Genetic diversity of an invasive earthworm, Lumbricus terrestris, *at a long-term trading crossroad, the Champlain Valley of Vermont, USA*

Erin L. Keller, Samantha T. Connolly, Josef H. Görres & Jos. J. Schall

Biological Invasions

ISSN 1387-3547
Volume 22
Number 5

Biol Invasions (2020) 22:1723-1735
DOI 10.1007/s10530-020-02215-7

Springer

Biol Invasions (2020) 22:1723–1735
https://doi.org/10.1007/s10530-020-02215-7

ORIGINAL PAPER

Genetic diversity of an invasive earthworm, *Lumbricus terrestris*, at a long-term trading crossroad, the Champlain Valley of Vermont, USA

Erin L. Keller · Samantha T. Connolly · Josef H. Görres · Jos. J. Schall

Received: 23 January 2019 / Accepted: 6 February 2020 / Published online: 12 February 2020

Abstract The invasive European earthworm, *Lumbricus terrestris*, is now widely distributed in North America. This success may result from high genetic diversity derived from multiple introductions from founder sources across Europe. Using a mitochondrial gene (COI) and microsatellite markers, *L. terrestris* from seven sites in the Champlain Valley of Vermont USA were scored for genetic diversity and population structure. This region has been a trading crossroads for centuries, thus likely to have received earthworms from multiple origins. COI sequences matched those reported for *L. terrestris* from Scotland, France, Austria, Denmark, Sweden, and Norway, and 2–5 haplotypes were found at each site. Genetic diversity (microsatellites) was great for each site, but not notably greater than for earthworm populations in general, possibly because there may be allele size homoplasy, or some restriction in the number of alleles possible at any locus. The earthworms were genetically differentiated among the Vermont study sites, even those 0.6–13 km distant. These results support the view that *L. terrestris* is a successful invasive earthworm because multiple introductions provided ample genetic variation for natural selection and local differentiation among locations in North America. Last, a large number of microsatellite markers is provided, including suggested PCR programs, for free use by future researchers.

Keywords *Lumbricus terrestris* · Genetic diversity · COI · Microsatellites · Multiple introductions

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10530-020-02215-7) contains supplementary material, which is available to authorized users.

E. L. Keller
School of Biological Sciences, Washington State University, Pullman, WA 99163, USA

S. T. Connolly
Department of Plant Biology, University of Vermont, Burlington, VT 05405, USA

J. H. Görres
Department of Plant and Soil Science, University of Vermont, Burlington, VT 05405, USA

Jos. J. Schall (✉)
Department of Biology, University of Vermont, Burlington, VT 05405, USA
e-mail: jschall@uvm.edu

Introduction

Invasive species are of great economic and conservation significance, but also provide insight into an ongoing natural, but unintended, experiment in biogeography. Alfred Wallace, the founder of the scientific study of biogeography (Wallace 1876), was particularly interested in long-distance introductions

onto islands (Wallace 1911), asking a question that is now a focus in the study of invasions. He asked, how do incomers enter an already established complex community? In modern terms, what characteristics of a species, especially its life history traits and genetic diversity, allow it to invade a complex community that may resist a species naïve to local conditions? For example, each invasion event opens a window into a central paradox: invasive species could originate as a small propagule and thus should suffer a demographic challenge (small populations face a high chance of being lost) and a genetic challenge of low genetic diversity, resulting inbreeding, and reduced ability to respond to selection when facing novel environments (Allendorf and Lundquist 2003; Kirk et al. 2013). Facing these odds, how do the incomers persist?

Roman and Darling (2007) reviewed aquatic invasions by small organisms and find no such paradox. For aquatic invasions, propagule pressure is great, both in number of incomers and invasive events. Genetic diversity is thus likely to be high, and perhaps even greater than the home region if propagules originate from multiple geographic regions. For larger organisms, Elton (1958) presents examples of very small propagule numbers that have established successful invasions (Japanese Beetle, *Popillia japonica*, European Starling, *Sturnus vulgaris*, and Gipsy Moth, *Lymantria dispar*, in NA, and African Giant Snail, *Achatina fulica*, in Hawaii). Mergeay et al. (2006) present an elegant study of a single clone of *Daphnia* that has invaded many freshwater African lakes in the past 70 years, displacing existing, and genetically diverse, *Daphnia* species. Likewise, a single clonal New Zealand snail, *Potamopyrgus antipodarum*, has spread over a large region in western North America (Dybdahl and Drown 2011). Thus, the interplay between propagule pressure (both number of individuals and number of founder sites), genetic diversity, and adaptation remains an open question in invasion biology (Sakai et al. 2001; Kirk et al. 2013).

We focus on the issue of genetic diversity in an invasive earthworm, *Lumbricus terrestris*, in a small spatial region in NE USA. The earthworms of central North America (northern USA and southern Canada) provide a model system to study how a species can enter an already well-established biotic community, become established, and even alter the environment in its favor. Glaciation presumably extirpated earthworms and all other soil organisms in these regions (except perhaps for extremophile microbes), so after the last glacial retreat ($\sim$ 12,000 ya) soils lacked earthworms. Earthworms in the glaciated regions of North America include a few species that have reentered from the south (such as *Eisenoides lonnbergi*, McCay et al. 2017), but most are invasive species, first from Europe beginning in the early 17th century (Tiunov et al. 2006) and more recently from Asia in the horticultural trade (Görres and Melnichuk 2012). The first literature record of *L. terrestris* in North America is Eisen (1872), but most likely the earthworm came centuries earlier in trade in grains and livestock, and is now known from 38 US states and 10 Canadian provinces (Gates 1976; Reynolds 2008). *L. terrestris* continues as an invasive taxon in Europe as well; in Romania it has moved into isolated areas and appears to have extirpated native earthworm species (Pop and Pop 2006). The species is now known far from its European source including Australia, New Zealand, and India (range map shown at https://www.cabi.org/isc/datasheet/109385). Thus, *L. terrestris* is a notably successful invasive species. Despite this broad range in Europe and now North America, and the role of *L. terrestris* as an important ecosystem engineer, surprisingly little has been reported on the population genetic structure of this species: these studies are from mainland Europe (Kautenburger 2006a, b; Richter 2009; Velavan et al. 2009), the Faroe Islands (Enckell et al. 1986), North America (Klein et al. 2017; Velavan et al. 2007), and comparisons between Europe and North America (Gailing et al. 2012; Porco et al. 2013).

