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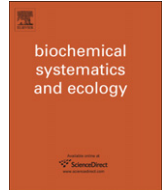
Genetic comparisons between North American and European populations of *Lumbricus terrestris* L.

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ABSTRACT

The common earthworm *Lumbricus terrestris* L. is an invasive species that was introduced to North America by European settlers. Subsequently, earthworms have been distributed by human activity, invaded a wide geographic range and changed previously earthworm-free ecosystems. In the present study we analyzed seven European and four North American populations from a wide geographic range at three formerly described nuclear microsatellite markers. All three markers produced multi-banding patterns and marker presence versus absence was scored in 88 narrow size intervals. Similar levels of genetic variation were observed for North American (Nei's gene diversity = 0.058, Shannon's $I = 0.100$) and European populations (Nei's gene diversity = 0.064, Shannon's $I = 0.104$). North American populations showed a higher similarity among each other than European populations in accordance with their recent introduction to North America. The relatively high level of genetic variation in North American populations and the high similarity among each other suggest their establishment from genetically diverse founder populations and rapid human-mediated population expansion. The source regions in Europe are still unclear from this analysis.

1. Introduction

Native North American earthworms have not fully recolonized North American forests since the last glaciation (Hendrix, 2006). The anecic earthworm, *Lumbricus terrestris* L. was first introduced to North America by early European settlers (Gates, 1976; Eisenhauer et al., 2007). Since then multiple introductions of *L. terrestris* to North America have occurred (Lindroth, 1957; Gates, 1976) and it has subsequently been distributed through human activities such as road constructions, transport of soils and release of unused fishing bait (Hendrix and Bohlen, 2002; Cameron et al., 2007). Today it is one of the most widely distributed exotic earthworms, having been reported in 38 US states and 10 Canadian provinces (Reynolds, 2008). *L. terrestris* is invasive over a wide geographic area, changing ecosystems that have evolved without earthworms (Bohlen et al., 2004; Hendrix, 2006; Scheu and Parkinson, 1994). Sequencing of the cytochrome oxidase I gene for nominal *L. terrestris*

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L. samples from Europe and North America revealed two species, *L. terrestris* and *Lumbricus herculeus*, in European samples, but only one species (*L. terrestris*) in North American specimen (James et al., 2010).

Earthworms are considered to be "ecosystem engineers" (Jones et al., 1994) because they modify the environment from its existing condition (Frelich et al., 2006; Lavelle et al., 2006). For example, anecic earthworms such as *L. terrestris* build permanent burrows, and pull large amounts of leaf litter into it, which they consume. They line their burrows with their casts, and deposit the rest of the cast on the soil surface (Zicsi et al., 2011) thus redistributing organic matter and minerals throughout the soil horizon. These alterations cause considerable changes in the composition and distribution of soil biota and ecosystem processes (Frelich et al., 2006; Szlavecz et al., 2011).

While the European origin of *L. terrestris* has been documented (Frelich et al., 2006; Hale et al., 2006), genetic studies comparing the level of genetic variation in potential source populations from Europe and North American populations are missing. Introductions from genetically diverse source populations are expected to maintain levels of genetic variation and thus the evolutionary potential ("invasiveness") of exotic species. Multiple introductions from genetically differentiated source populations can especially increase the level of genetic variation if formerly isolated populations come into secondary contact (e.g. Ellstrand and Schierenbeck, 2000; Kolbe et al., 2004). Genetic analyses can reveal the amount of genetic variation that exists within and among European and North American *L. terrestris* populations and provide insights into the geographic origin of North American earthworm populations and the importance of repeated introductions versus population expansion in the invasion of this species.

This project was designed to assess the population genetic structure of local North American and European earthworm populations. We hypothesize that North American earthworm populations (1) show no reduced genetic variation as compared to European populations due to their establishment from genetically diverse founder populations and subsequent population expansion, (2) are more similar to each other than European populations as result of a shorter divergence time, mixing via extensive transport, and the absence of severe genetic bottlenecks.

2. Material and methods

2.1. Sample collection

Earthworm samples were collected from 2004 to 2007 for seven European and for four North American regions in 2010 (Table 1). A varying number of individuals were sampled at each location by first identifying distinctive *L. terrestris* castings and burrows and then using different extraction techniques. Identification of earthworms was based on external characteristics (Schaefer, 1992). Sequencing of the cytochrome oxidase I gene (COI) in the European samples according to James et al. (2010) revealed the presence of only one species (Richter et al. in prep.). Electrical and chemical extraction, and hand sorting were performed for European populations, and mustard and formalin solutions were used for North American populations to extract earthworms from their subterranean burrows (Table 1). North American individuals located within the same sampling area were considered to belong to the same population and were taken from points spaced a minimum of 10 m apart along three straight line transects separated by 30 m. For European populations the sampling points were at least 5 m apart. All earthworms were transported within individual freezer bags supplied with leaf litter located within close proximity to the burrow from which they were taken. Upon collection, samples were stored in a cooler until returning to the lab where they were stored in a −80 °C freezer.

