one found to vary with lineage (significantly higher in Lineage B

429 individuals). Previous work has indicated that the two lineages differ in the genetic

430 mechanisms underlying the development of arsenic tolerance. Analysis of amplified

431 fragment length polymorphisms indicated that Lineage A showed differences in patterns of

432 nuclear markers indicating genetic tolerance, while Lineage B showed a difference in DNA

433 methylation patterning, but not genetic differences (Kille et al., 2013). The observed

434 difference here in As accumulation between lineages across sites suggests that these

435 genetic differences lead to phenotypic differences in the handling of As.

436

437 *5. CONCLUSIONS*

438 Earthworms represent 'super-sentinels' exploited for environmental monitoring and

439 ecotoxicology, as well as being keystone soil engineers essential for soil quality. The

440 identification of possible drivers of species and lineage distributions has potential

441 implications for their use in environmental assessment as well as in studies of ecosystem

442 service delivery. For example, when assessing biodiversity effects of pollution and land-use

443 change it may be valuable to consider the occurrence of different lineages to understand

444 how populations may adapt to change through changes in lineage frequency. This analysis

445 may be required because the two widespread cryptic lineages of *L. rubellus* differ in their

446 habitat preferences with frequencies changing as conditions change. Given that bacterial

447 communities are also known to differ in relation to soil pH, then difference in the nature and

448 strengths of earthworm and microbial interactions can be expected between lineages. These

449 relationships between soil macrofauna and microbes are key to soil carbon turnover, nutrient

450 cycling and soil structural characteristics and this aspect warrants further investigation.

451 Earthworms are also valuable for metal biomonitoring. Our results suggest that the lineages

452 behave identically with respect to metal bioaccumulation, with the exception of As. Thus,

453 selection of morphotype *L. rubellus* will provide a coherent picture of metal accumulation

454 independent of lineage, unless As is a specific focus of any assessment.

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626

627   LEGENDS TO FIGURES

628

629   Figure 1. Location of collection sites and the proportion of Lineage A (dark blue shading) and

630   Lineage B (light yellow shading) *L. rubellus* based on the total number of collected and

631   assigned genotyped individual (given in brackets) for the 26 sites visit over four separate

632   collection campaigns

633

634   Figure 2. Principal component analysis results show the ordination of site geographical,

635   climatic, biotic and soil chemical variables of sample sites showing the major related site

636   characteristic variables.

637

638   Figure 3. Boxplots showing median (centre line), upper and lower quartile (box limits) and

639   upper and 95% confidence intervals (whiskers) of trace metal concentrations measured

640   across 17 samples site for assigned Lineage A and Lineage B *L. rubellus*.