We examined *L. terrestris* at a small region in Vermont, USA, but one that has been the crossroads of trade for three centuries (see Albers [2000] for a review of historical geography of Vermont). Earthworm's eggs are encapsulated in "cocoons", which are resistant to desiccation, freezing, and other injury (Holmstrup and Westh 1994; Meshcheryakova and Berman 2014; Nouri-Aiin and Görres 2019). *L. terrestris* produces 3–4 cocoons per month (Butt et al. 1992). Thus, cocoons could readily move in bulk loads of agricultural product, even in dry or cold storage conditions, and propagule pressure would be substantial. More recently, Canadian populations have been exploited in the fishing bait trade which moves the earthworms over long distances (Tomlin 1983).

We asked two broad questions. First, were there multiple introductions of *L. terrestris* earthworms into this economically busy small region in Vermont from

different source regions of Europe? We sequenced the "barcoding" region of the mitochondrial cytochrome oxidase (COI) gene (Folmer et al. 1994), and compared results with sequences deposited in Genbank. The earthworms, though, have likely been moved about in Europe by both natural movements and human activities since Neolithic times, but today's locations should reflect occupation for centuries (Richter 2009). These data would indicate if the propagule pressure included multiple origins for even a small recipient region. Second, is the genetic diversity high within sites and is there geographic genetic structure among sites only meters to several km distant? Microsatellite markers were used to assess genetic diversity of the earthworms. If the earthworms originated from different regions in Europe, then the microsatellite allele diversity should be exceptionally high both in number of alleles and the range in allele lengths. Although the common name in North America of *L. terrestris*, the "nightcrawler", suggests it is a mobile species, in fact individuals build deep ($\sim$ 2.5 m) burrows and remain loyal to their homesites, anchoring with distinctive flat terminal body segments and moving only short distances (Nuutinen and Butt 1997, 2005). Thus, this behavior could lead to local inbreeding, and even fine-scale genetic differentiation among sites. Using the microsatellite markers to measure the genetic diversity within and among sites, we sought evidence of local inbreeding, and to determine if there is geographic genetic structure that could allow local adaptation. These data thus provide a resolution for the "genetic paradox", namely that multiple introductions into local sites provided high genetic variation, even higher than any single site in the source populations, for an important invasive species of North American soils. Last, we provide a large library of microsatellite markers for use by other researchers, including suggested PCR programs for each.

Methods

Collection sites and collecting methods

We sampled earthworms at seven sites, three at a remnant woodland on the University of Vermont campus (37–88 m separation); three in wooded areas at the Shelburne Farms, a display and research farm (683–2102 m distant); and one at the East Woods Natural Area, a wooded area managed by the University. These three areas were separated from 3 to 13 km. GPS coordinates and codes for the sites are given in Table 1. Historic and current use ranged from long-term farming and cattle raising for $>$ 100 years (SF sites), repeated logging until $\sim$ 75 ya (EW), and sheep grazing ($\sim$ 100 ya) followed by fallow woodlot (WM). The canopy of the current forest at all sites is dominated by sugar maple (*Acer saccharum*, Marshall). Thirteen collection days spanned early June to early August. Earthworms were located by searching for their burrows indicated by prominent excavation mounds, which comprise both mineral soil and vegetation, and plugged with tuffs of grass and leaves in the daytime (termed middens). Earthworms were flushed from burrows with a water-mustard solution funneled into the burrow (Gunn 1992). Upon emergence of a worm, it was immediately washed in water, and then stored in boxes of commercial earthworm bedding. Collections at each site covered $\sim$ 2–4 m^2. A morphologically indistinguishable species, *L. herculeus*, is widespread in Europe, so earthworms in our sample were confirmed as *L. terrestris* by the COI barcoding gene (James et al. 2010). Earthworms were collected with permission of relevant land managers.

Extracting DNA

Each earthworm was washed in water, then killed in 50% ethanol, washed in dH_2O, then the seminal vesicle (the organ of self-sperm storage) was extracted and frozen at $-20°$. That organ provided high density and quality DNA. Tissue DNA was extracted using the Qiagen (Valencia, USA) DNeasy kit and the kit's protocol and DNA stored at 4°.

COI sequences

The mitochondrial COI gene was amplified using standard barcoding primers (Folmer et al. 1994: LCO1490 GGT CAA CAA ATC ATA AAG ATA TTG G and HCO2198 TAA ACT TCA GGG TGA CCA AAA AAT CA, with PCR program 94° for 1 min, followed by 32 cycles of 94° for 1 min, 49° for 30 s, 72° for 1 min, and a final extension of 72° for 2 min. The resulting 710 bp amplicon was Sanger sequenced at the Yale University Keck genomics center using the ABI 3730xL analyzer with ABI Big

Table 1 Location of study sites used in collecting *Lumbricus terrestris* earthworms. Also indicated are genetic data for the earthworms at each site, COI (COI gene for barcoding) and MS (microsatellites). All worms used for the COI analysis were included for the MS study

Site	Site code	GPS (N, W)	Genetic
Shelburne Farms Entrance	SFEN	44.391512	COI
		−73.251089	MS
Shelburne Farms	SFCB	44.389529	COI
Coach Barn		−73.277435	MS
Shelburne Farms	SFAB	44.392006	COI
Animal Barn		−73.259495	
Wind Mill	WM1	44.47301	COI
		−73.191398	
	WM2	44.473982	COI
		−73.191442	MS
	WM4	44.474767	COI
		−73.191166	MS
East Woods	EW	44.447524	COI
		−73.198777	MS

Dye chemistry. All sequences reported here were taken from electropherograms that had clear, unambiguous peaks for all nucleotides. COI sequences were aligned using CLUSTALX (Larkin et al. 2007), to inspect quality, and then submitted to a BLAST search for matches to sequences in the Genbank data set (NCBI, Arlington, USA). A neighbor-joining gene tree was constructed for haplotypes recovered using CLUSTALX and Geneious Prime (www.geneious.com) programs.

Microsatellite markers

Previously published microsatellite primers (Velavan et al. 2007) did not amplify well for our samples. Other workers similarly had difficulty in using these primers perhaps because of the genetic variation among *L. terrestris* populations (Gailing et al. 2012; Souleman et al. 2016). Therefore, we identified new markers for use in this study. DNA of high molecular weight, concentration, and purity extracted from a single SFAB *L. terrestris* was submitted to the Cornell University Evolutionary Genetics Core Facility (Ithaca, New York). There using the method of Hamilton et al. (1999) DNA was restriction digested and enriched with a panel of 16 probes with variable repeat motifs, and fragments sequenced using the Titanium 454 platform (454 Life Sciences, Branford, USA).

