Genetic diversity and population structure in an invasive pantropical earthworm along an altitudinal gradient

Authors

[1*]Diana Ortíz-Gamino, [2]Luis Cunha, [3]Esperanza Martínez-Romero, [1]Norma Flores-Estévez and [1]Ángel I. Ortíz-Ceballos

Affiliations

[1]Instituto de Biotecnología y Ecología Aplicada (INBIOTECA), Universidad Veracruzana, Av. de las Culturas Veracruzanas No. 101 (Campus USBI) Col. Emiliano Zapata, 91090 Xalapa, Veracruz, México.

[2] Organisms and Environment Division, School of Biosciences, Cardiff University, Cardiff, CF103AT, UK.

[2] Embrapa Florestas, Estrada da Ribeira, Colombo, Paraná, Brazil

[3]Ecología Genómica, Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Av. Universidad s/n Col. Chamilpa 62210, Cuernavaca, Morelos, México.

*Correspondence: Diana Ortíz-Gamino, Email: diana.gamino@gmail.com,

Abstract

Population genetic analyses of the invasive pantropical earthworm *P. corethrurus* populations will contribute significantly to better understand the ecology and especially the reproductive system of this species. Using 34 polymorphic ISSR markers the genetic diversity and population structure was assessed for four populations of *P. corethrurus* along an altitudinal gradient, ranging from sea level up to ~1667 meters. Nuclear markers were able distinguish two genetic clusters, probably corresponding to two distinct genetic lineages, herein defined as A and B. Clones were detected in one population (Actopan at 480 masl) and its number was lower than expected for a parthenogenetic species. Nevertheless, low levels of genetic diversity and high number of intermediary genotypes were detected among the studied *P. corethrurus* populations with no apparent population structure related to the distinct geographic regions, which may indicate that human-mediated transference is prevalent, in particular, for the lower altitude regions. Hybridisation between the two genetic clusters was tested and pointed to 11 MLGs as being later-generation hybrids (B1 introgression) mainly associated with the three lower altitude regions. Still, most of the individuals seem to belong to lineage A and only five individuals seem to belong exclusively to the lineage B. Interestingly, these parental individuals were only found present at the highest altitude site, Naolinco (1566-1667 masl), which also showed the highest values of genotypic richness. During the biological invasion, multiple introduction of different genetic lineages can provide opportunities for admixture among genetically distinct clusters. The signatures of admixture among *P. corethrurus* populations along the altitudinal gradient in Mexico may have allowed the invasion success by directly increasing fitness. ISSR markers revealed to be useful for the study of genetic variation in the invasive pantropical earthworm, *P. corethrurus*.

45 **Keywords:** Invasive earthworms, *Pontoscolex corethrurus,* asexual reproduction, altitudinal

46 gradient.

1. Introduction

47

48 Earthworms are not only ubiquitous ecosystem engineers, but through their action the

49 functioning of the ecosystem is sustained (Lavelle et al. 1997; Brown et al. 2001; Barros et al.

50 2004). Even with the importance that soils organisms have into the ground, terrestrial ecosystem

51 seems to be the least studied (González et al. 2006; Decaëns 2010). In fact, for a long time, little

52 recognition has been given to invasions of soil organisms (Gates 1954); however during the last

53 30 years belowground invasion has become highly important (Hendrix et al. 2002; Hendrix and

54 Bohlen 2006; Craven et al. 2016; Cicconardi et al. 2017). The first records in Mexico about

55 exotic earthworms was made between 1900-1906, lumbricids (*Dendrobaena octaedra,*

56 *Lumbricus Terrestris*), megascolecids (*Dichogaster bolaui*) and rhinodrilids (*Pontoscolex*

57 *corethrurus*) (Eisen 1900; Bedard 1912), and until now, 51 exotic species were described by

58 classical taxonomy (Fragoso and Rojas 2014). The role involved into earthworm's invasion is

59 not fully understood, i.e. distribution and establishment of exotic earthworms are due to different

60 factors acting at different time-spatial scale (Marinissen and Van den Bosch 1992; Baker et al.

61 2006; Brown et al. 2006; González et al. 2006; Cameron and Bayne 2009; Marichal et al. 2012;

62 Narayanan et al. 2016). On the other side, the key of invasive earthworm's success could be

63 based on its molecular plasticity supported by high genomic promiscuity tightly associated with

64 reproductive strategies (Vitturi et al. 2002; Bengtsson 2009; Dupont et al. 2012; Fernández et al.

65 2013; Cunha et al. 2014; Pavlíček et al. 2016).

66 Parthenogenesis is not uncommon in earthworms and it is usually associated with a

67 dispersal advantage, mainly because a single propagule could be sufficient to stablish a new

68 population (Terhivuo and Saura 2006). Thus, a rapid adaptation of parthenogenetic clonal

69 populations may be an important mechanism for the success during the colonisation event

4

70   (González et al. 2006; Hendrix et al. 2006; Terhivuo and Saura 2006) as *Pontoscolex*

71   *corethrurus*.

72   *P. corethrurus* is a species of earthworms that was renamed into the Rhinodrilidae family

73   (James 2012). This species comes from South America, Guiana Shield area of the Amazon

74   (Gates 1954; Righi 1984) and it has been suggested that its reproduction is usually

75   parthenogenetic (Gates 1973; Buch et al. 2011). Its growth and reproduction is not limited

76   anymore by temperature (Ortíz-Gamino et al. 2016; Narayanan et al. 2016). In parthenogenetic

77   species such as *P. corethrurus*, the genetic variation in regions far from their natural dispersion

78   range may be low if only one or very few individuals were colonizers (Dupont et al. 2012). In

79   addition, there is evidence of a cryptic species complex of *P. corethrurus*, for which two deeply

80   divergent mitochondrial lineages have been detected (Cunha et al. 2014), although using nuclear

81   markers such divergence was not that conspicuous.

82   In a recent study (Ortíz-Gamino et al. 2016), it was found that the altitudinal distribution

83   of *P. corethrurus* in the central zone of the Veracruz State is extensive and goes from sea level to

84   around 1600 meters of altitude with an average temperature of 17 °C. In this study, it was

85   hypothesised that populations of *P. corethrurus* along the altitudinal gradient could be

86   distinguished into two different ecotypes, which could correspond to a different genetic

87   background or even to the presence of cryptic species (Cunha et al. 2014; Ortíz-Gamino et al.

88   2016). Also in India, after just 100 years since the introduction of *P. corethrurus* is locally

89   naturalized and shows a wide distribution along an altitudinal gradient (Narayanan et al. 2016),

90   similar to what was found in Veracruz State, Mexico. However, as a parthenogenetic species, its

91   dispersal seems to be strongly driven by human activities (González et al. 2006; Feijoo et al.

92   2007; Hendrix et al. 2008; Dupont et al. 2012).

93   *P. corethrurus* is considered an important ecosystem engineer (Lavelle et al. 1997;

94   Brown et al. 2001; Domínguez and Gómez-Brandón 2009), its role should not be neglected

95   (Lavelle et al. 1987; Pashanasi et al. 1992; Pashanasi 1994; Brown et al. 2004; Fragoso et al.

96   2009; Geissen et al. 2009; Huerta and Van der Wal 2012) but due to the fact that it can quickly

97   become a dominant species in human disturbed environments, sometimes becoming the only

98   earthworm species present (Lapied and Lavelle 2003; Feijoo et al. 2007; Uribe et al. 2012;

99   Hernández-Castellanos et al. 2013; Zavala et al. 2013; Gutiérrez and Cardona 2014) ecological

100   researches are necessary.

101   Population genetic analyses of the invasive pantropical earthworm *P.*

102   *corethrurus* populations will contribute significantly to better understand the ecology and

103   especially the reproductive system of this species (Marichal et al. 2012; Cunha et al. 2014), which

104   might be crucial for planning successful strategies for biological invasion mitigation or for future

105   assumptions.

106   In order to evaluate the clonal diversity of parthenogenetic earthworms, biochemical

107   markers have been used like allozyme (Terhivuo and Saura 1993; Terhivuo and Saura 2006), and

108   molecular markers like mitochondrial regions (Heethoff et al. 2004; Knott and Haimi 2010;

109   Fernández et al. 2011; Fernández et al. 2013; Cunha et al. 2014). Several molecular methods for

110   assaying nuclear genetic diversity have been used attempted to estimate the relationships and

111   variability in earthworms, through AFLP (Cunha et al. 2014), RADtag (Giska et al. 2015) and

112   RAPD, ISSR and URPs (Sharma et al. 2011) contributing significantly to unveil genetic

113   relationships within earthworms. Inter Simple Sequence Repeats (ISSR) are useful dominant

114   nuclear markers commonly used to study genetic variation and population structure within and

115   among populations. ISSR have been effective in detecting certain cryptic species in mosses, such

6

116   as *Aneura pinguis* and *A. maximus* (Buczkowska et al. 2016). For *Ae. aegypti*, ISSR exhibited

117   four unique banding profiles for each of the four genotypes (Abbot 2001). For blackfly species,

118   ISSR-PCR is a powerful technique for fingerprinting (Dušinský et al. 2006).

119   The main advantages of ISSR-PCR are associated to the fact that they do not require the

120   knowledge of genomic sequences, low cost, able to detect of high levels of polymorphism,

121   require simple operation, can be of high stability and reproducibility, and have a rapid turnover

122   (Wolfe and Liston 1998; Abbot 2001; Dušinský et al. 2006; Sharma et al. 2011; Ng and Tan

123   2015).

124   The objective of this work was to determine the genetic variation and population

125   structure in four populations of *P. corethrurus* along an altitudinal gradient in the central area of

126   the State of Veracruz using ISSR as nuclear markers. As a hypothesis, it is suggested that genetic

127   diversity would be higher in the intermediate zone of the chosen gradient, since the extremes of

128   the gradients are usually the most stressful environments and in the intermediary area a relatively

129   optimal environment could be found.