641

642   Figure 4. Scatterplots with fitted locally weighted scatterplot smoother line of proportion of

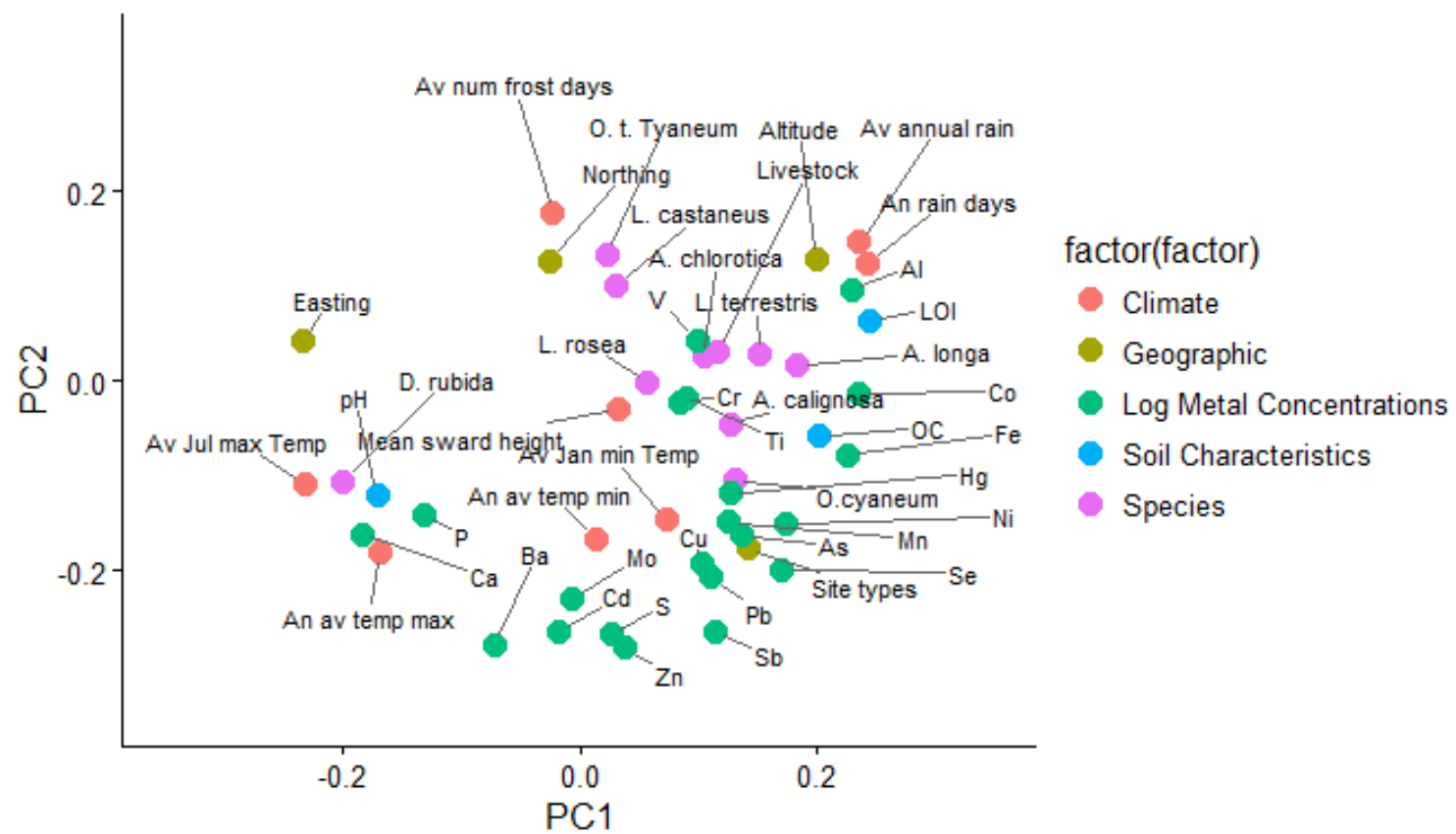643   Lineage A *L. rubellus* in relation