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Changes in the genetic structure of an invasive earthworm species (*Lumbricus terrestris*, Lumbricidae) along an urban – rural gradient in North America

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ABSTRACT

European earthworms were introduced to North America by European settlers about 400 years ago. Human-mediated introductions significantly contributed to the spread of European species, which commonly are used as fishing bait and are often disposed deliberately in the wild. We investigated the genetic structure of *Lumbricus terrestris* in a 100 km range south of Calgary, Canada, an area that likely was devoid of this species two decades ago. Genetic relationships among populations, gene flow, and migration events among populations were investigated using seven microsatellite markers and the mitochondrial 16S rDNA gene. Earthworms were collected at different distances from the city and included fishing baits from three different bait distributors. The results suggest that field populations in Alberta established rather recently and that bait and field individuals in the study area have a common origin. Genetic variance within populations decreased outside of the urban area, and the most distant populations likely originated from a single introduction event. The results emphasise the utility of molecular tools to understand the spatial extent and connectivity of populations of exotic species, in particular soil-dwelling species, that invade native ecosystems and to obtain information on the origin of populations. Such information is crucial for developing management and prevention strategies to limit and control establishment of non-native earthworms in North America.

1. Introduction

Invasive species are typically described by three general characteristics: range extension (Facon and David, 2006), high local abundance (Suarez et al., 1999), and disruption of ecosystem functions (Mooney and Hobbs, 2000). Invasions are often initiated by singular events that change current ecosystem conditions, like climatic changes with subsequent disturbance of ecosystems, or human activities as agriculture, urbanization, and pollution (Davis, 2009). An invasive population usually corresponds to a set of individuals that has been introduced into a new territory where individuals established, increased in number and subsequently spread (Estoup and Guillemaud, 2010), with some introductions being successful while others are not. The genetic structure of invading populations is assumed to strongly affect invasion success (Sakai et al., 2001), and studies on population genetics may provide critical information on founder size, number of introductions, and dispersal, which are important factors for successful invasions. For instance, populations originating from single introduction events are likely to have low genetic variation (Allendorf and Lundquist, 2003) and thus limited ability to adapt to local environments (Sakai et al., 2001), even though, in rare cases invasions can be successful when genetic variability is low (Tsutsui et al., 2000). Multiple introduction events, however, increase genetic diversity and therefore the probability of successful establishment and adaptation to novel environments by mixing genotypes (Kolbe et al., 2004).

The common European earthworm species *Lumbricus terrestris* (Linnaeus, 1758) was introduced into North America by European settlers and started its invasion at the east coast about 400 years ago (Gates, 1976). It is a well-known ecosystem engineer (Lee 1985; Edwards and Bohlen 1996; Lavelle et al., 1998; Eisenhauer, 2010) that influences physical and biotic properties of the soil by bioturbation and affects the density of other soil invertebrates, and plant community

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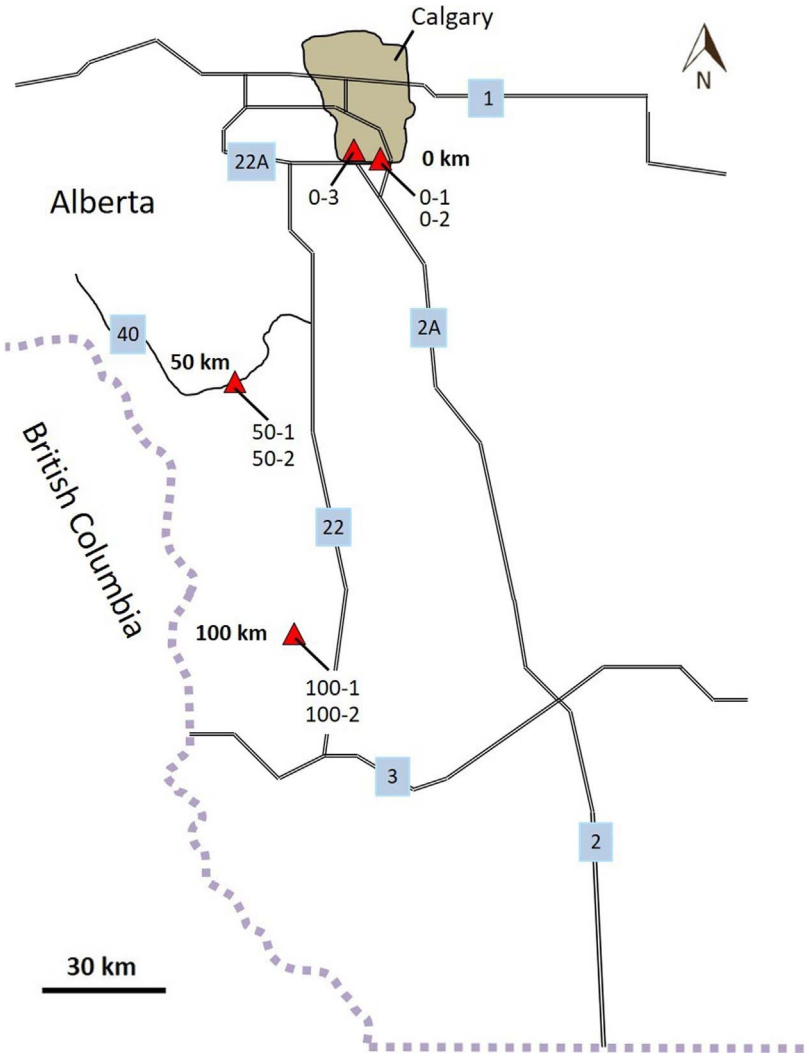