130   2. Material & Methods

131   2.1 Sampling Sites and Animal Collection

132   The altitudinal distribution of *P. corethrurus* along the central region of Veracruz State goes

133   from the sea level up to 1667 masl. Four sampling points were established at each of four sites

134   along this altitudinal gradient: Laguna Verde (LV), Actopan (AC), Ingenio La Concepción (LC)

135   and Naolinco (NA) at 24, 480, 973-1,036 and 1,566–1,667 masl, respectively (Table 1 and
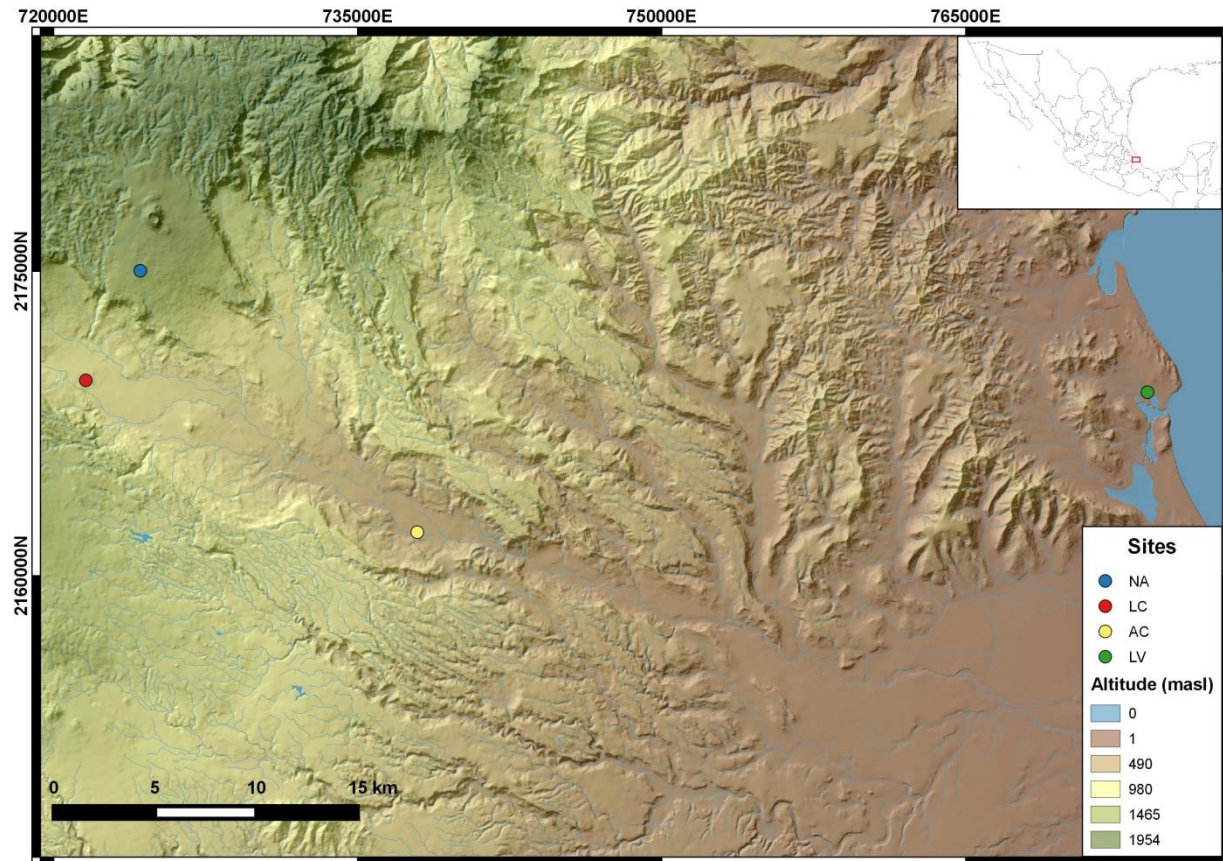
136   Figure 1) (Ortíz-Gamino et al. 2016).

137

Figure 1. Sampling sites along an altitudinal gradient in center region of Veracruz state, Mexico. LV, Laguna verde
(24 masl), AC, Actopan (480 masl), LC, Ingenio La Concepción (973-1036 masl) and NA, Naolinco (1566-1667
masl). The digital elevation model was created using data provided by Instituto Nacional de Estadística y Geografía,
México.

During September of 2013, 40 mature (clitellate) individuals were collected (n= 10 per
site). Animals were kept in plastic boxes with moistened soil and transported to the laboratory at
INBIOTECA for taxonomical/anatomy identification (Moreno and Borges 2004). Individual
were rinsed in water, washed from soil particles and posteriorly fixated in 96% ethanol. Animals
were conserved at -20 until further processing.

Table 1. Attributes of the sampling sites along an altitudinal gradient in Veracruz State, México.

Sampling Site	Altitude (masl)	Climate	Soil's texture		
			Clay (%)	Silt (%)	Sand (%)

8

Laguna Verde (LV)	24	Aw1(w)(i')g	25.6-27.6	21.8-34.4	40.0-50.6
Actopan (AC)	480	Aw0(w)(i')gw"	12.0-13.6	28.8-35.8	50.6-59.2
Ingenio La Concepción (LC)	973-1036	(A)Ca(f)(i')gw"	25.6-27.6	21.8-34.4	40.0-50.6
Naolinco (NA)	1566-1667	Cb (fm)(i')gw"	12.0-13.6	28.8-35.8	50.6-59.2

149   Notes:

150   Climate: Aw1(w)(i')g and Aw0(w)(i')gw" are used for warm and sub-humid climate; (A)Ca(f)(i')gw" for warm and

151   humid and Cb (fm)(i')gw" for wet and semi-humid climate. For more details (average temperature,

152   evapotranspiration, total annual precipitation, etc.) see Ortíz-Gamino et al. (2016).

153

2.2 DNA isolation and quantification

155   Tail wall tissue was used for extraction of genomic DNA. Total DNA was extracted using the

156   DNeasy Blood & Tissue kit (Qiagen) following the manufacturer's instructions. DNA was

157   checked for quality by gel electrophoresis and quantified using a spectrophotometer (ND-2000,

158   Nanodrop Technologies, Wilmington, DE).

2.3 ISSR protocol

160   Forty *Pontoscolex corethrurus* individuals (n = 10 per site) were used for ISSR screening. This

161   screen used 5 ISSR primers (Table 2), which were previously reported to produce polymorphic

162   and reproducible DNA fingerprints from *Eudrilus eugeniae* and *E. fetida* (Sharma et al. 2011).

163   Each PCR reaction contained 1X PCR buffer, 2 mM $MgCl_2$, dNTPs at 100 μM each, primers at

164   0.8 μM each, 1.5 U DNA Taq polymerase, 1ug/ul BSA and 30 ng template DNA. The PCR

165   reaction mix was adjusted to a final volume of 10 μL with water. PCR amplifications were

166   performed in an Eppendorf Mastercycler. Conditions included an initial step of 3 min at 95°C,

167   followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at primer-specific

168   temperature (Table 2) for 30 seconds, and elongation at 72°C for 1 min. A final extension was

169   performed at 72°C for 10 min. PCR products were visualized in 2% agarose gels (with ethidium

170   bromide at 1ul/ml). The initial screening used a total of 18 primers but only primers that were

171   polymorphic and reproducible were chosen (Table 2).

172

173   Table 2. Primers used for ISSR amplification.

Primer	Sequence	Ta (°C)	Maximum number of bands	Estimated size (bp)
840	GAGAGAGAGAGAGAGAYT	59.5	8	2000-200
834	AGAGAGAGAGAGAGAGYT	61	7	2000-300
866	CTCCTCCTCCTCCTCCTC	70	6	2000-400
810	GAGAGAGAGAGAGAGAT	52.4	6	2000-400
807	AGAGAGAGAGAGAGAGT	54.4	7	2000-300

174

175

2.4 Data analysis

177   The amplified DNA fragments were transformed into a binary matrix (1= presence, 0=

178   absence). A multilocus genotype (MLG) was constructed for each individual by combining data

179   of single ISSR fingerprints using the procedure available in the package POPPR (Kamvar et al.

180   2014) Isolates with the same MLG were considered clones, and some analyses were conducted

181   for the original and clone-corrected dataset (Gramaje et al. 2014).

182   The R package POPPR (Kamvar et al. 2014) was used to calculate the dissimilarity

183   distance matrices and to generate a minimum spanning network from the matrices. To assess the

184   possible evolutionary relationships among MLGs, a minimum spanning network was constructed

185   using the genotypes of each sampling location. Bootstrapping was performed with the R package

186   POPPR (Kamvar et al. 2014) with 1000 bootstrap resampling. Genotypic diversity, genetic

187   richness and the evenness index adapted from the Simpson index of genotypic diversity were

188   calculated for each population using the R package POPPR (Kamvar et al. 2014). The function

189   'rarecurve' from the R package VEGAN (Oksanen et al. 2007) was used to generate rarefaction

190   curves representing species richness. Curves were calculated to determine if the sampling

191   intensity was adequate to detect most of the *P. corethrurus'* MGLs present in each population.

192   Additionally, calculation for the minimum number of loci able to distinguish all MGLs was also

193   performed. Because sample size varied among populations we employed rarefaction to explore

194   the effect of sample size on observed species richness.

195   **2.5 Genetic differentiation, structure and clustering analyses**

196   The estimation of the genetic variance for all MLGs was evaluated by analysis of molecular

197   variance (AMOVA), using the GenAlEx v.6.5 software (Peakall and Smouse 2006). It was

198   calculated as PhiPT (analogue of F_{st} fixation index) for all populations and within populations

199   (Peakall and Smouse 2006) relative to the total variance. Significance was obtained by using

200   9999 permutations, and confidence interval at 95%, by 10,000 re-samplings. For this analysis,

201   only single copies of the different genotypes were used to give identical weight to MLGs. The

202   Mantel test was used to evaluate whether there was a correlation between the matrix of genetic

203   differentiation between the pairs of MLGs and the spatial distance matrix between the

204   populations, using Arlequin v.3.5 (Excoffier and Lischer 2010). The existence of association

205   between *P. corethrurus* individuals was assessed initially using a Principal Coordinates Analysis

206   (PCA), implemented in GenAlEx v. 6.51 (Peakall and Smouse 2006). As PCA is independent of

207   any genetic hypotheses it is suitable for the analysis of partially clonal species. Additionally,

208   Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrograms were also

209   created using the POPPR package in R (R core team 2004) Bootstrapping was performed with

210   the R package PVCLUST with 10,000 bootstrap resamplings (Suzuki and Shimodaira 2006).