to (a) Soil % OM and (b) soil pH.
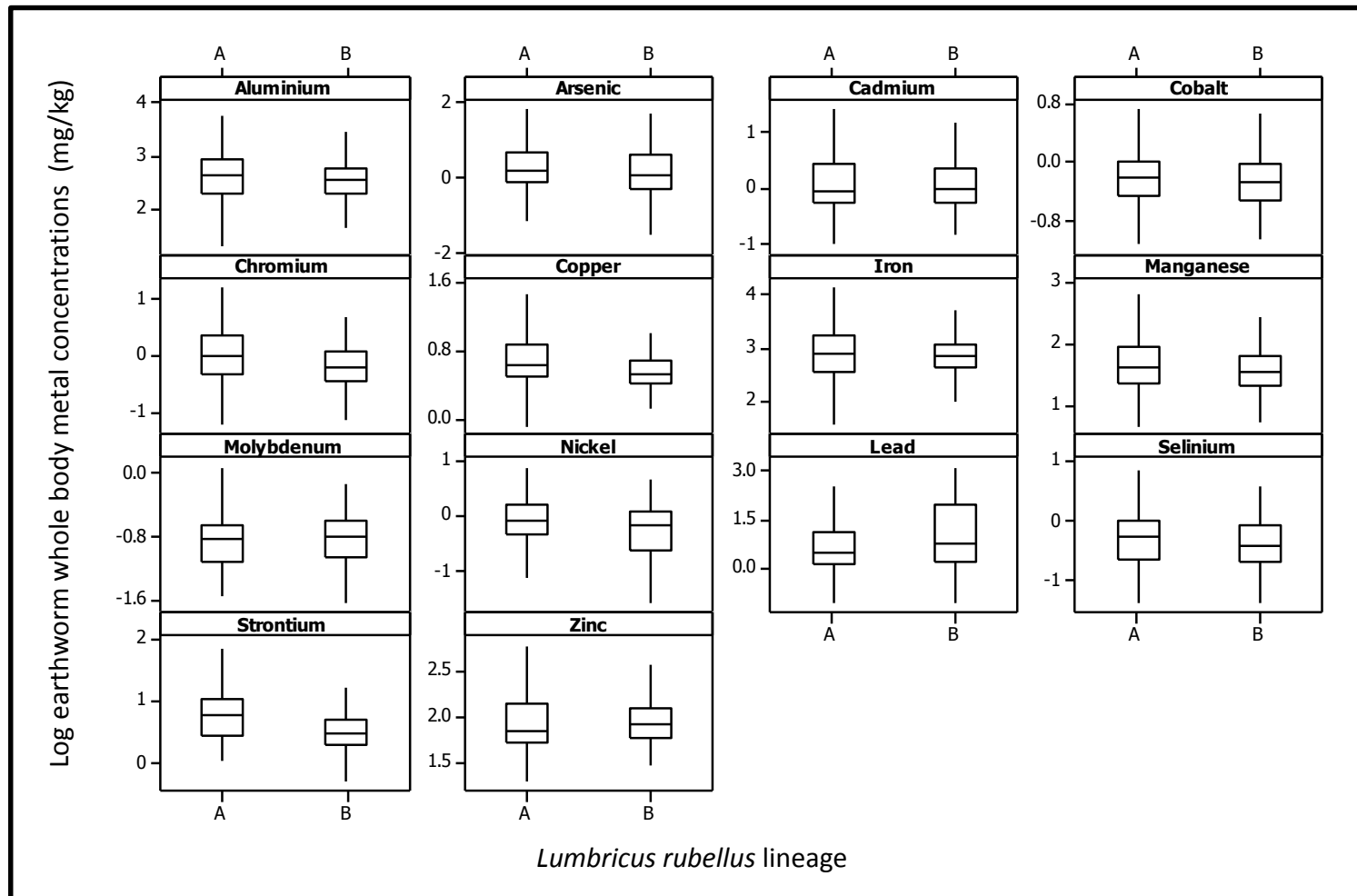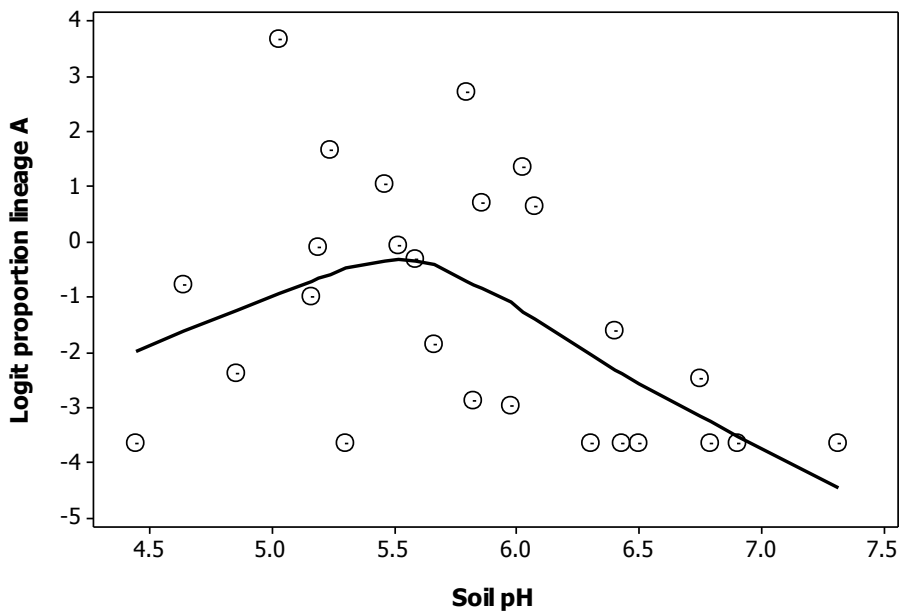
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645   FIG. 1