Microsatellite markers were amplified with forward primers fluorescently labeled with 6FAM dye (Integrated DNA Technologies, Coralville, USA) and the Qiagen TopTaq mix. A negative control was used for each PCR run. PCR product was diluted, and added with LIZ500 size standard to Hi-Di formamide (Life Technologies, Foster City, USA). The samples were then processed on the 3730xL Genetic Analyzer (ThermoFisher Scientific, Wilmington, USA) at the Cornell University Core Laboratory. Pherograms were inspected using PeakScanner 1.0 software (Applied Biosystems, Coralville, USA) to score microsatellite alleles.

Microsatellite scoring

Microsatellite scoring errors can give spurious results for population genetics studies (review in Pompanon et al. 2005). To reduce such errors we first explored $\sim$ 50 microsatellite markers, choosing those of 3 or 4 base motifs to reduce scoring errors due to stutter. A panel of five markers was chosen that amplified well, with no failed amplification (reducing possibility of null alleles as noted in Results), with minimal stutter, variable across earthworms, and produced clear, unambiguous peaks on the electropherograms to score. Details on primers, PCR program, and GenBank accession numbers for the microsatellite markers are given in Online Resource 1. All PCR programs include a long final extension to eliminate problems due to +A additions to the amplicons. A sample of earthworms (N = 20) were processed a second time to test for reproducibility of the results; all resulted in identical

allele scores. Genetic linkage across markers was tested with the GENEPOP online version (Rousset 2008), null alleles with MICRO-CHECKER (Van Oosterhout et al. 2004), and errors in calling alleles that could be due to stutter or large allele drop-out with MICRO-CHECKER.

Summary measures of genetic diversity for each microsatellite locus was calculated with GenAlEx (v6.501) (Peakall and Smouse 2012) to yield number of alleles (Na), the unbiased expected heterozygosity (uHe), the observed heterozygosity (Ho), and Shannon's Information Index (I). If the earthworms originated from multiple sites in Europe we expect a large number of alleles for each locus. Also, we calculated the range in number of repeats, expecting a wide range for worms of multiple origins.

If *L. terrestris* mates very locally, we expected evidence of inbreeding. First, the observed and expected heterozygosity were inspected. Expected heterozygosity is calculated on the observed proportions of each allele. Inbreeding should result in a reduced observed heterozygosity compared to expected under random association of alleles. Second, a formal measure of inbreeding (F) was calculated. Last, inbreeding in the sessile worms should result in deviation from Hardy–Weinberg expected proportions. This was tested with a Markov-chain method in GENEPOP (Rousset 2008), using a Bonferonni correction for multiple comparisons.

Population structure across sites was assessed with several methods (GenAlEx platform). First, we calculated number of private alleles for each locus (alleles found at only a single site), Nei's Unbiased Genetic Distance (a measure of genetic differentiation), Fst (the proportion of overall genetic diversity accounted for by site with values ranging from 0 for an overall panmixia across sites to 1.0 for complete differentiation which would require private alleles at each site), and AMOVA (Analysis of Molecular Variance that also measures the proportion of genetic variation accounted for by site). Tests for significant difference from no structure were performed using a permutation method of 999 draws from the overall data. Two other measures of structure, the G'st that uses G statistics, and Shannon sH(AP) that is based on information measures, gave very similar results and thus are not shown. Second, we asked how many migrants per generation would yield measured Fst values (Wright 1951). We used Wright's method to calculate Nm, also available in the GenAlEx platform. Third, we did assignment tests that ask if an individual can be placed to a site based on genotypes at the microsatellite loci: one in a maximum likelihood framework (Paetkau et al. 2004) in GenAlEx, and a Bayesian method followed in the R package given by François (2016). This last method is more accurate in the presence of inbreeding expected for *L. terrestris* than the more often used STRUCTURE program. Last, a cross-entropy criterion sought to score the likely number of ancestral populations across the sampling sites (François 2016).

Results

Clear COI sequences were obtained for 90 worms from the seven sites, resulting in seven haplotypes that were identical to sequences already known (Genbank results). Six of these were identified in Europe, and one known only from North America (Tables 2, 3). Assuming the European haplotypes represent worm populations of long residency, the Vermont worms ultimately originated from a broad geographic region from as far north as Scandinavia, south to Austria, and west to Scotland. Each of our sites had multiple earthworm haplotypes (2–5 haplotypes) despite some small sample sizes. For example, WM1 with a sample of ten worms sequenced, showed three haplotypes, and EW with six worms sequenced resulted in two haplotypes. Genetic distance for these haplotypes were a low of 1% (A vs. B). These were previously found in Ohio, USA and Denmark, so it is possible the two haplotypes are from the same original source. The greatest distance was for haplotype G versus A, B, and F (4.5–5%). Previously James et al. (2010) found a maximum distance of 3.37% across their sampled *L. terrestris* haplotypes. A neighbor-joining gene tree for the haplotypes, plus two others identified from Europe is given in Online Resource 2, and shows minimal geographic signal for the genetic similarity of the haplotypes. Note that all of the haplotypes were found multiple times in our sample, they were identical to those reported previously, the difference across haplotypes ranged from 6 (A vs. B) to 34 (F vs. G) nucleotides, and all electropherograms were clear and unambiguous. Thus, we conclude that the haplotypes detected represent real genetic lineages in the earthworms.

Table 2 COI haplotypes identified from Vermont sites. Location was given for collection location for identical sequences reported on Genbank. Those identification numbers are given

Haplotype	Location	Genbank
A	North America (Ohio)	HQ024590.1
B	Denmark	FJ214211.1
C	Norway	KX90479.1
D	Scotland, Austria, Sweden, France (2 locations)	LT900528.1
		JN869936.1
		HQ024547.1
		FJ937306.1
		FJ937305.1
E	Sweden, France	HM388351
		KU888617
F	Sweden, France	HM388352
		KU888614
G	Norway	HQ024542

Table 3 Distribution of COI haplotypes (Table 2) across sites in Vermont. Number of earthworms given

Site	A	B	C	D	E	F	G	Site total
WM1		8				1	1	10
WM2	3	7	1			3		14
WM4	3	8	1					12
EW	4		2					6
SF AB		13				7		20
SF CB		9			2		1	12
SF EN		6	1	4	4	1		16
Haplotype total	10	51	5	4	6	12	2	

The full set of *L. terrestris* genomic fragments containing microsatellites is presented in Online Resource 3 and 4, including FASTA files, suggested PCR primers, microsatellite motif, and expected amplicon size. This information may be useful for researchers studying *L. terrestris* both in North America and Europe.