Fig. 1. Sampling locations of *Lumbricus terrestris* in southern Alberta, Canada. Individuals were sampled in Calgary (shaded area) and 50 km and 100 km south of Calgary. Subpopulations (triangles) 0–1 and 0–2, 50-1 and 50-2, 100-1 and 100-2 are 5 m apart; subpopulation 0–3 is 5 km apart from subpopulations 0–1 and 0–2. Major road networks are marked (black lines) and labelled with national road numbers.

composition (Lee 1985; Edwards and Bohlen 1996; Eisenhauer et al., 2007, 2010; Craven et al., 2016). Consequently, earthworms cause massive changes in boreal and temperate forests in North America and are of major concern for conservation and management actions (Bohlen et al., 2004; Callaham et al., 2006; Hendrix et al., 2008). These earthworms live in vertical burrows deep in the soil, which they leave mostly at night for foraging. Active dispersal is very limited in *L. terrestris* (2–4 m y^{-1}; Marinissen and van den Bosch, 1992) making autonomous expansion of populations slow. It feeds on a variety of leaf litter materials and is tolerant to a broad range of climatic and other environmental conditions like habitat structure, disturbance or pollution, which contributes to its potential to invade new areas (Edwards, 2004; Frelich et al., 2006; Addison, 2009; James et al., 2010). Today, *L. terrestris* is distributed across the North American continent, though sometimes patchy and absent in the Great Plains and the states along the Gulf of Mexico, displaying a fast invasion over the continent in only a few hundred years (Reynolds, 2008).

Information on the distribution of *L. terrestris* is primarily based on presence-absence data, but the importance of human-mediated dispersal for the rapid and wide-range expansion of invasive earthworm species is evident (Hendrix et al., 2008). Disposal of fishing bait is common (Seidl and Klepeis, 2011; Cameron et al., 2013), and transport of earthworms and their cocoons associated with soil adhering to vehicles has also been identified as a key source of introduction and distribution for some European earthworm species (Holdsworth et al., 2007a,b; Cameron et al., 2008). However, little is known about the relevance of multiple and repeated introductions of earthworms for the genetic structure and sustainability of populations. Until today, few studies have investigated the genetic structure of exotic European earthworms in North America. These studies revealed the importance of multiple introduction events and human-mediated dispersal (jump dispersal) for the rapid spread of *Dendrobaena octaedra* (Cameron et al., 2008) and that North American populations of *L. terrestris* derived from genetically diverse European founder populations (Gailing et al., 2012).

Forests in the Canadian province Alberta likely have not been invaded by *L. terrestris* for much more than 20 years (Scheu and Parkinson, 1994). This new invasion provides a unique opportunity to investigate the genetic structure of invading earthworm populations. Here we used genetic information from microsatellite markers and mitochondrial 16S rDNA to investigate the genetic variability and structure of *L. terrestris* populations in the field.

Individuals were sampled at different distances from the city of Calgary. As *L. terrestris* is a poor disperser, we hypothesised that both the numbers of alleles and the most common alleles will differ significantly among earthworm populations from the different sampling sites. Further, we hypothesised that the genetic variance within populations declines with increasing distance to the city area because human-mediated introductions and transfer are more likely in the urban areas.

Additionally, we collected earthworms from one small local bait shop and the two largest and most popular bait distributors in Calgary to analyse the genetic diversity of bait and to examine whether bait

Table 1
Summary of sampling locations, number of sampled individuals (Pop. size) and coordinates of sampling sites of *Lumbricus terrestris* from southern Alberta. Average measures for genetic diversity (N_a, number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity) at seven microsatellite loci in this study are given for a) one population dataset for field, bait shop and both combined and for three populations assigned by STRUCTURE and b) for each sampling location.