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FIG. 2

FIG. 3
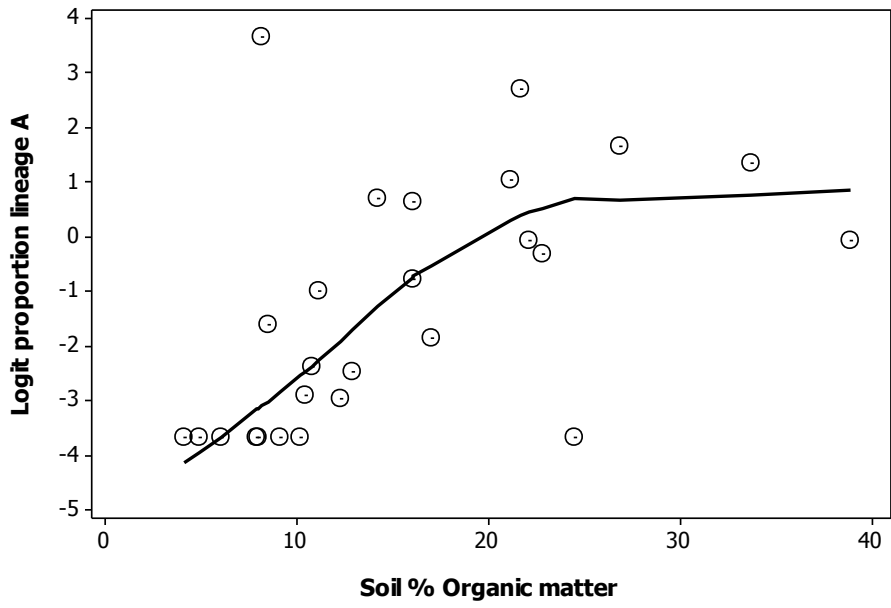
FIG. 4

Supplementary Table 1. Geographical locations and reported climatic conditions of the 23 sites used for the collection of morphotype *L. rubellus*.