Tests for microsatellite scoring errors showed no effect of stutter or large allele drop-out. Likewise no genetic linkage was detected across the markers. Three of the markers (17,360, 82,408, 21,433) were highlighted as possible problems with null alleles by the MICRO-CHECKER algorithm. However, this is most likely driven by a heterozygosity deficit due to inbreeding (below). All of the worms amplified for all markers, and thus with no possible null/null genotypes, arguing an absence or rarity of actual nulls for the markers.

A substantial number of alleles were detected in the earthworms (Tables 4, 5). For example, even the site with smallest sample size (WM4 = 18), where a maximum of 36 alleles would be possible for a diploid organism, 8–11 alleles were noted. The expected heterozygosity was also high for all markers, indicating the alleles were fairly even in proportions. The observed heterozygosity, however, tended to be substantially lower than expected. Likewise, a measure of inbreeding (F) was found for the markers at all sites, (Table 4). These results all are expected if significant inbreeding takes place in these sessile earthworms. A second measure of allele diversity is the range in number of repeats for each locus, also shown in Table 5.

The goal here was to determine if the genetic diversity of the earthworms at our study sites is low (if propagule pressure was low, followed by genetic drift) or high (if introductions came from multiple geographic sites). This required comparisons to other populations. Table 6 compares the results with all reported studies on other populations of *L. terrestris* and other earthworm species. Results for both number of alleles and range in allele sizes were similar for these other earthworm populations.

Genetic diversity, measured as expected heterozygosity (H), can be used to estimate the breeding, or effective population size (Ne) for a population sampled from a discrete spatial patch (Neal et al. 2016), and using the equation Ne $\mu = 1/8$ {(1/

Table 4 Sample sizes for number of earthworms (N), range in number of alleles (Na) across five microsatellite markers, and number of private alleles (summed over all markers) surveyed in *Lumbricus terrestris* at five sites (site codes in Table 1). Also given are measures of genetic diversity (uHe = unbiased estimate of heterozygosity; Ho = observed heterozygosity; I = Shannon's Information Index) and a measure of inbreeding (F = inbreeding coefficient), for these, the means (SE) across the loci are given

Site	N	Na	Private	uHe	Ho	I	F
EW	46	8–11	1	0.772 (0.046)	0.483 (0.079)	1.73 (0.14)	0.359 (0.106)
WM2	27	6–10	5	0.788 (0.047)	0.558 (0.085)	1.80 (0.16)	0.266 (0.119)
WM4	18	8–11	2	0.804 (0.048)	0.641 (0.083)	1.784 (0.153)	0.164 (0.117)
SFEN	27	7–10	4	0.760 (0.059)	0.556 (0.098)	1.712 (0.150)	0.267 (0.103)
SBCB	20	7–11	3	0.838 (0.012)	0.480 (0.120)	1.927 (0.075)	0.414 (0.144)

Table 5 Summery statistics for variation of microsatellite markers in the genome of the invasive (in NA) earthworm *Lumbricus terrestris* at five sites in Vermont, USA. Na = total number of alleles (range for sites in Table 4); Size Range = range in number of repeats across all alleles (all loci with 3 base motif, except 21433 with 4 base); uHe = unbiased estimate of heterozygosity; Ho = observed heterozygosity; I = Shannon's Information Index; P (H-W) = significance for deviation from Hardy–Weinberg equilibrium with Bonferonni correction

Locus	Na	Size range	uHe	Ho	I	P (H-W)
12065	12	31	0.881	0.761	2.34	0.301
17360	14	26	0.880	0.543	2.29	< 0.001
21433	17	37	0.834	0.386	2.62	< 0.001
74661	11	11	0.746	0.645	1.77	0.754
82408	11	41	0.877	0.326	2.18	< 0.001

$[1 - H)]^2) - 1\}$. For our sites, the spatial boundaries for each population could be viewed with confidence only for the WM sites because it was surrounded by roads and paved areas. Midden density ranged from 0 to $\sim$ 30/m^2 with median of $\sim$ 10. Measuring the site's area via a Google image (5544 m^2), the estimated census number of adult worms was 55,440. Mutation rates for microsatellites (μ) has not been estimated for earthworms, but using a typical rate for invertebrates as $\mu = 10^{-4}$, a calculation of 33,380 breeding earthworms is obtained, reasonably close to the census estimate. Midden density was similar for the other sites, so these earthworms likely had high population sizes at each location.

All measures of population structure revealed differentiation among sites (Table 7). These include Nei's Unbiased Distance, Wright's Fst, Shannon Diversity Statistic, G'st, and AMOVA. Only WM2 and WM4 were not significantly distinct. Number of effective migrants per generation that would maintain these Fst values, ranged from ∞ for the WM sites (that were 88 m distant), and 3–6 migrants for all other pairwise comparisons. The AMOVA analysis partitioned overall genetic variation, with 7% accounted for among sites. The Shannon Diversity statistics partitioned 17% of variation among sites (Log-Likelihood G). Private alleles (those found only in one sample) were detected at all sites (Table 4). Comparing the COI haplotype and microsatellite data, the WM sites and SFEN had both the greatest number of haplotypes detected (5 each) and private alleles (8 and 4). This is expected if worms from different origins carry different microsatellite allele distributions.

The two assignment tests placed individual worms to site. A maximum likelihood method placed 70% of worms to their actual site, and if the two WM sites are combined, 83% of worms were successfully placed. The Bayesian analysis converged on the number of sites (k) as four (Online Resource 5). Thus, both methods conclude there are four sites, with the two very nearby WM sites combined. The cross-entropy analysis estimates that a single ancestral site gave rise to the sampled worms (Online Resource 6).