(a)

Dataset	Sample size	N_A	H_o	H_e
1 Population				
Field	154	29.00 ± 3.53	0.69 ± 0.04	0.89 ± 0.02
Bait	36	16.71 ± 2.12	0.69 ± 0.06	0.87 ± 0.03
Combined	190	32.57 ± 4.21	0.69 ± 0.04	0.90 ± 0.02
3 Populations				
Bait, 0 km	104	26.71 ± 2.92	0.69 ± 0.03	0.89 ± 0.02
50 km	40	11.71 ± 1.30	0.68 ± 0.09	0.81 ± 0.03
100 km	46	17.86 ± 2.32	0.69 ± 0.05	0.88 ± 0.02
Total	190	18.76 ± 1.86	0.69 ± 0.03	0.86 ± 0.01

(b)

Location		Pop. no.	Pop. size	Sample size	Coordinates	N_A	H_o	H_e
Bait shops		Bait	36	36	n.a.	16.71 ± 2.12	0.69 ± 0.06	0.87 ± 0.03
Calgary	Fish Creek Park (south)	0 − 1	96	23	N 50,9 W 114,0	11.43 ± 0.75	0.69 ± 0.07	0.84 ± 0.02
	Fish Creek Park (south)	0 − 2	28	24	N 50,9 W 114,0	13.86 ± 1.18	0.67 ± 0.05	0.89 ± 0.01
	Fish Creek Park (north)	0 − 3	70	21	N 50,92 W 114,1	10.86 ± 0.77	0.66 ± 0.07	0.79 ± 0.03
50 km south	Eden Valley	50 − 1	37	21	N 50,4 W 114,5	11.14 ± 1.24	0.69 ± 0.05	0.83 ± 0.02
	Eden Valley	50 − 2	28	19	N 50,4 W 114,5	10.14 ± 1.55	0.76 ± 0.07	0.81 ± 0.03
100 km south	Maycroft	100 − 1	21	21	N 49,87 W 114,3	7.43 ± 0.48	0.70 ± 0.07	0.77 ± 0.02
	Maycroft	100 − 2	38	25	N 49,87 W 114,3	8.71 ± 1.02	0.67 ± 0.08	0.76 ± 0.04
	Total		353	190		11.29 ± 0.55	0.69 ± 0.02	0.82 ± 0.01

genotypes are present in populations sampled in the field. Two major distributors supply bait shops with earthworms, with *L. terrestris* being the most common bait species (A. Klein, unpubl. data). Both distributors started breeding earthworms with hand-collected individuals from Canadian soils, one was founded in 1965, the second in the 1980s in Michigan. Because of the independent formation of the two companies, we hypothesised that earthworm genotypes of the two distributors differ and that due to the long breeding history of at least 30 years, heterozygosity will be significant lower in populations of bait shop individuals compared to populations collected in the field.

2. Material and methods

2.1. Taxon sampling

Populations of *L. terrestris* were sampled in the region of Calgary, Alberta (Canada), in September 2012. A hierarchical sampling strategy was applied, i.e., three populations were sampled at three sampling locations 50 km and 100 km apart; two to three replicates were taken, representing subpopulations at 5 and 5000 m distance (Fig. 1, Table 1). Individuals were collected from quadrats of 0.5 × 0.5 m using mustard extraction (Gunn, 1992). Sampling plots were extended by additional 0.5 × 0.5 m quadrats until 20–30 individuals were collected from each subpopulation at each of the locations. Accordingly, single plot sizes covered a continuous area between 0.25 m^2 to 3 m^2. The first sampling area was within the urban area of Calgary (0 km), the second 50 km south in Eden Valley 216 between a river and a car park of a popular recreational area near Highway 40. The third sampling location was 100 km south of Calgary in a mountainous forest several hundred meters from a gravel road and about 20 km from the nearest paved road (Highway 22); the closest human settlements were a cattle farm and a camp ground more than 10 km away. All sampling locations were within 15 m of a river. Additionally, twelve individuals from each of the two largest Canadian bait shop distributors and one regional bait shop were sampled to collect the most frequently used baits in the sampling area. The majority of local bait shops sell artificial baits, and those selling life-bait obtained nightcrawlers from the two major distributors mentioned above. The shops were in close proximity to fishing spots along the lower Bow River, running through Calgary and the Glenmore Reservoir in the south west of Calgary, as well as to our sampling locations. Earthworms were stored in 95% ethanol and transferred to the laboratory at the University of Alberta, Edmonton, for determination of species. One centimetre of tail tissue was shipped to the University of Göttingen for molecular analyses; remaining body parts were stored as voucher specimens at the University of Alberta. Altogether, we analysed 190 earthworms, 154 specimens collected from seven sampling sites in the field and 36 individuals from bait shops.

To ensure that we analysed only *L. terrestris*, we applied a barcoding approach: first we used 16S to verify the identity of *L. terrestris* in adult and juvenile specimens, and second we compared COI sequences of all bait shop samples to ensure that no specimens of the morphologically similar species *L. herculeus* were present in the dataset (James et al., 2010). In total, we analysed 12 individuals from each of the three bait shops and compared their sequences with 11 *L. terrestris* and 30 *Lumbricus herculeus* individuals from the study of James et al. (2010).