2.2. Sample preparation, DNA isolation, and polymerase chain reaction

Earthworm samples were individually cleaned with warm water and a 70% ethanol solution in preparation for tissue extraction. Small pieces of tissue located at the anterior end of the specimen were removed with a scalpel and forceps and used for DNA isolation. The reagents and methodologies outlined in the Qiagen DNeasy Blood and Tissue Kit were used for this process.

Table 1
Sampling locations.

Abbreviation	Location	Description	Latitude	Longitude	Sample year	Sampling method	n
BOS	Bosnia Herzegovina	Garden, Sarajevo	43.84N	18.36E	2006	Handsorting	24
FRA-Bru	France	Meadow, near Bruz	48.40N	1.45W	2006	Electrical	11
FRA-Béd	France	Meadow, near Bédee	48.12N	2.10W	2007	Chemical	12
FIN	Finland	Lawn, Jokioinen	60.81N	28.48E	2004	Handsorting	12
SWE	Sweden	Lawn, Uppsala	59.80N	17.65E	2004	Electrical	8
GER-Tim	Germany	Pasture, near Timmendorfer strand	53.96N	10.76E	2004	Handsorting	12
GER-Bay	Germany	Lawn, near Bayreuth	49.94N	11.72E	2004	Electrical	11
MI-Huron	Michigan	Forest, Huron Mountain reserve	46.89N	87.87W	2010	Mustard solution	21
MI-Houg	Michigan	Forest, Houghton	47.53N	93.47W	2010	Mustard solution	20
MD	Maryland	Forest edge, near Baltimore	39.34N	76.63W	2010	Formalin extraction	19
ME	Maine	Hemlock, sugar maple forest, Unity	44.60N	69.33W	2010	Mustard solution	9

The microsatellite markers, LTM128, LTM163, LTM208, that were used in population genetic analyses in *L. terrestris* (Velavan et al., 2007, 2009) and additional microsatellite markers LTM26, LTM165, LTM193 and LTM278 originally developed for this species (Velavan et al., 2007) were tested for amplification and polymorphisms. Three microsatellites were polymorphic, exhibiting up to 6 bands per amplification reaction (5 for LTM163, 4 for LTM128, 6 for LTM165). Each of these bands exhibited typical microsatellite stutter and patterns were reproducible among samples. The three reproducible and polymorphic microsatellites LTM163, LTM128 and LTM165 were used to genotype all 159 specimen. The other markers did not amplify in our samples after repeated modifications of the PCR protocol. DNA amplification was conducted through polymerase chain reaction (PCR) in 15 μL reactions including 2 μL of both forward and reverse primers (each 5 pmol/μL), 7.5 μL of Qiagen HotStart mix (containing 10 mM Tris–HCl (pH 9.0), 1.5 mM MgCl$_2$, 50 mM KCl, 0.2 mM each of dNTPs, 0.6 U Taq DNA polymerase), 1.5 μL of ddiH$_2$O, and 2 μL of diluted template DNA ($\sim$20 ng). PCR reactions were conducted with the following temperature profile on the GeneAmp PCR system 2700 (Applied Biosystems): 15 min at 94 °C; 35 cycles consisting of 1 min stages at 94 °C, 60 °C for primer annealing, and 72 °C; 15 min of final elongation at 72 °C followed by an indefinite hold at 16 °C. PCR products were checked using gel electrophoresis in 2.5% agarose gels and ethidium bromide staining. All PCR products were electrophoretically separated on the QIAxcel Fast Analysis System (Qiagen) with the QIAxcel DNA high Resolution Kit for microsatellite analysis. Fragment sizes were determined and adjusted among runs including in each run the QX DNA size marker (25–450 bp, Qiagen) and reference samples as controls.