Discussion

Our interest in *Lumbricus terrestris* continues a long pedigree in biology. *L. terrestris* was the first earthworm to receive a taxonomic binomial by Linnaeus. The type specimen was long lost, but a neotype was described based on worms collected at the site where Linnaeus is likely to have taken his specimen in

Table 6 Microsatellite allele diversity by number of alleles per locus (Na Range) and the variation in number of repeats across alleles and across loci (Repeat Range, with NA = absent data) for a variety of earthworm studies. Notes on origin of worm sampled are given

Species	N Loci	Na range	Repeat range	Notes	References
Allolobophora chlorotica	8	6–21	NA	Two sites within 500 m; France	Dupont et al. (2015)
Allolobophora chlorotica	8	4–14	2–15	Two sites; UK and France, multiple cryptic species	Dupont et al. (2011)
Allolobophora icterica	8	3–6	NA	Two sites within 500 m; France	Dupont et al. (2015)
Amynthas corticus	9	2–16	4–37	1 site, Azore island	Cunha et al. (2017)
Eisenia fetida	16	3–9	5–31	Commercial worms from worm farm	Somers et al. (2011)
Hormogaster elisae	10	8–25	49–101	1 site; Spain	Novo et al. (2008)
Hormogaster elisae	4	12–24	NA	1 site; Spain	Novo et al. (2010)
Aporrectodea longa	11	3–15	2–24	1 site; Germany	Strunk et al. (2012)
Aporrectodea icterica	7	3–11	NA	7 sites; northern France	Torres-Leguizamon et al. (2014)
Lumbricus castaneus	8	7–29	NA	6 sites near Paris	Dupont et al. (2019)
Lumbricus rubellus	7	7–15	10–70	1 site, UK	Harper et al. (2006)
Lumbricus terrestris	7	7–13	NA	Multiple sites over 100 km; Canada Na here is range across loci by site	Klein et al. (2017)
Lumbricus terrestris	3	14–19	30–45	One meadow; Germany	Velavan et al. (2009)
Lumbricus terrestris	10	5–18	15–106	Single site; Canada	Velavan et al. (2007)
Lumbricus terrestris	8	5–25	NA	44 nearby (< 3 km); France	Souleman et al. (2016)
Lumbricus terrestris	5	6–11	11–41	Seven sites within 13 km; USA	This paper

Table 7 Population structure of *Lumbricus terrestris* among five sites in northern Vermont, USA, based on five microsatellite markers. Given is Nei Unbiased Genetic Distance (D) above diagonal, and Fst below diagonal. Not shown are G'st and Shannon sH(AP) values that give qualitatively same results. All pair-wise comparisons significant based on 999 permutations, except for WM2 versus WM4

	EW	WM2	WM4	SF EN	SF CB
EW		0.182	0.253	0.612	0.664
WM2	0.039		0.023	0.522	0.507
WM4	0.049	0		0.724	0.523
SF EN	0.109	0.093	0.107		0.372
SF CB	0.102	0.077	0.070	0.064	

Uppsala, Sweden (James et al. 2010). Darwin's (1881) classic study on earthworm behavior, his best-selling book during his lifetime, most likely centered on *L. terrestris* (although, oddly, he never formally identified his study species) (Butt et al. 2008). More recently, ecologists have been interested in *Lumbricus terrestris* because it is a highly successful invasive earthworm, able to enter complex soil communities and becoming essentially naturalized in many areas of North America. *L. terrestris* continues to spread in both North America and Europe (James et al. 2010; Pop and Pop 2006). In North America the earthworm can reach high densities in forests, and removed the entire autumn leaf drop each year, thus substantially altering the environment (James et al. 2010).

In molecular biology, *L. terrestris* was the first earthworm, and one of the first animals, to have its complete mitochondrial genome sequenced (Boore and Brown 1995). Use of the mitochondrial COI gene, the taxonomic barcoding region (Folmer et al. 1994), now allows insight into colonization history of the worms from Europe to North America (Porco et al. 2013). All of the earthworms we sequenced were *L. terrestris*. The morphologically very similar cryptic

species, *L. herculeus*, was absent; it has never been found in North America, a perplexing question of why that very similar species has not become established (James et al. 2010). The COI data revealed that the Vermont earthworms originated from a broad range of locations in Europe, even if they were not directly introduced from each of those locations. The great geographic range of *L. terrestris* in Europe may well mask a cryptic diversity of insipient species and the introductions into Vermont could represent significantly distinct genetic lines.

Trade from Europe to our study region over three centuries came from via the Connecticut River, and after canals were built in 1823 and 1843, trade emerged from the Hudson and St. Lawrence rivers into Lake Champlain (http://www.uvm.edu/place/burlingtongeographic/stories/wf-2.php). By 1808, one of the first commercial steamships built traveled Lake Champlain delivering goods and people (Albers 2000). Most of the widespread locations in Europe that are likely sources for our worms (Scotland, Sweden, Norway, France) were active trading partners with the Lake Champlain region. The cross-entropy analysis of the nuclear microsatellite markers suggests a single ancestral population gave rise to all Vermont earthworms, even if the sites now are genetically differentiated. Perhaps all of the mitochondrial haplotypes came to the study area from a single source, such as maritime England where trade and invasions introduced worm lineages from throughout Europe. For example, the invasion from what is now modern Denmark by Ivar the Boneless in the 9th century could have introduced earthworms from Scandinavia long ago (Jones 2001). A full study of the genetic lineages across Europe could well match trading and conquest history, thus linking cultural history with the history of an invasive species. We chose our sites, current or past farmland or woodlots, away from fishing locations where bait worms could have been released, so it is likely that the introductions came from early trade.

Despite the importance of *L. terrestris* as an invasive species in both North America and Europe, rather few studies on the population genetics of the species have previously been reported. The genetic diversity and population structure have been probed by biochemical methods (allozymes, or protein variation as a surrogate for genetic variation; Enckell et al. 1986), two methods that scan anonymous variable regions in the genome, Random Amplified Polymorphic DNA (RAPD) (Kautenburger 2006a, b) and Restriction fragment length polymorphism (RFLP) (Richter 2009), gene sequences (Klein et al. 2017; and most recently regions in the genome with variable repeats, typically three to six genetic nucleotides (microsatellites) (Gailing et al. 2012; Klein et al. 2017; Souleman et al. 2016; Velavan et al. 2007, 2009).

The goal of these studies has been to understand geographic structure across the landscape of the earthworms and the role of invasion history on their genetic structure. The general conclusion based on this range of techniques is that all populations of *L. terrestris* surveyed, both in Europe and North America, are genetically diverse, including a German meadow (Velavan et al. 2009), six of the Faroe Islands where the worms may have come many centuries ago (Enckell et al. 1986), in Alberta, Canada, where the worms most likely arrived only 20 years ago (Klein et al. 2017; Velavan et al. 2007), multiple sites in Germany (Kautenburger 2006a, b), multiple sites in Germany, France, Finland, Sweden, Bosnia in Europe and Michigan, Maryland, and Maine in North America (Gailing et al. 2012), and multiple sites in Finland, Sweden, Austria, Bosnia-Herzegovina, France, and Germany (Richter 2009). Although sample sizes for some of these studies were small, and a variety of techniques were used (above), the overall conclusion is that the earthworms display high levels of genetic variation at all sites, and that invasion by *L. terrestris* into North America did not result in lower genetic diversity compared to potential source areas. However, two studies showed some influence of invasion history. Richter (2009) found lower genetic diversity in European populations distant from assumed refugia sites during the last glaciation (up to 14,000 ya) compared to a crossroads region (Germany). In Alberta, Canada, where *L. terrestris* seems to have been introduced only $\sim$ 20 years before the study (Klein et al. 2017), the greatest number of microsatellite alleles was found in an urban area where the fishing bait industry most likely introduced worms multiple times over the past two decades (Klein et al. 2017).