211   Populations structure was explored using the Bayesian clustering method implemented in

212   STRUCTURE v.2.3.4 (Pritchard et al. 2000) and distance based approach, a Discriminant

213   analysis of principal components (DAPC) implemented in Adegenet R package (Jombart et al.

214   2010). STRUCTURE v.2.3.4 (Pritchard et al. 2000) was used to identify the number of genetic

215   clusters within the dataset, and to assign individuals to the defined clusters using an admixture

216   model. For this, 15 independent runs for each K (1 - 8) to confirm consistency. A burn-in period

217   of 100,000 iterations and further 200,000 MCMC generations. To determine the most likely

218   value of K, we used the "BestK" implemented in CLUMPAK (Evanno et al. 2005), which uses

219   ΔK method of Evanno et al. (2005) Results from the 15 replicate runs were combined using

220   CLUMPAK online version (Evanno et al. 2005). Since DAPC allows assessment of structure of

221   populations without making assumptions of panmixia makes it appropriated to populations that

222   may show partial clonality. Moreover, this technique provides a robust alternative to compare to

223   the Bayesian clustering methods like the one implemented in STRUCTURE (Pritchard et al.

224   2000). Relative dissimilarity distances were calculated according to the index of association

225   (Brown et al. 1980; Smith et al. 1993). The approach returns a distance reflecting a ratio of the

226   number of observed differences by the number of possible differences.

227   The hybridisation status of individuals according to the Bayesian genetic clusters defined in

228   Structure (defined as putative Lineage A and Lineage B) was further investigated using

229   NEWHYBRIDS v1.1 (Anderson and Thompson 2002) which also uses a Bayesian assignment,

230   by implementing a multilocus allele frequency model-based approach. This approach performs

231   clustering of MGLs without any a priori knowledge of parental allele frequencies, and also has

232   the advantage of specifically assuming a mixture of parental and several hybrid and parental

233   classes, such as the genotypes corresponding to parental lineages, F1, F2 (F1 x F1) and later

234   generation or introgressive hybrids B1 (e.g. parental 1 x F1) and B2 (e.g. parental 2 x F1).

235   Individual posterior probabilities belonging to each hybrid category were estimated using the

236 MCMC method in a Bayesian framework using Jeffreys-type priors and a burn-in period of

237 100000 iterations followed by 50000 sweeps from the posterior distribution sampling (Anderson

238 and Thompson 2002). Linkage disequilibrium as an indication of random mating was calculated

239 and tested for significance with 1,000 randomizations using the R package POPPR (R core team

240 2004) measures of gametic disequilibrium tested were the index of association (I_A) (Brown et al.

241 1980; Smith et al. 1993), and a standardized alternative of the I_A ($\bar{r}_d$) (Agapow and Burt 2001).

242 The null hypothesis for this test is that there is random association among alleles at different loci

243 and $I_A=0$; the null hypothesis for random mating is rejected where $I_A>0$.

3. Results

3.1 Genotypic diversity, genetic richness and evenness index

246 Table 3. Genetic parameters of genetic variation in *Pontoscolex corethrurus* along an altitudinal gradient in central
247 Veracruz state, México.

Sampling Site	N	MLG	eMLG	Pb	Tb	H	G	E_5	H_{exp}	Ia	rbarD
LV	9	9	9	20	34	2.20	9	1	0.30	1.98*	0.10*
AT	10	8	8	22	34	2.03	7.14	0.93	0.30	3.23*	0.15*
LC	6	6	6	27	34	1.79	6	1	0.39	1.16*	0.04*
N	10	10	10	24	34	2.30	10	1	0.29	4.08*	0.18*
Total	35	33	9.85			3.48	31.41	0.97	0.40	1.65	0.05

248 Notes:
249 N, Number of individuals observed; MLG, Number of multilocus genotypes observed; Pb, polymorphic bands; Tb,
250 total number of bands present H, Shannon-Wiener Index of MLG diversity; G, Stoddart and Taylor's Index of MLG
251 diversity (Stoddart and Taylor 1988); Lambda, Simpson's Index; E_5, Evenness, (Pielou 1975; Ludwig and Reynolds
252 1988; Grünwald et al. 2003); H_{exp}, Nei's unbiased gene diversity (Nei1978); Ia, the index of association (Brown et
253 al. 1980; Smith et al. 1993); rbarD, the standardized index of association ($\bar{r}_d$) (Agapow and Burt 2001); * shows
254 significant value with $p < 0.01$

255

256 All populations showed high genotypic diversity, but without significant differences between the

257 four populations. The five ISSR primers generated a total of 33 MLGs among the 35 *P*.

13

258 *corethrurus* individuals. Two Actopan MLG were observed twice in that population. The resting

259 31 MLGs were observed only once. Overall, MLG diversity (H) varied across populations

260 defined by different altitudes in Veracruz (Table 3). Evenness values were higher in Laguna

261 Verde (24 masl), La Concepcion (973-1036 masl) and Naolinco (1566-1667 masl), and this is

262 due to the fact that all genotypes found were unique, compared with the population from

263 Actopan (480 masl), that showed repeated genotypes (0.93). Nei's unbiased gene diversity

264 (Hexp) values varied from the highest found in La Concepcion (0.39) and to a lowest in

265 Naolinco (0.29). According to the rarefaction curves (Figure 2A), Naolinco had a higher number

266 of samples and observed MGLs relative to the other populations. Comparison of rarefaction

267 curves showed significant differences in diversity among these populations with Naolinco

268 population being more diverse than the others, and with La Concepcion having a lower level of

269 MLG diversity. Interestingly, the minimum number of loci to define the total number of MLGs

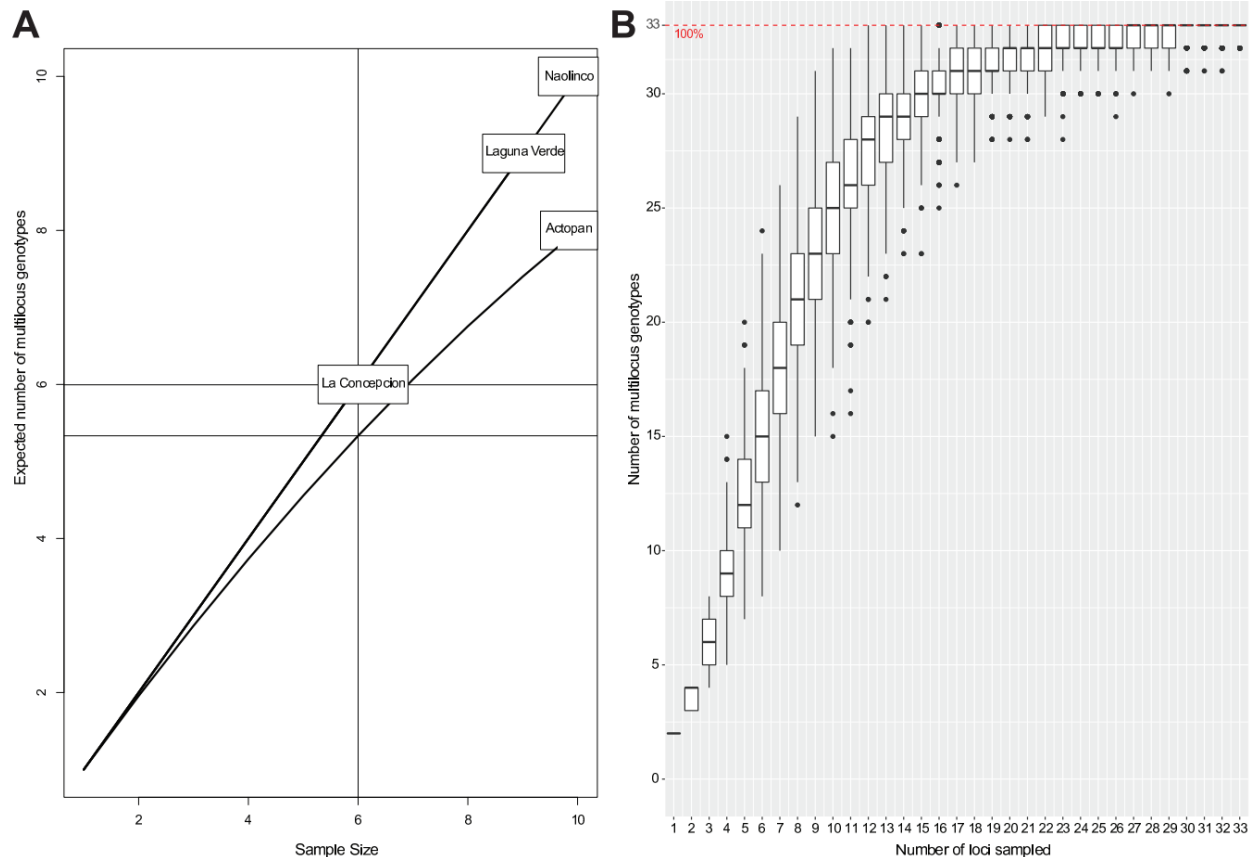270 found reached a plateau after 18 loci (Figure 2B).

Figure 2. Rarefaction curve of expected number of multilocus genotypes captured per individuals sampled (A), and a multilocus genotype accumulation curve according to the number of loci sampled (B).

3.2 Genetic differentiation

The AMOVA results (Table 4), showed 25% of the variation among populations and 75% within populations. The overall PhiPT was 0.25 (with a $PhiPT_{max}$ of 0.686 and a Phi'PT of 0.362) with an associated permutation p-value <0.001.

Table 4. Analysis of molecular variance (AMOVA)

	Level of variation	d.f.	SS	MS	Est. Var.	Proportion (%)
a)	Among Populations	3	59.079	19.693	1.760	25%
	Within Populations	29	154.497	5.327	5.327	75%
	Total	32	213.576		7.088	100%

15

	Population	Laguna Verde	Actopan	La Concepcion	Naolinco	
b)	Laguna Verde	0.000				
	Actopan	0.229**	0.000			
	La Concepcion	0.069	0.161**	0.000		
	Naolinco	0.335**	0.347**	0.199*	0.000	

280 Notes:

281 (a) testing for genetic differentiation between four locations in central Veracruz state in Mexico with populations

282 defined by altitude, and the PhiPT pairwise comparisons (b); d.f., degrees of freedom; SS, sum of squares; MS,

283 mean squares; Est. Var.,estimated variance; %, proportion of molecular variation. Significance levels as follow * $p <$

284 0.05 and ** $p < 0.01$.