2.2. DNA extraction, gene amplification, and genotyping

Genomic DNA was extracted using the Qiagen Blood & Tissue kit (Qiagen; Hilden, Germany). The mitochondrial gene 16S rDNA was used as a species marker to identify juvenile individuals. An 800 bp fragment of 16S was amplified using the primers 16S-LumbF2 (5′-CGA CTG TTT AAC AAA AAC ATT GC-3′) and Ho_16Sra (5′-GCA CTA TTC TGC CAY CTT GT-3′) (Pérez-Losada et al., 2009). The standard barcoding gene cytochrome-oxidase-subunit I (COI) was used to check for the presence of the cryptic species *L. herculeus* using the standard barcoding primers LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) (Folmer et al., 1994). All PCR reactions for sequencing (16S and COI) were performed in 25 µl volumes containing 11.75 µl ultrapure H_2O, 1.25 µl BSA (~4%), 2.5 µl Buffer with KCl, 1 µl dNTPs (10 mM), $MgCl_2$ (25 mM), *Taq* polymerase (5 U/µl; Thermo Scientific; Schwerte, Germany), 1 µl of each primer (10 mM), and 2.5 µl template DNA. The PCR protocol consisted of an initial activation step at 95 °C for 3 min, 40

amplification cycles (denaturation at 95 °C for 30 s, annealing at 53 °C for 60 s, elongation at 72 °C for 60 s), and a final elongation step at 72 °C for 10 min. PCR products were checked on a 1% agarose gel for successful amplification, and positive products were purified using the QIAquick PCR Purification kit (Qiagen) and were sequenced at the Göttingen Genome Sequencing Laboratory (Georg August University Göttingen). All sequences are available at NCBI (http://www.ncbi.nlm.nih.gov/genbank, accession numbers KM986892-KM987009).

To assess fine-scale-resolution of local genetic diversity and gene flow among populations, individuals were genotyped at seven highly polymorphic microsatellite loci (LTM 026, 128, 163, 187, 193, 252, and 278; Velavan et al., 2007). Microsatellite markers were amplified using a *Taq* polymerase (Thermo Scientific) and a HotStarTaq Mastermix (Genaxxon; Ulm, Germany) following the protocol of Velavan et al. (2007); deviations from their protocol are listed in Tables A.1 and A.2 in Supplementary material. For genotyping, forward primers were fluorescence labelled with FAM (Sigma Aldrich; Munich, Germany) and analysed at the Department of Animal Sciences, Georg August University Göttingen. Microsatellite DNA-fragment analysis was performed with Genemapper (Life Technologies; Carlsbad, California, USA) and Geneious 8.0.5 (Biomatters Ltd; Auckland, New Zealand; Kearse et al., 2012). To exclude genotyping errors we analysed two datasets independently in the laboratory, which were processed by different persons and with some samples being repeated in both datasets. Allelic patterns at all loci were consistent in both runs. Positive and negative control samples were included in every run, data were analysed by semi-automated followed by manual gene calling in Genemapper and cross-checked with Geneious. Microsatellite profiles were tested for scoring errors and the presence of null alleles with MICRO-CHECKER v2.2.3 (Van Oosterhout et al., 2004).

2.3. Sequence and microsatellite analyses

Sequences of 16S and COI were checked with Sequencher 4.9 (Gene Codes Corporation, USA). Consensus sequences were assembled in BioEdit 7.0.1 (Hall, 1999) and aligned with the integrated ClustalW software using multiple alignment parameters: 10.0 for gap opening and 0.1 for gap extension. The sequence alignment was transformed into a haplotype alignment using the FaBox 1.41 online tool (Villesen, 2007). The best fit model of sequence evolution was estimated with TOPALi 2.5 (Milne et al., 2004). Phylogenetic trees were calculated with MrBayes 3.2 (Ronquist et al., 2012). Mean number of pairwise differences, gene and nucleotide diversity were calculated using the distance methods in Arlequin 3.5.2.2 (Excoffier 2015). Spatial structure was analysed based on the 16S alignment using a median-joining haplotype network (Bandelt et al., 1999) in Network 5.0 (Fluxus Technology Ltd., Suffolk, England). Population structure software STRUCTURE (Pritchard et al., 2000a,b; Falush et al., 2003, 2007) that uses genotypic data to assign individuals into K populations. Three independent runs were performed for each K, with K ranging from one to ten, the most likely number of K was inferred with STRUCTURE HARVESTER (Earl and von Holdt, 2012). All analyses for genetic diversity and genetic structure were conducted in GenAleX 6.5 (Peakall and Smouse, 2006) with 9999 permutations and 1000 bootstrap replicates for the most likely population assignment. Genetic diversity was calculated for the three genetic populations assigned by STRUCTURE using the mean number of alleles (*Na*) and observed (*Ho*) and expected (*He*) heterozygosity for all loci combined. The genetic variance between populations was estimated by the mean inbreeding (F_{IS}) and overall inbreeding (F_{IT}) coefficients and the fixation index (F_{ST}). Genetic structure and gene flow were investigated by calculating deviations from Hardy-Weinberg equilibrium for each loci and all genetic populations separately were calculated using a Chi-Square test. Pairwise genetic differentiation among the assigned genetic populations and between subpopulations at the eight separate sampling locations were calculated and analysed by comparing pairwise F_{ST} values as well as by using hierarchical analysis of molecular variance (AMOVA).