We also found substantial genetic diversity in *L. terrestris* at our study sites, with mean expected heterozygosity values across sites from 0.760 to 0.802, and even higher values if data are combined for the sites (Tables 4, 5). These values reflect the typically

high number of microsatellite alleles, but also their rather even distribution in frequency.

The finding of a broad geographic range for the source populations suggested to us that the worms should be very diverse, more so than at any single population in Europe. Better measures to compare across earthworm populations are the number of alleles and their variation in length. Both number of alleles and the range in allele lengths did not differ from other *L. terrestris* samples, nor for earthworms in general (Table 6). There thus seems to be missing diversity, at least for microsatellite alleles. One model of microsatellite evolution, the K-alleles model, posits that each locus has some limit in the number of alleles either from selection or the mutational process (review in Estoup et al. 2002). Thus, there would be a fairly constant number of alleles across sites for *L. terrestris*. A second possibility is size homoplasy in which the introduced earthworms from each source could well have brought their own set of alleles which were not homologous across those donor sites. That is, a particular microsatellite allele we identified could have had multiple origins, and not be homologous. Models of molecular evolution show that over a few hundred generations, size homoplasy would be common at each locus for isolated sites (Estoup et al. 2002). Also, Kirk et al. (2013) catalogue a list of factors that can shape the genetic structure of an introduced species beyond founder effect or multiple introductions. Use of allele diversity to compare source and recipient populations should therefore be considered with care.

The movement of *L. terrestris* earthworms among our sites most likely has been slight in recent years, both because their natural ability to move distances as adults is minimal and because farming activities that would move cocoons across the landscape is likely also to be low in recent years and some sites. For example, no agricultural activity has taken place in the sites on the university campus (WM) and a nature preserve in the city (EW) for many years. This sets the stage both for inbreeding at each site and genetic differentiation over time across the sites. Inbreeding varied across sites (Table 4), but reached 0.414 at one site. Measured by standard population genetics methods (for example, Fst values) and population assignment test, there appears to be differentiation across sites. The differentiated sites were separated by only 0.6 to 13 km. Even for the experimental farm sites only 2 km distant (SFEN vs. SFCB), the earthworms were differentiated, suggesting low movement of either the earthworms or cocoons across these sites.

Previous studies have found population structure for *L. terrestris* in both Europe and North America at a variety of spatial scales, although not as fine-grained as found in our study: at sites in Ontario, Canada over 50–100 km (Klein et al. 2017), across the Faroe Islands (Enckell et al. 1986), in German sites 4.5–245 km (Kautenburger 2006a), and across sites in Europe from 75 to 225 km (Richter 2009). Kautenburger (2006b) found differentiation across sites in Germany, not based on distance, but on agricultural use of the land. Velavan et al. (2009) found that the *L. terrestris* was not differentiated over a very fine scale, from 1 to 50 m in a German meadow, despite the low vagility of these earthworms. However, this view of low movement of *L. terrestris* earthworms is contradicted by observations of the movement of invasion fronts in North American hardwoods suggesting movement of up to 10 m/year (Hale et al. 2005), a dispersion rate that might explain low fine-scale variation. A single contrasting study, Gailing et al. (2012), found no population structure even between their sites in Europe and North America, but this may have been a result of most sites having small sample sizes (< 20), and only three microsatellite markers assessed.

We scored only five microsatellite markers in our study; is it possible the results were biased by so few markers? Several methods of analysis found differentiation among sites 0.7–13 km distant, but not two sites 88 m distant, including the standard Fst analysis and two methods to assign worms to sites. Five markers were therefore sufficient to detect differentiation. For the more pressing issue of number of alleles found, the markers could have been biased toward a low number of alleles by chance, and the apparent "missing diversity" for *L. terrestris* earthworms may be spurious. Thus, the results for our study needed to be compared with those for other earthworms and particularly for *L. terrestris*. Only 16 previous studies on earthworms looked at microsatellite allele diversity. To be able to make valid comparisons, we chose the median number of markers used in other studies. Table 6 reveals that across studies the number of markers used is not associated with the maximal number of alleles detected, nor for the range in number of alleles. Overall, though, the results suggest that

rather few microsatellite markers may reveal the true genetic diversity within earthworm populations. We find it striking that so few studies on the population genetics of earthworms have been conducted, especially because of the importance of earthworms for soil ecology, and as invasive species.

We now propose a possible history of *L. terrestris* based on our findings and previous studies reviewed above. The two morphologically cryptic species, *L. terrestris* and *L. herculeus* diverged prior to the last glaciation in Europe > 22,000 ya (James et al. 2010). *L. terrestris* was extirpated over most of Europe during the last glacial period except for refugia in present Balkans, Italy, and southern France (Richter 2009). The subsequent range expansion, still at its stressed northern limit, led to geographic genetic differentiation (measured as COI sequences). Movement of people and livestock from Neolithic to modern times then moved these now-differentiated earthworms across Europe (Richter 2009). (One haplotype we detected in Vermont has been found in sites as diverse as Scotland, Austria, Sweden, and France). *L. terrestris* continues to expand its range in Europe (Pop and Pop 2006). Earthworms were also eliminated from the soils of areas of North America by glaciers, and remained earthworm-free for thousands of years after glacier retreat (Hale 2008). Over the past three centuries, *L. terrestris* and other earthworms were introduced into North America, and with the most recent introductions in some parts of western Canada (Klein et al. 2017). Propagule pressure has been great, both in number of individual worms and in the form of cocoons that survive harsh environments (Görres et al. 2018; Meshcheryakova and Berman 2014; Nouri-Aiin and Görres 2019) and travel readily (Tiunov et al. 2006), and mitochondrial lineages arriving by introductions from multiple geographic sites (Gailing et al. 2012). At trading crossroads, such as the Champlain Valley of Vermont, multiple lineages were introduced, and then genotypes adapted to different environmental conditions. Genetic variation in North America is not reduced compared to European sites, including very local habitat patches as shown for the Vermont sites. However, the diversity of nuclear microsatellite alleles seems lower than expected based on the diversity of mitochondrial lineages, perhaps due to lack of homology of the alleles scored. Other explanations for this discrepancy, though, need be explored. The earthworms in recent years do not seem to be moving among close-by sites, resulting in population structure and local inbreeding. Finally, the genetic diversity and population structure sets the stage for local adaptation when the earthworms become established in a complex soil community, and reach very high densities with major changes in forest structure (James et al. 2010). This story would thus explain the success of *L. terrestris* as an invasive species. Each of these many steps in our proposed history of this important invasive species deserves scrutiny, in studies of ecology, phylogeny, and population genetics.