285

3.3 Clustering and structure analysis

287 The principal component analysis (PCA) showed that, although some individuals cluster together

288 per site (e.g. animals in Naolinco), most of the individuals scattered in a non-uniform clustering

289 (Figure 3A). Axis 1 and 2 of the PCA accounted for 26% and 15% of total genetic variability.

290 The global minimum spanning network showed that all populations have MLGs that are closely

291 related (Figure 3B). The comparison of the Euclidian genetic distance matrix with the matrix of

292 geographic distances using the Mantel test showed that there is no correlation between these two

293 matrices suggesting that data in the genetic distance matrix is not explained by the geographic

294 positioning of the populations. The optimal UPGMA tree for the individuals and by populations

295 is presented with bootstrapped values (Figure 4A and 4B respectively). In both analyses, the

296 PCA and dendrogram there is no clear discrimination related to the geographic distances. Still,

297 La Concepcion and Laguna Verde seem to be closely associated although geographically distant.
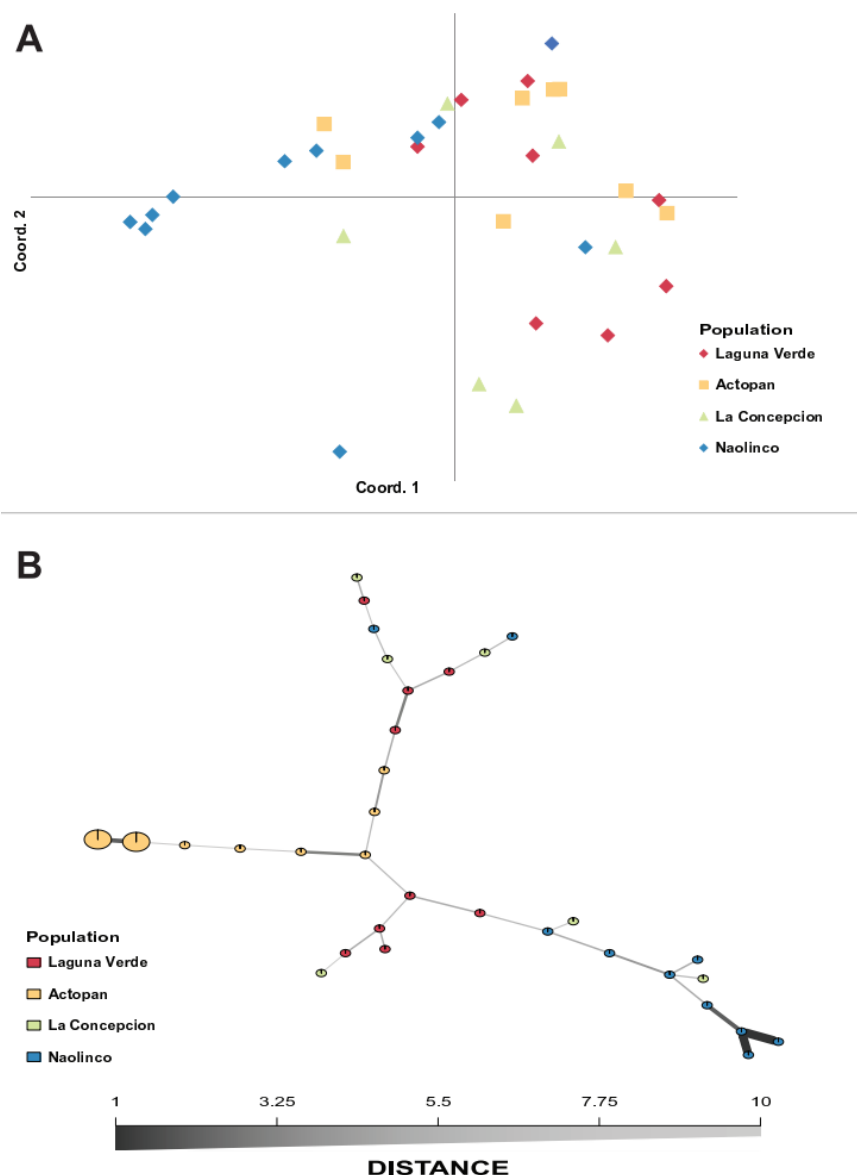
16

A

B

298

Figure 3. A Principal Component Analysis (A) and a Minimum Spanning Network (B) showing the relationship
among multilocus genotypes (MLGs) observed along an altitudinal gradient in central Veracruz State, Mexico in
four distinct populations. Each node signifies a different MLG. Node sizes and colours match to the number of
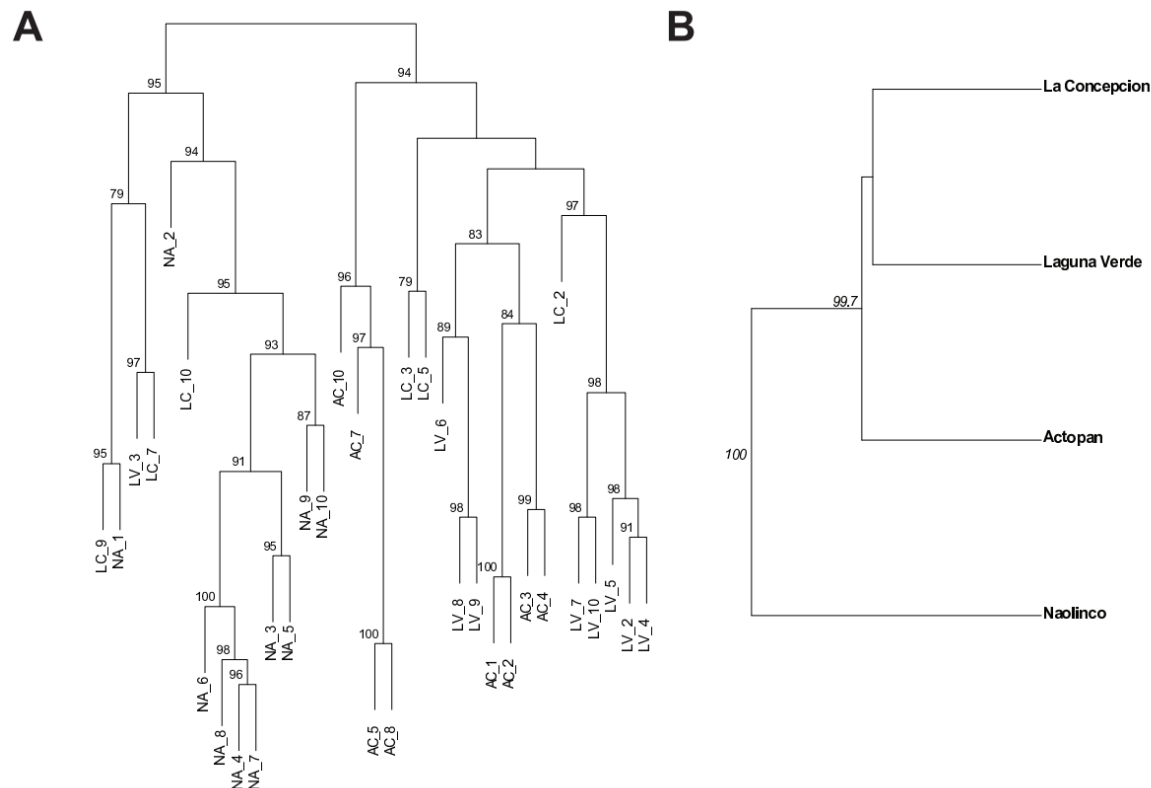individuals and population membership, respectively. Edge thickness and colour are proportional to absolute genetic
distance. Edge lengths are arbitrary.

304

305

306

17

Figure 4. UPGMA dendrogram of genetic distance among MGLs (A) and among Populations (B) observed along an altitudinal gradient in central Veracruz State, Mexico. Only values with support greater than 70% using 1000 bootstrap samples are shown.
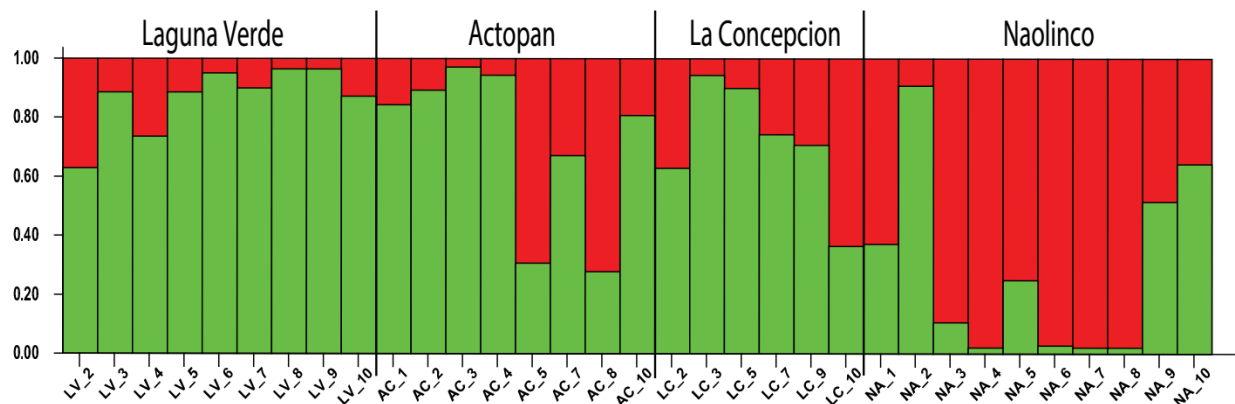

Figure 5. The estimated population structure with a summary plot of the estimates of Q based on the ISSR data observed along four populations along an altitudinal gradient in central Veracruz State, Mexico. Each individual is

18

316   represented by a vertical line, which is partitioned into K coloured segments representing the individual's estimated

317   membership fractions in K clusters (%).

318

319   The Bayesian analysis of population structure estimated two distinct genetic clusters (K =

320   2, Ln P(D)= -557.89 ± 0.31, s.d.) distributed along the four geographic populations (Fig. 5).