Site name	Site types	Land use	Ordnance Survey Grid reference	Easting	Northing	Altitude	Annual Average temp Max	Annual average temp min	Average Jan Min Temp	Average Jul Max Temp	Average Annual Rain	Annual rain days	Average No Forst Days
Alice Holt ECN Control	Unpolluted	Broadleaf woodland	SU 80060 39821	480060	139821	88.6	14.1	6.4	1.6	21.9	755	121	45.9
Avonmouth Control	Unpolluted	Improved pasture	ST 57006 82149	357006	182149	7	14.2	7	2.2	21.5	802	126	34.9
Avonmouth Incinerator	Industrial polluted	Rough grassland	ST 54099 81659	354099	181652	6	14.2	7	2.2	21.5	802	126	34.9
Avonmouth Savalco	Industrial polluted	Rough grassland	ST 53859 79411	353859	179411	6	14.2	7	2.2	21.5	802	126	34.9
Caradon Mine	Mining polluted	Rough grassland	SX 25624 69792	225624	69792	226	13.2	7	3	19.1	1385	172	30.6
Carrick Fell Mine	Mining polluted	Rough grassland	NY 32211 32982	332211	532982	661	13	5.8	1.6	19.7	1521	176	56.5
Castell Mine	Mining polluted	Rough grassland	SN 77415 81254	277415	281254	297	11.9	5.2	1	18.2	186	191	58.4
Clydach Smelter	Industrial polluted	Broadleaf woodland	SN 69587 01409	269587	201409	25	13.5	8.5	4	19.6	999	148	9.7
Cwmystwyth control	Unpolluted	Rough grassland	SN 79598 74222	279598	274222	198	11.9	5.2	1	18.2	1856	191	58.4
Cwmystwyth mine	Mining polluted	Rough grassland	SN 80852 75166	280852	275166	177	11.9	5.2	1	18.2	1856	191	58.4
Devon Great Consouls Control	Unpolluted	Improved pasture	SX 42560 74019	242560	74019	133	14	8.1	4	19.9	1007.4	142	16.3
Devon Great Consouls Mine	Mining polluted	Broadleaf woodland	SX 42385 73152	242385	73152	133	14	8.1	4	19.9	1007.4	142	16.3
Didcot Power Station	Industrial polluted	Broadleaf woodland	SU 51645 91402	451645	191402	53	14.4	5.9	1.2	22.6	661	112	57.7
Drayton ECN Control	Unpolluted	Improved pasture	SP 16391 55061	416391	255061	66	14.5	5.9	1.3	22.8	614	114	52.2
Dinas Powys	Unpolluted	Broadleaf woodland	ST 15868 70431	315868	170431	57	14.7	7	2.3	21.7	1151.9	149	35.7
Ecton Mine	Mining polluted	Broadleaf woodland	SK 09698 58263	409698	358263	103	13.9	6	1.2	22.1	598	112	49.1
Parys Mountain	Mining polluted	Rough grassland	SH 43829 89971	243829	389971	117	13.2	7.7	3.6	18.8	841	143	20.3
Port Talbot Control	Unpolluted	Rough grassland	SS 83690 84574	283690	184574	150	13.5	8.5	4	19.6	999	148	9.7
Port Talbot Blast Furnace	Industrial polluted	Rough grassland	SS 79001 85463	279001	185463	6.1	13.5	8.5	4	19.6	999	148	9.7
Porton Down ECN	Unpolluted	Improved pasture	SU 19575 37692	419575	137692	102	14.1	6.2	1.4	21.9	749	122	47.6
Roman Gravels Mine	Mining polluted	Rough grassland	SJ 33592 00339	333592	300339	368	14.1	5.6	1.3	21.6	668	126	51.8
Scunthorpe blast furnace	Industrial polluted	Arable	SE 94800 15840	494800	415835	19.7	13.4	5.7	0.9	21.3	613	115	49.8
Scunthorpe Control	Unpolluted	Arable	SE 93156 12000	493156	412000	10.7	13.4	5.7	0.9	21.3	613	115	49.8
Shipham control	Unpolluted	Improved pasture	ST 46312 59409	346312	159409	54	14.6	7	2.6	21.7	899	134	28.9
Shipham mine	Mining polluted	Improved pasture	ST 44799 57273	344799	157273	169	14.6	7	2.6	21.7	899	134	28.9
Snowdown ECN control	Unpolluted	Rough grassland	SH 63674 55116	263674	355116	748	12	5.9	1.8	18.1	2612	199	50.1

Supplementary Table 2. Vegetation and presence (shaded) or absence (unshaded) for earthworm species at the 23 sites used for the collection of morphotype *L. rubellus*.