Acknowledgements We thank Dana Bishop, Woodlands Manager, Shelburne Farms, and Rick Paradis, University of Vermont Natural Areas Director, for aid in use of property under their stewardship. The research was funded by a USDA-Hatch grant from the University of Vermont Agricultural Research Station.

References

Albers J (2000) Hands on the land. MIT Press, Cambridge

Allendorf FW, Lundquist LA (2003) Introduction: population ecology, evolution and control of invasive species. Conserv Biol 17:24–30

Boore JL, Brown WM (1995) Complete sequence of the mitochondrial DNA of the annelid worm *Lumbricus terrestris*. Genetics 141:305–319

Butt KR, Frederickson J, Morris RM (1992) The intensive production of *Lumbricus terrestris* L. for soil amelioration. Soil Biol Biochem 24:1321–1325

Butt KR, Lowe CN, Beasley T, Hanson I, Keynes R (2008) Darwin's earthworms revisited. Eur J Soil Biol 44:255–259

Cunha L, Thornber A, Kille P, Morgan AJ, Novo M (2017) A large set of microsatellites for the highly invasive earthworm *Amynthas corticus* predicted from low coverage genomes. Appl Soil Ecol 119:152–155

Darwin CR (1881) The formation of vegetable mould through the action of worms with observations on their habitats. Murray, London

Dupont L, Lazrek F, Porco D, King RA, Rougerie R, Symondson WOC, Livet A, Richard B, Decaën T, Butt KR, Mathieu J (2011) New insight into the genetic structure of the *Allolobophora* chorotica aggregate in Europe using microsatellite and mitochondrial data. Pedobiologia 54:217–234

Dupont L, Grésille Y, Richard B, Decaëns T, Nathier J (2015) Dispersal constraints and fine-scale spatial genetic structure in two earthworm species. Biol J Linn Soc 114:335–347

Dupont L, Pauwels M, Dume C, Deschins V, Audusseau H, Gigon A, Dubs F, Vandenbulke F (2019) Genetic variation of the epigenic eartheorm *Lumbricus castaneus* populations in urban soils of the Paris region (France) revealed using eight newly developed microsatellite markers. Appl Soil Biol 135:35–37

Dybdahl MF, Drown DM (2011) The absence of genotypic diversity in a successful parthenogenetic invader. Biol Invasions 13:1663–1672

Eisen GA (1872) Om nagra arkiska Oligochaetre. Öfv. Vet.-Akad. Förhandl Stockholm 29:119–124

Elton CS (1958) The ecology of invasions by animals and plants. Chapman and Hall, London

Enckell PH, Bengston S-A, Duwes P, Niklasson M, Stille B, Wiman B (1986) The dispersal pattern of an anthropochorous species: genetic variation in populations of *Lumbricus terrestris* L. (Lumbricidae) in the Faroe Islands. Hereditas 104:253–261

Estoup A, Jarne P, Cornuet J-M (2002) Homoplasy and mutation model at microsatellite loci and their consequences for population genetics analysis. Mol Ecol 11:1591–1604

Folmer O, Black M, Hoch W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3:294–299

François O (2016) Running structure-like population genetic analysis with R. R Tutorials in Population Genetics, U Grenoble-Alpes, pp 1–9

Gailing O, Hickey R, Lilleskov E, Szlavecz K, Richter K (2012) Genetic comparisons between North American and European populations of *Lumbricus terrestris* L. Biochem Syst Ecol 45:23–30. https://doi.org/10.1016/j.bse.2012,07.018

Gates GE (1976) More on earthworm distribution in North America. Proc Biol Soc Wash 89:467–476

Görres JH, Melnichuk RDS (2012) Asian invasive earthworms of the genus *Amynthas* Kinberg in Vermont. Northeast Nat 19:313–322

Görres JH, Connolly ST, Chang C-H, Carpenter NR, Keller EL, Nouri-Aiin M, Schall JJ (2018) Winter hatching in New England populations of invasive pheretimoid earthworms *Amynthas agrestis* and *Amynthas tokioensis*: a limit on population growth, or aid in peripheral expansion? Biol Invasions 20:1651–1655

Gunn A (1992) The use of mustard to estimate earthworm populations. Pedobiologia 36:65–67

Hale CM (2008) Evidence for human-mediated dispersal of exotic earthworms: support for exploring strategies to limit further spread. Mol Ecol 17:1165–1169

Hale CM, Frelich LE, Reich PB (2005) Exotic European earthworm invasion dynamics in northern hardwood forests of Minnesota, USA. Ecol Appl 15:848–860

Hamilton MB, Pincus EL, DiFiore A, Fleisher RC (1999) Universal linker and ligation procedures for construction of genomic SNA libraries enriched for microsatellites. Biotechniques 27:500–507

Harper GL, Casey CP, Morgan AJ, Kille P, Bruford MW (2006) Microsatellite markers for the earthworm *Lumbricus rubellus*. Mol Ecol Notes 6:325–327

Holmstrup M, Westh P (1994) Dehydration of earthworm cocoons exposed to cold: a novel cold hardiness mechanism. J Comp Phys B 164:312–315

James SW, Porco D, Decaens T, Richard B, Rougerie R, Erseus C (2010) DNA barcoding reveals cryptic diversity in *Lumbricus terrestris* L., 1758 (Clitellata): Resurrection of *L. herculeus* (Savigny, 1826). PLoS ONE 5:15629

Jones G (2001) A history of the vikings. Oxford University Press, Oxford

Kautenburger R (2006a) Genetic structure among earthworms (*Lumbricus terrestris* L.) from different sampling sites in western Germany based on random amplified polymorphic DNA. Pedobiologia 50:257–266. https://doi.org/10.1016/j.pedobi.2006.02.005