3. Results

3.1. Population characterisation

In total, 190 individuals were genotyped at seven loci and analysed as a single population to estimate the overall genetic diversity. We analysed the field collected individuals (n = 154, field) and bait shops (n = 36, bait) separately and in a combined dataset (n = 190, complete). The numbers of alleles (*Na*) for the complete dataset were 22 (minimum), 53 (maximum), and 32.6 (mean), with all field individuals having more alleles than all bait individuals (Table 1a). The level of heterozygosity was very similar between bait and field populations with 0.216 and 0.218, respectively, and slightly lower than in the combined dataset (0.229; Fig. 3). All loci were one hundred percent polymorphic and deviated significantly ($p < 0.001$) from Hardy-Weinberg-Equilibrium (*HWE*). Genetic diversity was high in field populations, but highest within the urban area of Calgary. Whether inbreeding or linkage disequilibrium affected population structure in our study cannot be answered because many factors (recombination, genetic drift, inbreeding, mutation and gene flow) that influence linkage disequilibrium are unknown in earthworms.

Comparing COI sequences of different 16S haplotypes with COI sequences of *L. herculeus* showed that all 190 individuals from Alberta were *L. terrestris* (Fig. A.1 in Supplementary material). The 190 sequences of 16S rDNA consisted of 26 haplotypes (Table A.3 in Supplementary material) that were very similar with a mean nucleotide diversity of 1.73% (± 0.87).

The analysis in STRUCTURE identified three genetic populations (k = 3, deltaK = −6238.9; Fig. A.2 in Supplementary material) that are conform to the three sampling locations and showed a very clear genetic structure (Fig. 2). Population 1 included all 68 individuals from Calgary (0 km) and all bait shop individuals, population 2 comprised all 40 individuals from the recreational area 50 km south of Calgary in Eden Valley 216, and population 3 included the 46 individuals from the mountain area 100 km south of Calgary. Only subpopulation 0_1 shared a considerable number of alleles with population 2 and only some individuals in subpopulation 100_1 .

3.2. Population structure

The mean number of alleles in the three genetic populations assigned by STRUCTURE was highest in the population at the Calgary urban area (bait shop and 0 km) with 27 alleles, lower at 100 km with 18, and lowest at 50 km with 12 alleles (Table 1a). Mean observed heterozygosity (*Ho*) was 0.69 and nearly identical among populations. Just as for the whole dataset, all loci of genetic populations were one hundred percent polymorphic and deviated significantly from *HWE*, and expected heterozygosity (*He*) was higher than *Ho*. Fixation indices were 0.197 (F_{IS}), 0.226 (F_{IT}), and 0.036 (F_{ST}) (Fig. 4), and genetic differentiation (pairwise F_{ST}) between populations was generally very low, with F_{ST} values between populations ranging between 0.020 and 0.032 (Table 2a). Four alleles (LTM 128, 187, 193 and 252) showed evidence for the possible occurrence of null alleles in all three assigned populations, which is probably caused by population substructure (Wahlund effect). However, this potential substructure was not analysed further, as this would require a more intensive sampling. A second STRUCTURE analysis including only the three unsuspicious markers (LTM 163, 278 and 026) produced the identical population structure.

Mean genetic variance (AMOVA) among all markers was highest within subpopulations (71%), considerably lower among subpopulations (25%), and lowest among populations (4%). The number of shared genotypes among the three genetic populations was also low; 19% of all haplotypes occurred in more than one population (Table 3). The inbreeding coefficient was lowest in the 50 km site (F_{IS} = 0.149)