321   Similarly, as the PCA and dendrogram, there is no apparent structuring according to the

322   geographic location, however, the population at Naolinco seems to belong mostly to cluster 2

323   while Laguna Verde to cluster 1, intermediary altitude sites, Actopan and La Concepcion seem to

324   be strongly admixed, having a more heterogenous proportion of each cluster among individuals.

325   Apparently, two distinct genetic lineages are present among the populations, and will be herein

326   defined as Lineage A (mostly present in Laguna Verde) and Lineage B (mostly present in

327   Naolinco). The DAPC analysis (Figure 6) confirmed the relation found on UPGMA dendrogram

328   where La Concepcion and Laguna Verde, although geographically distant they seem to be

329   closely related in terms of structuring, potentially associated with the heterogenous proportions

330   of each genetic cluster as defined in the Bayesian analysis, while Actopan is detached (Lineage

331   A) as in the Bayesian analysis, which also could explain the detachment of Naolinco population

332   (Lineage B). Notable, both clone-corrected (n = 33) and uncorrected data (n = 35) reject the

333   hypothesis of no linkage among markers as the probabilities (both I_A and $\bar{r}_d$ show significant p-

334   values, see Table 3), these results support asexuality in populations (Supplementary Figure 1).

335   Interestingly, the Bayesian clustering according putative hybridisation status (Figure 7) showed

336   12 MLGs as being later-generation hybrids B1 (Lineage A x F1 introgression) mainly associated

337   with the three lower altitude regions. In general, individuals belonging to parental Lineage A

338   dominated (16 individuals) followed by later-generation B1 (12 individuals).
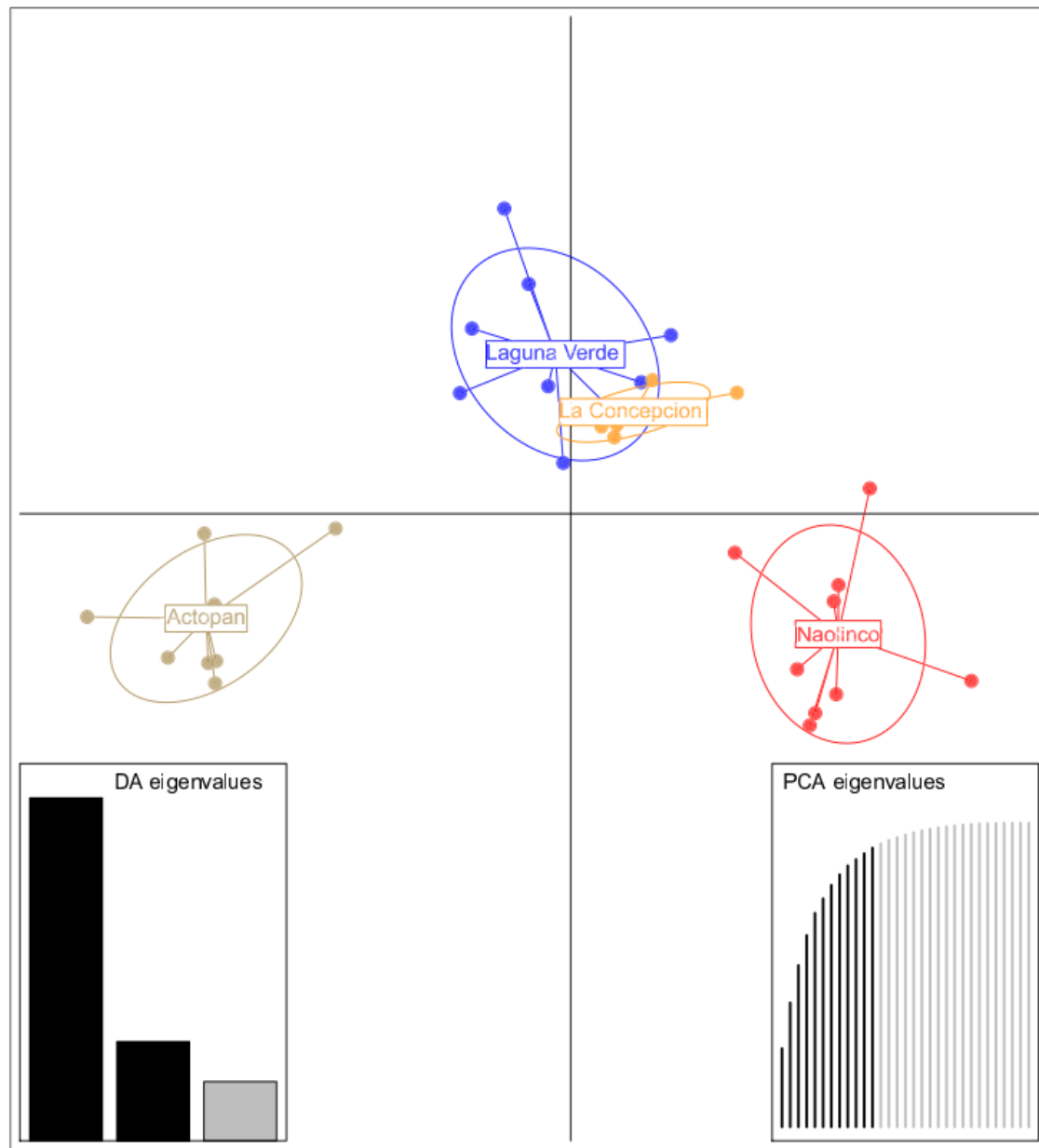
19

339

Figure 6. Genetic structure using the ISSR data in 35 *Pontoscolex corethrurus* individuals based on discriminant analysis of principal components (DAPC). Proportion of Eigenvalues in discriminant analysis is given on bottom left graphic and bottom right shows the PCA Eigenvalues, where the first 12 significant principal components are highlighted in black.
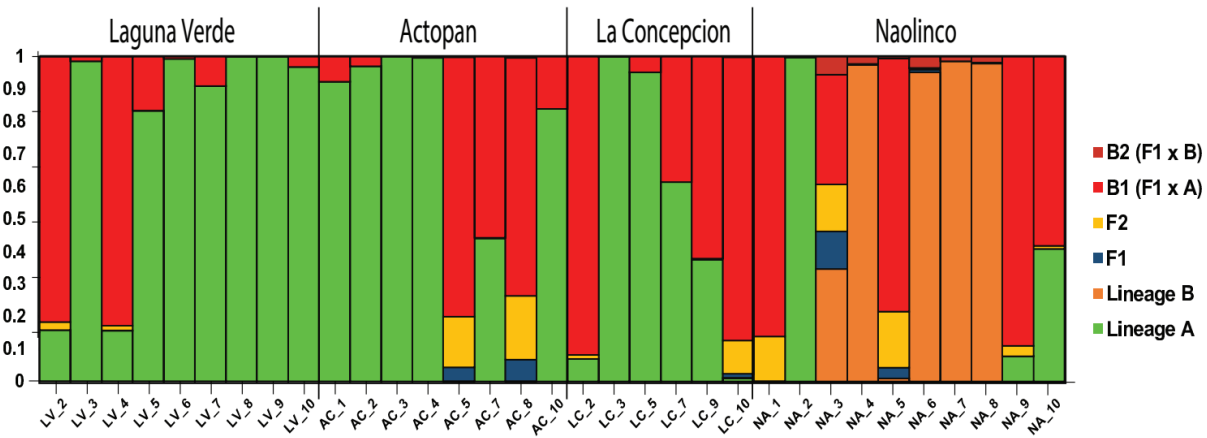
20

Figure 7. Classification of *P. corethrurus* individuals according to a Bayesian assignment algorithm implemented in NEWHYBRIDS (Anderson and Thompson, 2002) to detect gene flow. Each unit represents an individual corresponding to parental lineages (Lineage A and Lineage B), F1 generation, F2 (F1 x F1) and later generation or introgressive hybrids B1 (Lineage A x F1) and B2 (e.g. Lineage B x F1).

4. Discussion

The factors that are playing a role in the earthworms' invasion processes are soil texture and chemical parameters (Marichal et al. 2012) but also other ecological variables (Hendrix and Bohlen 2006; Craven et al. 2016) and features inherent to the species (González et al. 2006; Cameron et al. 2008; Marichal et al. 2012). Among factors that are known to influence genetic structure in earthworms such as the slow dispersion capacity, life history and reproduction mode, and the environmental filters, which may contribute for population isolation, may favour the intra-specific relationships as strong factor acting on population structure (Baker 1998; Novo et al. 2010; Fernández et al. 2013). Additionally, in tropical regions temperature may also be imposing range restrictions in species complexes (Janzen 1967) which build up the assumption that temperature in tropics may promote isolation of earthworm's species.

Genetic variability in asexual organisms may be high (Terhivuo and Saura 2006; Bengtsson 2009), however for *P. corethrurus*, even within its proposed endemic range shows

363   low genetic variation (Dupont et al. 2012). These results are the first showing genetic diversity

364   and population structure of four *P. corethrur*us populations located along an altitudinal gradient

365   through ISSR markers including when in Sharma et al. (2011) the best nuclear markers with *E.*

366   *eugeniae* and *E. fetida* were URPs which showed 98.3% of polymorphism. Our hypothesis was

367   expect to find higher genetic diversity in intermediary areas resulting from selection and more

368   extreme environmental conditions, however, movement of MLGs along the altitudinal gradient

369   seem more prevalent and more conspicuous, in particular along the lower altitude regions

370   (Laguna Verde, Actopan and La Concepcion). This could be only explained by an intense

371   human-mediated transference through road networks (Ortíz-Gamino et al. 2016) or probably

372   associated with agriculture and other human activities (Lapied and Lavelle 2003; Baker et al.

373   2006; González et al. 2006; Feijoo et al. 2007; Dupont et al. 2012).