Kautenburger R (2006b) Impact of different agricultural practices on the genetic structure of *Lumbricus terrestris*, *Arion lusitanicus*, and *Microtus arvalis*. Anim Biodiv Conserv 29:19–32

Kirk H, Dorn S, Mazzi D (2013) Molecular genetics and genomics generate new insights into invertebrate pest invasion. Evol Appl 6:842–856

Klein A, Cameron EK, Heimburger B, Eisenhauer N, Scheu S, Schaefer I (2017) Changes in the genetic structure of an invasive earthworm species (*Lumbricus terrestris*, Lumbricidae) along an urban–rural gradient in North America. Appl Soil Ecol 120:265–272. https://doi.org/10.1016/j.apsoil.2017.08.009

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) ClustalX. Bioinformatics 23:2947–2948

McCay TS, Pinder RA, Alvarado E, Hanson WC (2017) Distribution and habitat of the endemic earthworm*Eisenoides lonnbergi* (Michaelsen) in the northeastern United States. Northeast Nat 24:239–248

Mergeay J, Verschuren D, De Meester L (2006) Invasion of an sexual American water flea clone throughout Africa and rapid displacement of a native sibling species. Proc R Soc B 2783:2839–2844

Meshcheryakova EN, Berman DI (2014) Cold hardiness and geographic distribution of earthworms (Oligochaeta, Lumbricidae, Moniligastridae). Entomol Rev 94:486–497

Neal AT, Ross MS, Schall JJ, Vardo-Zalik AM (2016) Genetic differentiation over a small spatial scale of the sand fly *Lutzomyia vexator* (Diptera: Psychodidae). Parasite Vector 9:550

Nouri-Aiin M, Görres JH (2019) Earthworm cocoons: the cryptic side of invasive earthworm populations. Appl Soil Ecol 141:54–60

Novo M, Velavan TP, Almodovar A, Schulenberg H, Diaz-Cosin DJ, Michiels NK (2008) Microsatellite markers for the drought-resistant earthworm *Hormogaster elisae*. Mol Ecol Resour 8:901–903

Novo M, Almodóvar A, Fernández RM, Gutiérrez M, Díaz Cosín DJ (2010) Mate choice of an endogeic earthworm revealed by microsatellite markers. Pedobiologia 53:373–379

Nuutinen V, Butt KR (1997) The mating behavior of the earthworm *Lumbricus terrestris* (Oligochaeta: Lumbricieae). J Zool Lond 242:783–798

Nuutinen V, Butt KR (2005) Homing ability widens the sphere of influence of the earthworm *Lumbricus terrestris* L. Soil Biol Biochem 37:805–807

Paetkau D, Slade R, Burden M, Estoup A (2004) Genetic assignment methods for the direct, real-time estimation of migration rate: a simulation-based exploration of accuracy and power. Mol Ecol 13:55–65

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. Bioinformatics 28:2537–2539

Pompanon F, Bonin A, Bellemain E, Taberlet P (2005) Genotyping errors: causes, consequences and solutions. Nat Rev Genet 6:847–859

Pop VV, Pop AA (2006) Lumbricid earthworm invasion in the Carpathian Mountains and some other sites in Romania. Biol Invasions 8:1219–1222

Porco D, Decaëns T, Deharveng L, James SW, Skarzynski D, Erséus C, Butt KR, Richard B, Hebert PDN (2013) Biological invasions in soil: DNA barcoding as a monitoring tool in a multiple taxa survey targeting European earthworms and springtails in North America. Biol Invasions 15:899–910

Reynolds JW (2008) Terrestrial Oligochaeta (Annelida: Clitellata) in North America, including Mexico, PuertoRico, Hawaii, and Bermuda. Megadrilogica 12:157–210

Richter K (2009) Genetic structure in European populations of the earthworm *Lumbricus terrestris*. Dissertation, Kassel University, Kassel, Germany

Roman J, Darling JA (2007) Paradox lost: genetic diversity and the success of aquatic invasions. Trends Ecol Evol 22:454–464

Rousset F (2008) Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. Mol Ecol Resour 8:103–106

Sakai AK, Allendorf FW, Holt JS, Lodge DM, Molofsky J, With KA, Baughman S, Cabin RJ, Cohen JE, Ellstrand NC, McCauley DE, O'Neil P, Parker IM, Thompson JN, Welle SG (2001) The population biology of invasive species. Ann Rev Ecol Syst 32:305–332

Somers CM, Neudorf K, Jones KL, Lance SL (2011) Novel microsatellite loci for the compost earthworm *Eisenia fedida*: a genetic comparison of three North American vermiculture stocks. Pedobiologia 54:111–117

Souleman D, Grumiaux F, Frérot H, Vandenbulcke F, Pauwels M (2016) Isolation and characterization of eight polymorphic microsatellites markers for the earthworm *Lumbricus terrestris*. Eur J Soil Biol 74:76–80

Strunk H, Hochkirch A, Veith M, Hankeln T, Emmerling C (2012) Isolation and characterization of eleven polymorphic microsatellite markers for the earthworm *Aporrectodea longa*. Eur J Soil Biol 48:56–58

Tiunov AV, Hale CM, Holdsworth AR, Vsevolodova-Perel TS (2006) Invasion patterns of Lumbricidae into the previously earthworm-free areas of northeastern Europe and the western Great Lakes region of North America. Biol Invasions 8:1223–1234

Tomlin AD (1983) The earthworm bait market in North America. In: Satchell JE (ed) Earthworm ecology. Springer, Dordrecht

Torres-Leguizamon M, Mathieu J, Decaens T, Dupont L (2014) Genetic structure of earthworm populations at a regional scale: inferences from mitochondrial and microsatellite markers in *Aporrectoda icterica*. PLOS ONE 9:e1015971-11

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 4:535–538

Velavan TP, Schulenburg H, Michiels NK (2007) Development and characterization of novel microsatellite markers for the common earthworm (*Lumbricus terrestris* L.). Mol Ecol Notes 7:1060–1062

Velavan TP, Weller S, Schulenburg H, Michiels NK (2009) High genetic diversity and heterogeneous parasite load in the earthworm *Lumbricus terrestris* on a German meadow. Soil Biol Biochem 41:1591–1595

Wallace AR (1876) The geographical distribution of animals; with a study of the relations of living and extinct faunas as elucidating the past changes of the earth's surface, 2 vols. Macmillan, New York

Wallace AR (1911) Island life, 3rd edn. Macmillian, London

Wright S (1951) The genetical structure of populations. Ann Eugen 15:323–354