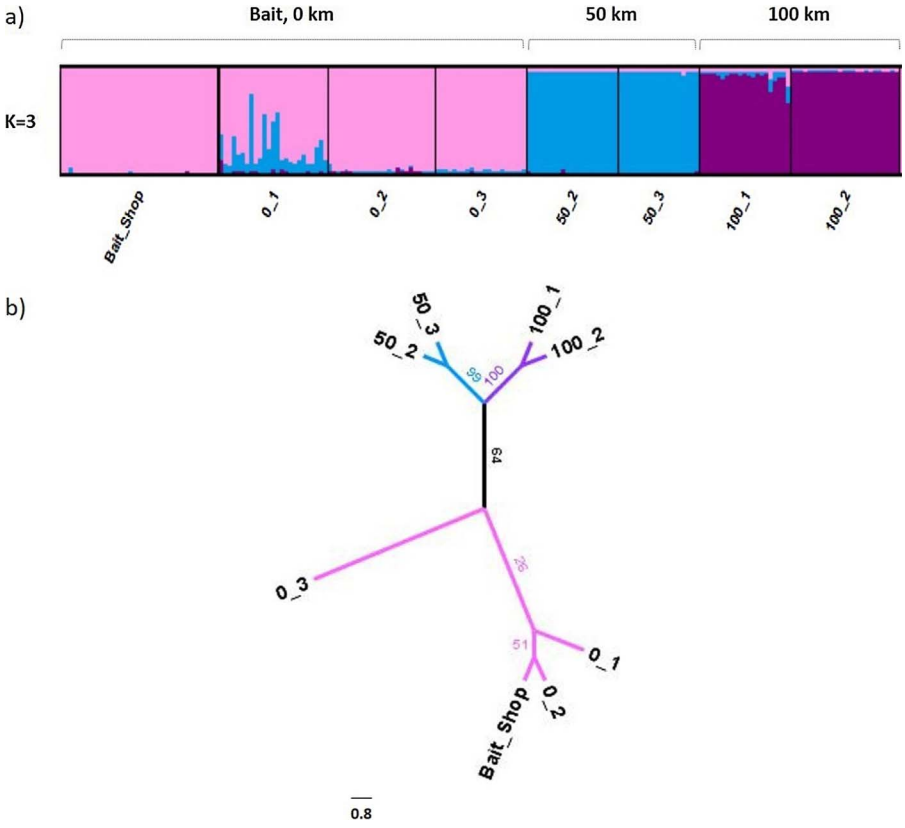

Fig. 2. a) Bar plots depicting the assignment of individuals of *Lumbricus terrestris* from three sampling areas to a specified number of clusters (k). Assigned clusters are shown on top, the number of genetic clusters (k) on the left, sampling locations below. Each individual is represented by a thin vertical bar. b) Unrooted population tree based on Nei's D_A genetic distance. Subpopulations (triangles) 0–1 and 0–2, 50-1 and 50-2, 100-1 and 100-2 are 5 m apart; subpopulation 0–3 is 5 km apart from subpopulations 0–1 and 0–2.

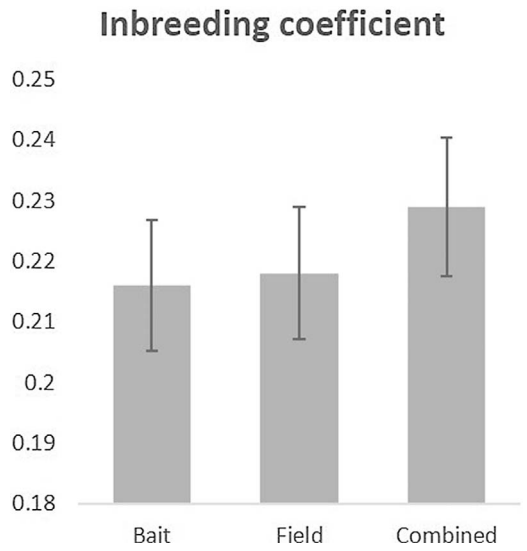

Fig. 3. Mean inbreeding coefficients for field, bait shop, and the combined datasets of field and bait shop populations of *Lumbricus terrestris*. Error bars are standard errors.

followed by the combined population of bait shop and urban area of Calgary individuals at 0 km (F_{IS} = 0.217), which was very similar to the variance within the mountain area 100 km south of Calgary (F_{IS} = 0.221).

Some structure emerged when comparing F_{ST} values between the eight subpopulations, i.e. combined bait shop individuals, sampling points 0_1-0_3 (Calgary), 50_1, 50_2 (50 km south), and 100_1, 100_2 (100 km south). Pairwise F_{ST} values were highest between the Calgary area at 0 km and the 100 km site (0.06–0.09; Table 2b), with subpopulation 0_3 being the most divergent in the whole dataset. The Calgary area and bait shop individuals were moderately differentiated (0.03-0.04), and subpopulations within the 50 km sites were rather

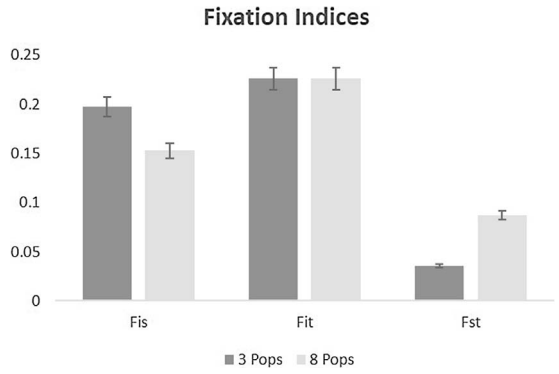

Fig. 4. Fixation indices (F_{IS}, $F_{IT,}$ *and* F_{ST}) for the three populations of *Lumbricus terrestris* assigned by STRUCTURE and eight subpopulations from the seven sampling points as well as the pooled bait shop samples. Error bars are standard errors.

similar (0.02) and moderately different to all other subpopulations (0.04-0.07).