Site name	Mean sward height	Livestock	Exposure	*Aporrectodea caliginosa*	*Aporrectodea rosea*	*Lumbricus castaneus*	*Dendrobaena rubida*	*Aporrectoda longa*	*Lumbricus terrestris*	*Lumbricus festivus*	*Octolasion cyaneum,*	*Allobophora chlorotica*	*Octolasion tyrtaeum tyaneum*
Alice Holt ECN Control	-	Deer	shaded										
Avonmouth Control	20 cm	None	part-shaded	■				■				■	
Avonmouth Incinerator	10 cm	None	open			■			■				■
Avonmouth Savalco	35 cm	None	open		■				■				
Caradon Mine	5 cm	horses	open										
Carrick Fell Mine	10 cm	sheep	open						■				
Castell Mine	5 cm	None	open			■				■			
Clydach Smelter	5 cm	None	shaded	■	■		■			■			
Cwmystwyth control	10 cm	None	open										
Cwmystwyth mine	10 cm	sheep	open										
Devon Great Consouls Control	10 cm	None	open	■	■				■			■	
Devon Great Consouls Mine	10 cm	None	part-shaded					■					
Didcot Power Station	-	None	Shaded	■		■		■	■		■		
Drayton ECN Control	15 cm	None	open	■	■	■		■	■		■		
Dinas Powys	-	None	shaded	■	■				■			■	
Ecton Mine	-	None	part-shaded						■				
Parys Mountain	5 cm	None	part-shaded	■									
Port Talbot Control	10 cm	None	part-shaded	■	■	■			■			■	
Port Talbot Blast Furnace	10 cm	horses	open										
Porton Down ECN	8 cm	None	part_shaded										
Roman Gravels Mine	3 cm	horses	open										
Scunthorpe blast furnace	5 cm	None	open	■		■		■	■			■	
Scunthorpe Control	6 cm	None	part-shaded	■		■			■		■	■	
Shipham control	10 cm	None	open						■		■	■	
Shipham mine	10 cm	cattle	part-shaded			■			■				
Snowdown ECN control	15 cm	sheep	open	■		■	■						

Supplementary Table 3. Arithmetic mean of measured soil chemical properties for pH, loss on ignition and concentrations of a suite of trace lements based on analysis of three samples collected from sites the 23 sites used for the collection of morphotype *L. rubellus*.