374   Clustering analysis (PCA and dendrogram based on genetic distances and bootstrapping)

375   identified a huge admixture among all populations, but was possible identify at least two

376   divergent and well-differentiated genetic clusters, potentially corresponding to different clonal

377   lineages (Lineage A and Lineage B). Also in French Guiana, 80 individuals of *P. corethrurus*

378   were grouped into two major clusters (Dupont et al. 2012), but this pattern was not found in Sao

379   Miguel Island because the PCA constructed using AFLP markers was spread rather than

380   clustered (Cunha et al. 2014). Here in Naolinco (1566-1667 masl) population showed the best

381   distinguishable cluster of individuals. The unique Naolinco signature may be associated with the

382   contrasting environmental conditions found at such higher altitude, which may be acting as an

383   environmental filter over this population. Possibly, at the highest altitude found in Naolinco, and

384   although, MGLs predominantly result from successive introductions, the temperature dispersion

385   (year average of 17 °C) may be the main barrier to MGLs until now (Ortíz-Gamino et al. 2016),

22

386   the Lineage B could be adapted to lower temperatures. Nevertheless, under a global change

387   scenario with intensive land-use change and alarming global warming (Jiménez and Decaëns

388   2000; Eisenhauer et al. 2014; Gutiérrez and Cardona 2014) this barrier could decrease and

389   benefit invasive pantropical earthworm's species. Otherwise, the lineage A seems spread since

390   the sea level until LC (973-1036 masl) which could indicate a wide ecological tolerance and

391   probably well-adapted to warm temperatures. The factors that are shaping this dispersion pattern

392   in clonal lineages are multivariable; some examples have been documented, in *Octolasion*

393   *tyrtaeum* some widespread and restricted clonal lineages have been identified (Terhivuo and

394   Saura 1993) also showed that one single haplotype was widespread and found in all sampling

395   stations. In *Aporrectodea trapezoides*, the clonal lineages seem to tend to stay close to their

396   original areas, which can indicate some level of local adaptation and/or strong interspecific

397   relations (Fernández et al. 2013). But for peregrine species, the evidence indicates that human

398   activities are strongly shaping dispersion patterns and MLG introduction through accidental

399   transfer (Cameron et al. 2008; Dupont et al. 2012; Ortíz-Gamino et al. 2016).

400   The AMOVA analyses showed that most genetic diversity (75%) was found within

401   populations and only 25% among populations. Similar results were found by Cameron et al.

402   (2008) in the earthworm *Dendrobaena octaedra* from Alberta, Canada. Cunha et al. (2014),

403   using AFLP markers on *P. corethrurus* found that most of the variability was also found within

404   populations and only a few proportion was associated between populations. The two

405   distinguishable genetic clusters revealed here by the Bayesian analysis would then correspond to

406   an established asexual lineage intermixed with individuals derived at least from two different

407   asexual lineages (Gramaje et al. 2014). Indeed, multiple introductions of different genetic

408   lineages is highly probable, there are at least three well-stablished *P. corethrurus* lineages, the

23

409   original from South America (Dupont et al 2012), other that probably has 100 years in India

410   (Narayanan et al. 2016), and the oldest one described until now in Sao Miguel Island in the

411   Azores Archipelago (Portugal) with ~200 years but even there the 28 haplotypes that were

412   found, all belonging to a single genetic lineage (Cunha et al. 2014). The accidental transfer of *P.*

413   *corethrurus* between Mexico and these three sites (French Guiana, India and Azores) can happen

414   (Cuatrecasas 1964; Schultes 1984).

415   The standardized index of association ($\bar{r}$d) supported the hypothesis for clonal population

416   structure due the widespread of several intermediary and similar MLGs along the altitudinal

417   gradient, but also by the linkage disequilibrium tests, where the null hypothesis of random

418   mating was rejected for all populations. Nevertheless, the interpretation of the results requires

419   caution because it is difficult to demonstrate the presence of linkage disequilibrium with small

420   sample sizes (Hagenblad et al. 2006; Du et al. 2007; Gramaje et al. 2014). The high levels of

421   genotypic diversity found in this study could be expected because there is evidence of high

422   diversity found in populations of *P. corethrurus* using AFLPs that may be indicative of

423   polyploidy (Cunha et al. 2014), but also the results are due because the evolutionary rate of

424   change within repeat regions compared with other nuclear regions like AFLPs, RAPD or URPs

425   (Sharma et al. 2011; Gramaje et al. 2014; Seyedimoradi and Talebi 2014; Ng and Tan 2015).

426   Even with several studies showing high percentage of polymorphic loci using ISSR (Abbot

427   2001; Dušinský et al. 2006; Luan et al. 2006; Sharma et al. 2011; Seyedimoradi and Talebi 2014;

428   Ng and Tan 2015; Buczkowska et al. 2016). Our decision to use ISSR markers was supported by

429   their potential of discrimination among geographically more or less isolated populations

430   (Dušinský et al. 2006), the capacity of ISSR to distinguish among cryptic species (Buczkowska

431   et al. 2016), but also ISSR amplify only nuclear regions of eukaryote (Seyedimoradi and Talebi

24

432   2014) and as an endogeic earthworm, *P. corethrurus* is commonly associated with Bacteroidetes

433   phylum (Bernard et al. 2012).

434   Finally, sexual reproduction is a rare event in *P. corethrurus*, is widely accepted that its

435   reproduction occurs mainly by parthenogenesis (Gates 1973). In earthworms, parthenogenesis

436   was associated with polyploidy as well to high levels of DNA methylation (Regev et al. 1998).

437   Therefore, is also plausible that methylation may be fostering the epigenetic control of

438   phenotypic plasticity, which is crucial for colonisation success (Stürzenbaum et al. 2009).

439   Assuming this hypothesis, *P. corethrurus* response to temperate environments (Ortíz-Gamino et

440   al. 2016) may be sustained by asexual reproduction, polyploidy and epigenetic control.

441

442   **5. Conclusion**

443   Over the last hundred years *P. corethrurus* has been shown to be involved with human

444   disturbance in many regions worldwide. We exposed the capability of ISSR to show high genetic

445   diversity in four populations of the invasive earthworm *P. corethrurus* along an altitudinal

446   gradient in tropical areas. These results revealed the existence of at least two well-differentiated

447   genetic clusters, potentially corresponding to different clonal lineages. Continued genotyping of

448   *P. corethrurus* individuals from same and other populations will be necessary to confirm the

449   results of genotype diversity, to track the movement and diversification of the lineages and to

450   identify new dominant genotypes or newly introduced lineages. Our evidence spotlight the

451   necessity of more studies about the ecology of the invasive species, because its establishment is

452   so complex, other parameters could help to understanding why *P. corethrurus* populations occur

453   in some site and not in others (Marichal et al. 2012) but we emphasize in particular about

454   reproductive behaviour of this animal, but this also applies for any peregrine species (Baker et al.

25

455 2006; González et al. 2006; Hendrix et al. 2006; Dupont et al. 2012). Furthermore, a

456 comprehensive taxonomic revision of *P. corethrurus* cryptic species complex (Cunha et al. 2014)

457 is needed in order to assess the precise taxonomic placement and rank. This would be helpful to

458 dissect the within variability found in our studied populations and precisely define any lineages

459 present.

References

469 Abbot P (2001) Individual and population variation in invertebrates revealed by Inter-simple

470 Sequence Repeats (ISSRs). Journal of Insect Science1: 8.

471 Agapow PM, Burt A (2001) Indices of multilocus linkage disequilibrium. Molecular Ecology

472 Notes 1:101-102. doi: 10.1046/j.1471-8278.2000.00014.x

473 Anderson EC, Thompson EA (2002) A model-based method for identifying species hybrids

474 using multilocus genetic data. Genetics 160: 1217-1229.

475 Avise JC, Arnold J, Ball RM, Bermingham E, Lamb V, Neigel JE, Reeb CA, Saunders NC

476 (1987) INTRASPECIFIC PHYLOGEOGRAPHY: The Mitochondrial DNA Bridge Between

477 Population Genetics and Systematics. Ann. Rev. Ecol. Syst. 18: 89-522.

478   Baker GH (1998) The ecology, management, and benefits of earthworms in agricultural soils,

479   with particular reference to southern Australia. In: Edwards CA (ed) Earthworm ecology. St

480   Lucie Press, Boca Raton, USA, pp 1229–257.

481   Baker GH, Brown G, Butt K, Curry JP, Scullion J (2006) Introduced earthworms in agricultural

482   and reclaimed land: their ecology and influences on soil properties, plant production and other

483   soil biota. Biol Invasions 8: 1301–1316. doi 10.1007/s10530-006-9024-6.

484   Barros E, Grimaldi M, Sarrazin M, Chauvel A, Mitja D, Desjardins T, Lavelle P (2004) Soil

485   physical degradation and changes in macrofaunal communities in Central Amazon. Applied Soil

486   Ecology 26:157-168.

487   Beddard FE (1912) Earthworms and Their Allies. University Press. Cambridge, UK.

488   Bernard L, Chapuis-Lardy L, Razafimbelo T, Razafindrakoto M, Pablo A-L, Legname E, …

489   Blanchart E. (2012). Endogeic earthworms shape bacterial functional communities and affect

490   organic matter mineralization in a tropical soil. The ISME Journal, 6(1), 213–222.

491   Bengtsson BO (2009) Asex and Evolution: A very Large-Scale Overview. In Schön I, Martens K

492   and Van Dijk P (eds) Lost Sex: The Evolutionary Biology of Parthenogenesis. New York.

493   Brown GG, Fragoso C, Barois I, Rojas P, Patrón JC, Bueno J, Moreno AG, Lavelle P, Ordaz V,

494   Rodríguez C (2001) Diversidad y rol funcional de la Macrofauna edáfica en los ecosistemas

495   tropicales mexicanos. Acta Zoológica Mexicana (n.s) 1: 79-110.

496   Brown AHD, Feldman MW, Nevo E (1980) Multilocus structure of natural populations of

497   *Hordeum spontaneum*. Genetics 96 (2): 523-536.

498   Brown GG, James SW, Pasini A, Nunes DH, Benito NP, Trigo P, Sautter KD (2006) Exotic,

499   Peregrine, and Invasive Earthworms in Brazil: Diversity, Distribution, and Effects on Soils and

500   Plants. Caribbean Journal of Science 42: 339-358.

27

501 Brown GG, Moreno A, Barois I, Fragoso C, Rojas P, Hernández B, Patrón JC (2004) Soil

502 macrofauna in SE Mexican pastures and the effect of conversion from native to introduced

503 pastures. Agriculture, Ecosystems & Environment 103: 313-327.

504 Buch A, Brown GG, Niva CC, Sautter KD, Lourençato LF (2011). Life cycle of *Pontoscolex*

505 *corethrurus* (Müller, 1857) in tropical artificial soil. Pedobiologia 54: 19-25.