3.3. Tree representation and haplotype networks of the mitochondrial marker

Haplotypes separated into four well-supported [posterior probabilities (pp): 0.98-1] and one moderately supported clades (pp: 0.92) (Fig. A.3 in Supplementary material). The genetic populations inferred from microsatellite markers mixed in most clades but the structure of the phylogeny was largely recovered by the haplotype network (Fig. 5). The largest cluster (Clade 1) was dominated by individuals from 50 km (green) and shared one haplotype with 13 individuals from location 100 km south (turquoise) and nine individuals from the Calgary urban area (0 km; blue), and another haplotype with one individual each from 0 km and 100 km. Individuals from bait shops (grey) dominated in clade 2, but shared two haplotypes with individuals from the city area.

Table 2
Pairwise F_{ST} values calculated from microsatellite data a) between three populations of *Lumbricus terrestris* and b) eight subpopulations; bait = bait shop individuals.

(a)

Population	Bait, 0 km	50 km	100 km
Bait, 0 km	0		
50 km	0.031	0	
100 km	0.02	0.032	0

(b)

Population	Pop1_0_1	Pop2_0_2	Pop3_0_3	Pop4_50_2	Pop5_50_3	Pop6_100_1	Pop7_100_2	Pop8_Bait
Pop1_0_1	0							
Pop2_0_2	0.031	0						
Pop3_0_3	0.041	0.04	0					
Pop4_50_2	0.04	0.042	0.046	0				
Pop5_50_3	0.051	0.049	0.061	0.018	0			
Pop6_100_1	0.062	0.058	0.061	0.051	0.052	0		
Pop7_100_2	0.074	0.07	0.086	0.072	0.07	0.05	0	
Pop8_Bait	0.037	0.029	0.037	0.046	0.056	0.053	0.068	0

Table 3
Population assignment of genotypes for three populations of *Lumbricus terrestris*, representing the number of genotypes that are unique to each population (self) and present in other populations (other) for each assigned population. The total number and percentage of genotypes unique or shared among populations in this dataset are also given; bait = bait shop individuals.

Population	Self	Other
Bait, 0 km	81	23
50 km	37	3
100 km	35	11
Total	153	37
Percentage	81%	19%

Individuals from the most isolated area 100 km south dominated clade 3 with 14 individuals and shared one of two haplotypes with a bait shop individual. Clade 4 was dominated by individuals from the Calgary urban area, but all three haplotypes included one (HT 1) or several (HT 4) individuals from the other locations. Clade 5 was genetically isolated from the other clades and consisted of only 5 individuals from the Calgary urban area. Haplotypes 6 and 7 from Calgary (0 km) were isolated haplotypes.

4. Discussion

All individuals were genetically very similar with mean Nei's genetic distance values of 0.043 among populations, suggesting that individuals are more similar to each other compared to the datasets of Gailing et al. (2012), who investigated four North American (d = 0.058) and seven European (d = 0.064) populations. However, Gailing et al., 2012 used in part different microsatellite marker assemblies hampering a direct comparison. The similarity among individuals in this study supports our assumption of very recent introductions and spread of propagules in the sampling area. Subpopulations sampled 5 m apart from each other always represented a single population, but genetic variance and allelic richness differed among populations at 5 km distance and was significantly different at 50 km distance.

The population from Calgary and the bait shop individuals were very similar, with one subpopulation from the city area being slightly different, suggesting either bait disposal or a common origin of field and bait individuals in this area. Though genetically very similar, populations at the three sampling areas differed significantly with alleles reoccurring at different frequencies. This suggests that all earthworms have a common origin and that bottlenecks due to releases of few individuals from a genetically diverse source generated the present genetic structure. Long-distance dispersal of *L. terrestris* is not well understood, but our data indicate that migration and gene flow between populations, for example by unintentional human transport, is unlikely. However, the release or distribution of few individuals apparently suffices to establish field populations; whether these are singular or repeated events needs further investigation. The three sampled bait shops only represent a subset of all potential bait shops within the vicinity of Calgary, and other bait shop populations could theoretically be genetically more closely related to the two other field locations south of Calgary, in particular the 50 km population. We tried to minimise this sampling bias by covering two bait shops providing earthworms from both dominant distributers in North America as well as one shop offering potential locally obtained baits. Nonetheless, with over ten bait shops in the area, more research on the different sources and genetic diversity of bait shop earthworms is needed.

Bait individuals showed a relatively high number of alleles at microsatellite markers and several genetic lineages of the 16S gene, thereby representing a genetically diverse source population. The importance of human activities to dispersal and introductions of earthworms into the wild has been demonstrated by several studies (Gundale et al., 2005; Keller et al., 2007; Holdsworth et al., 2007a,b; Cameron et al., 2007). Human dispersal of earthworms combined with the genetic diversity of bait individuals likely facilitates the successful establishment of *L. terrestris*.