Site name	Soil pH	% Soil loss on ignition	Al (mg/kg)	As (mg/kg)	Ba (mg/kg)	Cd (mg/kg)	Co (mg/kg)	Cr (mg/kg)	Cu (mg/kg)	Fe (mg/kg)	Hg (mg/kg)	Mn (mg/kg)	Mo (mg/kg)	Ni (mg/kg)	Pb (mg/kg)	Sb (mg/kg)	Se (mg/kg)	Ti (mg/kg)	V (mg/kg)	Zn (mg/kg)	Ca (mg/kg)	P (mg/kg)	S (mg/kg)
Alice Holt ECN Control	5.16	11.15	8737	13.2	19.3	0.1	6	12.6	12.4	16500	0.17	78	0.3	9.5	27.1	0.4	0.5	7	24.6	43	1640	424	202
Avonmouth Control	5.86	14.23	11130	20.9	356	2.3	10	46.3	75.1	26133	0.76	539	1.2	25.1	207	1.3	1.7	32.9	26.9	697	68633	524	1029
Avonmouth Incinerator	6.5	7.94	11000	36.9	439	50.6	12.3	23.6	204.3	26133	1.07	1010	2	25.7	1943	14.9	5.6	34.4	31.8	4640	39267	1600	3190
Avonmouth Savalco	6.3	24.51	14733	7.8	75.1	2.6	8.1	25.1	29.8	19233	0.25	663	0.6	18.4	99.6	1.2	0.6	95.5	29.6	299	4777	1233	734
Caradon Mine	4.64	16	5147	407	33.7	1.17	2.03	3.9	609	20633	0.33	221	2.57	4.27	69.1	3.17	1.49	27.4	13.8	43.5	342	776	884
Carrick Fell Mine	4.85	10.8	16300	737	33.5	3.07	8.18	16.4	59	33300	2.06	837	18.10	11.4	173	7.01	3.85	165	92.6	282	1607	961	1457
Castell Mine	5.03	8.15	14333	14.7	15.7	9.03	12.5	16.4	60	46267	0.5	844	1.16	16.5	210	2.28	1.35	8.1	26.8	1792	321	506	312
Clydach Smelter	5.3	10.2	6780	44.1	142	1.61	90.1	32.3	465	25067	0.34	1369	2.68	1799	278	3.57	14.43	70.9	47.2	370	39277	945	1230
Cwmystwyth control	5.23	26.89	16167	19.8	14.6	0.1	6.7	18.7	19.7	38033	0.12	467	0.9	14.3	626	1.6	1.2	6.6	24.6	116	209	688	709
Cwmystwyth mine	5.46	21.13	20033	49	29.8	0.2	41.2	24.1	28.8	50433	112	2597	1.5	23.3	657	2.5	1.4	22	33.3	127	551	689	534
Devon Great Consols Control	5.58	22.79	21533	310	45.5	0.4	14.8	31.7	107.3	45800	0.05	585	1	27.5	68	1.4	2.3	41.8	38.7	140	2213	1177	919
Devon Great Consols Mine	5.19	38.9	17300	6270	45.9	0.2	25.7	17.8	2647	79600	0.64	630	1.3	22.3	225	13.9	2.6	38.4	38.5	277	3340	286	1503
Didcot Power Station	7.31	4.17	10570	14.5	139	0.53	8.89	14.7	41	22900	0.34	309	0.93	24.1	102	2.42	1.40	15.1	26.9	181.00	37600	901	1250
Drayton ECN Control	5.79	21.65	12833	25.1	48.4	0.4	4.9	15.8	46	28533	0.1	805	2.3	10.7	30.2	0.6	0.5	34.3	32	176	35000	2000	1183
Dinas Powys	6.9	8	17166	23	85.4	1.2	16.1	39	42.6	24933	0.1	1120	1.7	45.1	109	*	1.2	41.5	45.7	770	12766	2070	*
Ecton Mine	5.82	10.4	1403	136	537	61.4	33.2	5.4	5787	12267	0.35	965	101	72.4	1553	92.1	5.42	20.1	20.2	6047	116200	203	9280
Parys Mountain	4.44	4.95	681	1480	96	8.77	7.78	5	3673	175667	3.34	35.7	37.5	3.17	29033	210	42	54.4	19	2333	1749	26.7	22467
Port Talbot Control	5.66	16.99	15300	14.5	127	0.6	11.3	37.7	33.8	31467	0.27	1287	0.9	17.4	39.1	0.8	1.2	15.7	62.9	480	13903	1427	1083
Port Talbot Blast Furnace	5.98	12.3	10323	14.5	287	0.8	4.7	129	35.5	34967	0.33	3550	1.7	16.8	117	2.2	1.1	230.7	140	341	89133	1227	1680
Porton Down ECN	6.75	12.93	6153	21	88.3	0.7	8	20	39.2	13567	0.37	611	0.9	17.3	109	1.4	0.5	46.3	17.2	184	148333	3100	1423
Roman Gravels Mine	6.4	8.48	10633	13.7	123	14.2	14.3	13.3	99	30133	0.33	582	1.17	24.8	1125	2.48	1.33	15.7	18.4	1788	6367	455	878
Scunthorpe blast furnace	6.43	9.14	10803	40.6	90.3	0.3	13.1	42	25.4	59900	0.33	1227	1.5	29.5	124	1.8	1	68.3	128.7	183	13500	1497	453
Scunthorpe Control	6.79	6.04	7197	24.6	47.4	0.4	4.9	15.4	20.2	21767	0.12	535	2.3	11.2	30.0	0.6	0.5	33.6	30.7	69.1	130667	1253	1167
Shipham control	6.07	16.1	12967	37.1	302	2.1	7.5	23.1	19.1	26500	0.03	465	0.7	16.4	163	0.9	0.4	53.9	34.8	328	3520	972	584
Shipham mine	6.03	33.7	9907	867	1526	404	12.5	21	85.1	85100	12.3	2277	5.1	31.6	7260	48.8	3.8	51.4	28.5	31833	16117	2370	3547
Snowdown ECN control	5.52	22.07	38600	17.5	10.1	0.3	30.6	78.2	19.5	70733	0.19	1350	0.5	30.4	37	0.5	1.4	1530	239	114	1111	524	658