506 Buczkowska K, Rabska M, Gonera P, Pawlaczyk EM, Wawrzyniak P, Czolpinska M,

507 Bączkiewicz A (2016). Effectiveness of ISSR markers for determination of the *Aneura pinguis*

508 cryptic species and *Aneura maxima*. Biochemical Systematics and Ecology 68: 27-35.

509 Cameron E, Bayne EM, Coltman DW (2008) Genetic structure of invasive earthworms

510 *Dendrobaena octaedra* in the boreal forest of Alberta: insights into introduction mechanisms.

511 Molecular Ecology 17: 1189-1197.

512 Cameron EK, Bayne EM (2009) Road age and its importance in earthworm invasion of northern

513 boreal forest. Journal of Applied Ecology 46: 28-36.

514 Cicconardi F, Borges PA, Strasberg D, Oromí P, López P, Pérez-Delgado H, Casquet

515 AJ, Caujapé-Castells J, Fernández-Palacios J, Thébaud B, Emerson BC (2016) MtDNA

516 metagenomics reveals large-scale invasion of belowground arthropod communities by

517 introduced species. Molecular Ecology. doi: 10.1111/mec.14037.

518 Cuatrecasas J (1964) Cacao and its allies: a taxonomic revision of the genus Theobroma. Contri

519 US Herbarium 35:379-614.

520 Cunha L, Montiel R, Novo M, Orozco-terWengel P, Rodrigues A, Morgan AJ, Kille P (2014)

521 Living on a volcano's edge: genetic isolation of an extremophile terrestrial metazoan. Heredity,

522 1-11.

28

523   Craven D, Thakur MP, Cameron EK, Frelich LE, Beauséjour R, Blair RB, Blossey B, Burtis

524   J, Choi A, Dávalos A, Fahey TJ, Fisichelli NA, Gibson K, Handa IT, Hopfensperger K, Loss

525   SR, Nuzzo V, Maerz JC, Sackett T, Scharenbroch BC, Smith SM, Vellend M, Umek

526   LG, Eisenhauer N (2016) The unseen invaders: introduced earthworms as drivers of change in

527   plant communities in North American forests (a meta-analysis). Global change biology 23 (3):

528   1065-1074.

529   Domínguez J, Aira M, Gómez-Brandón M (2009) El papel de las lombrices de tierra en la

530   descomposición de la materia orgánica y el ciclo de nutrientes. Ecosistemas 18: 20-31.

531   Du FX, Clutter AC, Lohuis MM (2007) Characterizing Linkage Disequilibrium in Pig

532   Populations. Int J Biol Sci. 3(3): 166-178. doi:10.7150/ijbs.3.166.

533   Dupont L, Decaëns T, Lapied E, Chassany V, Marichal R, Dubs F, Maillot M, Ro V (2012)

534   Genetic signature of accidental transfer of the peregrine earthworm *Pontoscolex corethrurus*

535   (Clitellata, Glossoscolecidae) in French Guiana. European Journal of Soil Biology 53: 70-75.

536   Dušinský R, Kúdela M, Stloukalová V, Jedlička L (2006) Use of inter-simple sequence repeat

537   (ISSR) markers for discrimination between and within species of blackflies (Diptera:

538   Simuliidae). Biologia 61(3): 299-304.

539   Eisenhauer N, Stefanski A, Fisichelli NA, Rice K, Rich R, Reich PB (2014) Warming shifts

540   'worming': effects of experimental warming on invasive earthworms in northern North America.

541   Scientific Reports 4:6890. doi: 10.1038/srep06890.

542   Eisen G (1900) Researches in the American Oligocheta, with special reference to those of the

543   Pacific coast and adjacent islands. Proceedings of California Academy of Science. 2:85–276

544   Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the

545   software STRUCTURE: a simulation study. Molecular ecology 14(8):2611-2620.

546 Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform

547 population genetics analyses under Linux and Windows. Molecular ecology resources 10(3):

548 564-567.

549 Feijoo A, Zúñiga M, Quintero H, Lavelle P (2007) Relaciones entre el uso de la tierra y las

550 comunidades de lombrices en la cuenca del río La Vieja, Colombia. Pastos y Forrajes 30(2).

551 Fernández R, Almodóvar A, Novo M, Gutiérrez M, Díaz Cosín DJ (2011) A vagrant clone in a

552 peregrine species: Phylogeography, high clonal diversity and geographical distribution in the

553 earthworm *Aporrectodea trapezoides* (Dugès, 1828). Soil Biology & Biochemistry xxx:1-9.

554 Fernández R, Almodóvar A, Novo M, Simancas B, Díaz Cosín DJ (2012) Adding complexity to

555 the complex: New insights into the phylogeny, diversification and origin of parthenogenesis in

556 the *Aporrectodea caliginosa* species complex (Oligochaeta, Lumbricidae). Molecular

557 Phylogenetics and Evolution. 64:368–379.

558 Fernández R, Almodóvar A, Novo M, Gutiérrez M, Díaz Cosin DJ (2013) Earthworms, good

559 indicators for palaeogeographical studies? Testing the genetic structure and demographic history

560 in the peregrine earthworm *Aporrectodea* trapezoides (Dugès, 1828) in southern Europe. Soil

561 Biology & Biochemistry. 58:127-135.

562 Fragoso C, Coria-Martínez ML, Camarena LM (2009) An update of the earthworm fauna of Los

563 Tuxtlas Ver. and adjacent regions: are native species in the risk of extinction? In: Barois I,

564 Huising EJ, Okoth P, Trejo D, De los Santos M (eds) Below-Ground Biodiversity in Sierra San

565 Marta, Los Tuxtlas, Veracruz, México. Instituto de Ecología, A.C., Xalapa, 219–228.

566 Fragoso C, Rojas P (2014) Biodiversidad de lombrices de tierra (Annelida: Oligochaeta:

567 Crassiclitellata). Revista Mexicana de Biodiversidad, Supl. 85:S197-S207.

568   Gates GE (1954) Exotic earthworms of the United States. Bulletin of the Museum of

569   Comparative Zoology at Harvard College. 111: 217-258.

570   Gates GE (1973) Contributions to North American Earthworms (Annelida) No. 6 Contributions

571   to a revision of the earthworm family Glossoscolecidae. I. *Pontoscolex corethrurus* (Müller,

572   1857). Bulletin of Tall Timbers research Station 14:1-12.

573   Gramaje D, León M, Santana M, Crous PW, Armengol J (2014) Multilocus ISSR Markers

574   Reveal Two Major Genetic Groups in Spanish and South African Populations of the Grapevine

575   Fungal Pathogen *Cadophora luteo-olivacea.* PLOS one.

576   Geissen V, Peña-Peña K, Huerta E (2009) Effects of different land use on soil chemical

577   properties, decomposition rate and earthworm communities in tropical Mexico. Pedobiologia 53:

578   75–86. doi: 10.1016/j.pedobi.2009.03.004

579   Giska I, Sechi P, Babik W (2015) Deeply divergent sympatric mitochondrial lineages of the

580   earthworm *Lumbricus rubellus* are not reproductively isolated. *BMC evolutionary*

581   *biology* 15(217).

582   González, G., Huang, C.Y., Zou, X., Rodríguez, C (2006). Earthworm invasions in the tropics.

583   Biological Invasions. 8,1247–1256. doi: 10.1007/s10530-006-9023-7.

584   Gutiérrez MC, Cardona CM (2014) Caracterización ecológica de las lombrices (*Pontoscolex*

585   *corethrurus*) como bioindicadoras de suelos compactados bajo condiciones de alta humedad del

586   suelo con diferentes coberturas vegetales (Zipacón, Cundinamarca). REVISTA CIENTÍFICA.

587   2(19).

588   Grünwald NJ, Goodwin SB, Milgroom MG, Fry WE (2003) Analysis of genotypic diversity data

589   for populations of microorganisms. Phytopathology. 93:738-746.

31

590   Hagenblad J, Bechsgaard J, Charlesworth D (2006) Linkage Disequilibrium Between

591   Incompatibility Locus Region Genes in the Plant Arabidopsis lyrata. Genetics, 173(2), 1057–

592   1073.

593   Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis

594   program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41:95-98.

595   Heethoff M, Etzold K, Scheu S (2004) Mitochondrial COII sequences indicate that the

596   parthenogenetic earthworm *Octolasion tyrtaeum* (Savigny 1826) constitutes of two lineages

597   differing in body size and genotype. Pedobiologia. 48:9–13.

598   Hebert P, Penton EH, Burns JM, Janzen DH, Hallwach W (2004) Ten species in one: DNA

599   barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*.

600   PNAS,101.41.

601   Hendrix PF (2006) Biological invasions belowground—earthworms as invasive species.

602   Biological Invasions Belowground: Earthworms as Invasive Species. Springer Netherlands, 1-4.

603   Hendrix PF, Bohlen PJ (2002) Exotic Earthworm Invasions in North America: Ecological and

604   Policy Implications Expanding global commerce may be increasing the likelihood of exotic

605   earthworm invasions, which could have negative implications for soil processes, other animal

606   and plant species, and importation of certain pathogens. Bioscience 52(9): 801-811.

607   Hendrix P, Baker G, Callaham M, Damof G, Fragoso C, Gonzalez G, James S, Lachnicht S,

608   Winsome T, Zou X (2006) Invasion of exotic earthworm into inhabited by native earthworm.

609   Biological Invasions 8:1287-1300.

610   Hendrix PF, Callaham Jr.MA, Drake JM, Huang ChY, James SW, Snyder BA, Zhang W (2008)

611   Pandora's box contained bait: the global problem of introduced earthworm. Ann.Rev. Ecol. Evol.

612   S. 39:593-613.

613   Hernández-Castellanos B, Zavala-Cruz J, Martínez-Hernández S, Dendooven L, Contreras-

614   Ramos SM, Noa-Carrazana JC, Fragoso C, Ortíz-Ceballos AI (2013) Earthworm Populations in

615   an Aged Hydrocarbon Contaminated Soil. Research Journal of Environmental Sciences. 7(1):27-

616   37. doi: 10.3923/rjes.2013.27.37

617   Huerta E, Van der Wal H (2012) Soil macroinvertebrates' abundance and diversity in home

618   gardens in Tabasco, Mexico, vary with soil texture, organic matter and vegetation cover.