Compared to other European earthworms that are now common in North America, *L. terrestris* is relatively sensitive to frost (Tiunov et al., 2006), but the presence of juveniles in all sampling locations indicates that populations established and reproduce successfully, despite long and cold winters. Although earthworms are thought to be limited by harsh climatic conditions (Bohlen et al., 2004; Frelich and Reich 2010), and minimal winter soil temperatures has been suggested to determine the northern boundary of earthworm distribution (Tiunov et al., 2006), the thick snow pack often present during winter months in the study region may insulate the soil from cold air temperatures and thus may allow earthworm survival.

This is the first study to investigate the population structure of the invasive earthworm species *L. terrestris* with microsatellite markers at fine spatial scales and to assess mechanisms of introduction and spread. Whether genetic diversity and structure among populations are low, moderate, or high at broader spatial scales can only be inferred by further studies in other areas of North America and preferably Europe, to compare allelic richness among wild populations. However, repeated

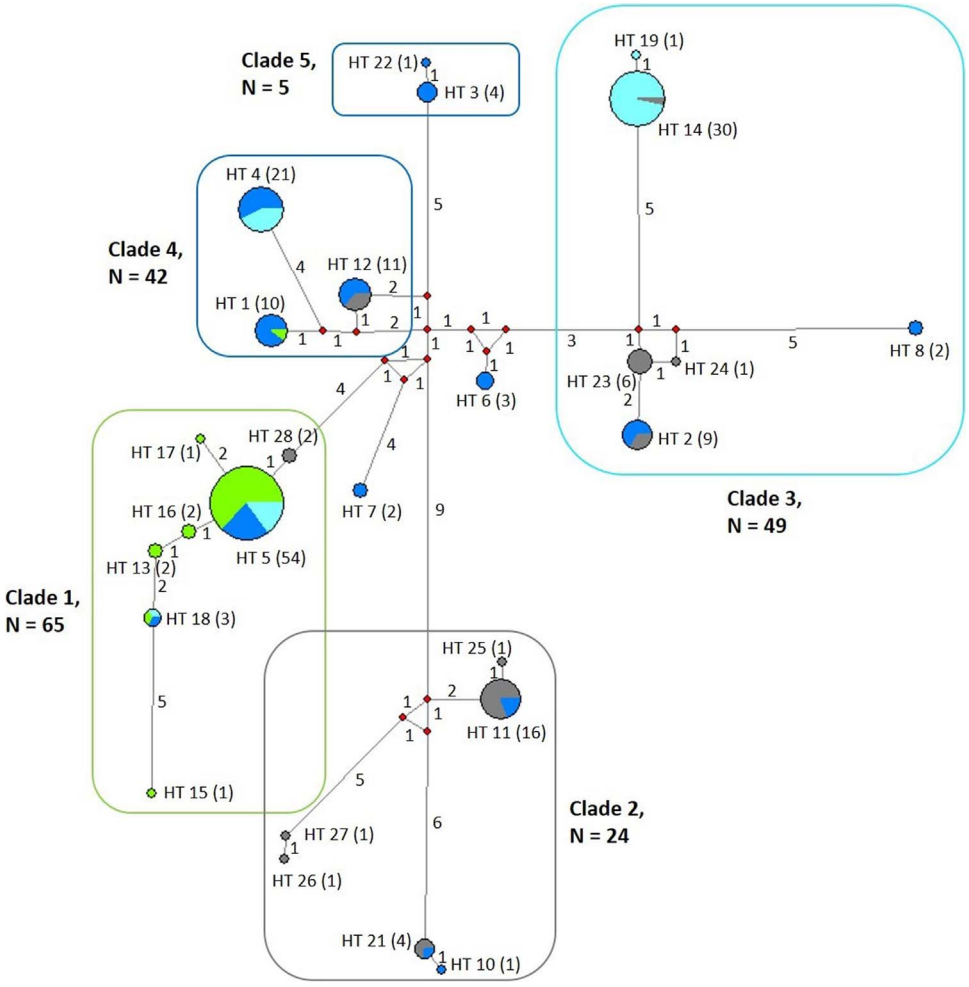

Fig. 5. Haplotype network based on 16S rDNA of *Lumbricus terrestris* from sampling locations in Calgary (blue) and 50 km (green) and 100 km (turquoise) south of Calgary as well as from three bait shops (grey). Clades were assigned in accordance with branch/node support by posterior probabilities. Numbers on branches are distance steps. The size of the pie charts indicate the number of individuals represented by the haplotype, the concrete numbers are indicated in brackets. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

introductions from a genetically diverse source or from several source populations, likely facilitated the successful establishment of earthworms in Alberta. As this species only recently started invading forests in Alberta (Scheu and Mclean, 1993), it provides an ideal model system to investigate population dynamics and adaptive processes during early invasion of an anecic earthworm species and to monitor potential management strategies for controlling further spread of earthworms into remote forested areas.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2017.08.009.

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