619   European Journal of Soil Science 50:68–75. doi: 10.1016/j.ejsobi.2011.12.007.

620   James SW (2012) Re-erection of Rhinodrilidae Benham, 1890, a senior synonym of

621   Pontoscolecidae James, 2012 (Annelida: Clitellata). Zootaxa. 3540: 67-68.

622   Janzen DH (1967) Why Mountain Passes are Higher in the Tropics? The American Naturalist.

623   101 (919):233-249.

624   Jiménez JJ, Decaëns T (2000) Vertical distribution of earthworms in grassland soils of the

625   Colombian Llanos. Biol. Fertil. Soils. 32:463–473.

626   Jombar T, Devillard S, Balloux F (2010) Discriminant analysis of principal components: a new

627   method for the analysis of genetically structured populations. BMC genetics 11(1):94.

628   Kamvar ZN, Tabima JF, Grünwald NJ (2014) Poppr: an R package for genetic analysis of

629   populations with clonal, partially clonal, and/or sexual reproduction. PeerJ 2: 281.

630   King RA, Tibble AL, Symondson WOC (2008) Opening a can of worms: unprecedented

631   sympatric speciation within British lumbricid earthworms. Mol. Ecol. 17:4684–4698.

632   Knott K, Haimi J (2010) High mitochondrial DNA sequence diversity in the parthenogenetic

633   earthworm *Dendrobaena octaedra*. Heredity. 105:341-347.

634   Lapied E, Lavelle P (2003) The peregrine earthworm *Pontoscolex corethrurus* in the East coast

635   of Costa Rica. Pedobiologia 47:471–474.

33

636 Lavelle P, Barois I, Cruz I, Fragoso C, Hernández A, Pineda A, Rangel P (1987) Adaptative

637 strategies of *Pontoscolex corethrurus* (Glossoscolecidae, Oligocheta), a peregrine geophagous

638 earthworm of the humid tropics. Biol Fertil Soils 5:188-194.

639 Lavelle P, Bignell D, Lepage M, Wolters V, Roger P, Ineson P, Heal OW, Dhillion S (1997) Soil

640 Function in a changing world: the role of invertebrate ecosystem engineers. Eur. J. Soil 33:159-

641 193.

642 Luan S, Chiang T-Y, Gong X (2006) High Genetic Diversity vs. Low Genetic Differentiation

643 in *Nouelia insignis* (Asteraceae), a Narrowly Distributed and Endemic Species in China,

644 Revealed by ISSR Fingerprinting. Annals of Botany. 98(3):583–589.

645 Ludwig JA, Reynolds JF (1988) Statistical ecology: a primer in methods and computing (Vol. 1).

646 John Wiley & Sons.

647 Narayanan SP, Sathrumithra S, Christopher G, Thomas AP, Julka JM (2016) Current distribution

648 of the invasive earthworm *Pontoscolex corethrurus* (Müller, 1857) after a century of its first

649 report from Kerala state, India. Opusc. Zool. Budapest. 47(1):101–107.

650 Ng WL, Tan SG (2015) Inter-Simple Sequence Repeat (ISSR) Markers: Are We Doing It Right?

651 ASM Sci. J., 9(1):30–39.

652 Marichal R, Grimaldi M, Mathieu J, Brown GG, Desjardins T, Lopes da Silva JrM, Praxedes C,

653 Martins MB, Velasquez E (2012) Is invasion of deforested Amazonia by earthworm *Pontoscolex*

654 *corethrurus* driven by soil texture and chemical properties? Pedobiologia, S5:233–240. doi:

655 10.1016/j.pedobi.2012.03.006.

656 Mantel N (1967) The detection of disease clustering and a generalized regression approach.

657 Cancer Res. 27: 209–220.

34

658   Marinissen JCY, Van den Bosch F (1992) Colonization of new habitats by earthworms.

659   Oecologia, 91: 371–376.

660   Miller MP (1997) Tools for Population Genetic Analyses (TFPGA) V.1.3. A Windows Program

661   for the Analysis of Allozyme and Molecular Population Genetic Data. Utah State University:

662   Logan, UT, USA.

663   Moreno AG, Borges S (2004) Avances en taxonomía de lombrices de tierra (Annelida:

664   Oligochaeta). Editorial Complutense. Madrid, España.

665   Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of

666   individuals. Genetics. 89(3):583-590.

667   Novo M, Almodóvar A, Fernández R, Trigo D, Díaz Cosín DJ (2010) Cryptic speciation of

668   hormogastrid earthworms revealed by mitocondrial and nuclear data. Molecular Phylogenetics

669   and Evolution 56: 507–512.

670   Ortiz-Gamino D, Pérez-Rodríguez P, Ortiz-Ceballos AI (2016) Invasion of the tropical

671   earthworm *Pontoscolex corethrurus* (Rhinodrilidae, Oligochaeta) in temperate grasslands. PeerJ.

672   4, e2572. doi.org/10.7717/peerj.2572

673   Oksanen J, Kindt R, Legendre P, O'Hara B, Stevens MH (2007) The vegan package. Community

674   ecology package. 10:631-637.

675   Pashanasi B, Lavelle P, Alegre J (1994) Efecto de las lombrices de tierra (*Pontoscolex*

676   *corethrurus*) sobre el crecimiento de cultivos anuales y características físicas y químicas en

677   suelos Yurimaguas. Folia Amazónica. 6:5-45.

678   Pashanasi B, Melendez G, Szott L, Lavelle P (1992) Effect of inoculation with the endogeic

679   earthworm *Pontoscolex corethrurus* (Glossoscolecidae) on N availability, soil microbial biomass

35

680   and the growth of three tropical fruit seedlings in pot experiment. Soil Biol. Biochem. 24:1655-

681   1659.

682   Pavlíček T, Cohen T, Yadav S, Glasstetter M, Král P, Pearlson O (2016) Aneuploidy Occurrence

683   in Oligochaeta. Ecology and Evolutionary Biology. 1(3):57-63

684   Peakall ROD, Smouse PE (2006) GENALEX 6: Genetic analysis in Excel. Population genetic

685   software for teaching and research. Mol. Ecol. Notes. 6:288–295.

686   Pielou EC (1975) Ecological diversity. Wiley-Interscience publication. New York. 165.

687   Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus

688   genotype data. Genetics.155(2):945-959.

689   Regev A, Lamb MJ, Jablonka E (1998) The role of DNA methylation in invertebrates:

690   developmental regulation or genome defence? Mol. Biol. Evol. 15:880–891.

691   Righi G (1984) Pontoscolex (Oligochaeta, Glossoscolecidae), a new evaluation. Studies on

692   Neotropical Fauna and Environment 19(3): 159-177.

693   R Core Team (2014) R: A language and environment for statistical computing. R Foundation for

694   Statistical Computing, Vienna, Austria. Available: http://www.R-project.org/.

695   Schultes RE (1984) Amazonian cultigens and their northward and westward migrations in

696   preColumbian times. In: Stone D (ed) Pre-Columbian plant migration, Papers of the Peabody

697   Museum if Archaeology and Ethnology 76 Harvard University Press. pp 69-83.

698   Seyedimoradi H,Talebi R, (2014). Detecting DNA polymorphism and genetic diversity in Lentil

699   (Lens culinaris Medik.) germplasm: comparison of ISSR and DAMD marker. Physiology and

700   Molecular Biology of Plants, 20(4), 495–500.

701   Smith JM, Smith NH, O'Rourke M, Spratt BG (1993) How clonal are bacteria? Proceedings of

702   the National Academy of Sciences 90(10): 4384-4388.

36

703   Sharma A, Sonah H, Deshmukh RK, Gupta NK, Singh NK, Sharma TR (2011) Analysis of

704   Genetic Diversity in Earthworms Using DNA Markers. Zoological Science. 28:25–31.

705   Stoddart JA, Taylor JF (1988) Genotypic diversity: estimation and prediction in samples.

706   Genetics. 118 (4):705-711.

707   Stürzenbaum SR, Andre J, Kille P, Morgan AJ (2009) Earthworm genomes, genes and proteins:

708   the   (re)discovery   of   Darwin's   worms.   Proc.   R.   Soc.   B.   276,   789–797

709   doi:10.1098/rspb.2008.1510.

710   Suzuki R, Shimodaira H (2006) Pvclust: an R package for assessing the uncertainty in

711   hierarchical clustering. Bioinformatics 22:1540–1542.

712   Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary

713   Genetics Analysis version 6.0. Molecular Biology and Evolution, 30: 2725-2729.

714   Terhivuo J, Saura A (1993) Clonal and morphological variation in marginal populations of

715   parthenogenetic earthworms *Octolasion tyrtaeum* and *O. cyaneum* (Oligochaeta, Lumbricidae)

716   from eastern Fennoscandia. Boll. Zool. 60: 87-96.

717   Terhivuo J, Saura A (2006) Dispersal and clonal diversity of North-European parthenogenetic

718   earthworms. Biol Invasions. 8:1205–1218. doi 10.1007/s10530-006-9015-7.

719   Uribe S, Huerta E, Geissen V, Mendoza M, Godoy R, Jarquin A   (2012)   *Pontoscolex*

720   *corethrurus* (Annelida: Oligocheta) indicador de la calidad del suelo en sitios de *Eucalyptus*

721   *grandis* (Myrtacea) con manejo tumba y quema. Revista de Biología Tropical, 60:1543–552. doi:

722   10.15517/rbt.v60i4.2072.

723   Vitturi R, Colomba MS, Pirrone AM, Mandrioli M (2002) rDNA (18S–28S and 5S)

724   colocalization and linkage between ribosomal genes and (TTAGGG)(n) telomeric sequence in

37

725   the earthworm, *Octodrilus complanatus* (Annelida: Oligochaeta: Lumbricidae), revealed by

726   single-and double-color FISH. Journal of Heredity 93(4):279-282.

727   Wolfe AD, Liston A (1998) Contributions of PCR-based methods to plant systematics and

728   evolutionary biology. In: Soltis DE, Soltis PS, Doyle JJ (eds). Plant Molecular Systematics II.

729   Kluwer Academic Press, Dordrecht, The Netherlands. Pp 43–86.

730   Zavala-Cruz J, Trujillo-Capistrán F, Ortiz-Ceballos G, Ortiz-Ceballos AI (2013) Tropical

731   endogeic earthworm population in a pollution gradient with weathered crude oil. Res. J. Environ.

732   Sci. 7:15-26.

733

734

735

736