2.3. Data analysis

The Biocalculator Fast software associated with the QIAxcel System was used to determine fragment sizes. Since each microsatellite primer pair amplified multiple fragments, fragment presence/absence was assessed in 88 size intervals (28 for LTM128, 27 for LTM163, 33 for LTM33) and a 0/1 matrix was established. The software GenAlEx 6.41 (Peakall and Smouse, 2006) was used to obtain a summary statistics of genetic variation parameters including the mean and standard errors for percentage of polymorphic loci ($p_{max} < 1.0$), the mean number of different alleles N_a, unbiased Nei's genetic diversity UH_e according to Nei (1973) and Shannon's information index I (Brown and Weir, 1983; Sherwin et al., 2006) for each geographically distinguished population. Nei's unbiased genetic diversity using a small sample size correction was calculated as $UH_{e(a)} = \left(\frac{2}{2n-1} \right) \left(1 - \sum_i p_{i(a)}^2 \right)$, where p_i is the frequency of the ith allele and n is the sample size. For the calculation of UH_e the frequency of the null allele at each locus was estimated as the square root of the proportion of individuals in which the fragment was absent (Nei, 1987). Shannon's information index was calculated as $I = -\sum p_i \ln p_i$, where p_i is the frequency of the ith fragment. Genetic distances among populations were calculated using Nei's unbiased genetic distance (Nei, 1978) in GenAlEx. Unweighted pair-group method with arithmetic means (UPGMA) and neighbor-joining (NJ) trees were generated from these results using the neighbor-joining program within Phylip 3.69 (Felsenstein, 1989). The dendrogram was visualized in TreeView (Page, 1996) and shows the relationships between populations using Nei's unbiased genetic distance (Hedrick, 2000; Nei, 1978). *F*-statistics were calculated by analysis of molecular variance (AMOVA) using Φ_{pt} as analog of F_{ST} for binary data (Huff et al., 1993; Weir and Cockerham, 1984) to describe genetic differentiation among populations, between geographic regions (Europe vs. North America) and differentiation among populations within geographic regions.

Population structure was analyzed with the program Structure 2.2 (Pritchard et al., 2000), which utilizes Bayesian statistics via the Markov Chain Monte Carlo (MCMC) algorithm to detect the genetic structure of populations for individuals across multiple markers by quantitatively clustering individuals based on the proportion of their genome belonging to a recognized population. A parameter set was defined as having a burn-in period length of 100,000 followed by 1,000,000 MCMC repetitions. Five runs were completed for each iteration for a range of K values (number of clusters) between 2 and 15.

UPGMA and NJ trees based on pairwise character differences among individuals were calculated with the software PAUP 4.0b10 (Swofford, 1998) and statistical support of clades was evaluated with 1000 bootstrap replications. A principal component analysis based on pairwise characters differences was performed with GenAlEx 6.41 to identify groups of related individuals and geographic structure in the data. Fine scale spatial genetic structure was analyzed for the North American populations using spatial autocorrelation analysis (Smouse and Peakall, 1999) with even samples sizes per distance class in the program GenAlEx 6.41. Upper and lower bounds of the 95% confidence intervals were determined by 999 random permutations of individuals among distance classes.

3. Results

3.1. Genetic variation within populations

On average Nei's gene diversity and Shannon's information index were slightly higher in European populations ($UH_e = 0.064$, $I = 0.104$), as compared to North American populations ($UH_e = 0.058$, $I = 0.100$) (Table 2). Directly observed genetic variation measures N_a and PPL showed similar values in both geographic regions with slightly higher values for North American populations with larger sample sizes.

The highest genetic variation was observed in the French population FRA-Bru ($UH_e = 0.082$, $I = 0.128$). Among European populations the lowest genetic variation was found in the Finnish population FIN ($UH_e = 0.053$, $I = 0.088$), while among North

Table 2
Summary of genetic variation calculated for individual populations (means and standard errors).

Populations	n	N_a	I	UH_e	PPL
European					
BOS	24	0.773 ± 0.104	0.105 ± 0.017	0.060 ± 0.011	38.64%
FRA-Bru	11	0.727 ± 0.103	0.128 ± 0.021	0.082 ± 0.014	36.36%
FRA-Béd	12	0.591 ± 0.098	0.103 ± 0.019	0.064 ± 0.013	29.55%
FIN	12	0.545 ± 0.098	0.088 ± 0.017	0.053 ± 0.011	27.27%
SWE	8	0.523 ± 0.096	0.095 ± 0.018	0.061 ± 0.012	26.14%
GER-Tim	12	0.523 ± 0.094	0.092 ± 0.019	0.058 ± 0.013	26.14%
GER-Bay	11	0.727 ± 0.103	0.113 ± 0.019	0.069 ± 0.012	36.36%
Mean	*11.43*	*0.630 ± 0.037*	*0.104 ± 0.007*	*0.064 ± 0.004*	*31.49% ± 2.06%*
North American					
MI-Huron	21	0.932 ± 0.107	0.102 ± 0.014	0.054 ± 0.008	47.06%
MI-Houg	20	0.841 ± 0.106	0.108 ± 0.016	0.061 ± 0.010	42.04%
MD	19	0.795 ± 0.105	0.107 ± 0.017	0.061 ± 0.011	39.77%
ME	9	0.443 ± 0.088	0.082 ± 0.018	0.054 ± 0.013	21.59%
Mean	*17.25*	*0.750 ± 0.052*	*0.100 ± 0.008*	*0.058 ± 0.005*	*37.50% ± 5.49%*
Overall mean		*0.674 ± 0.030*	*0.102 ± 0.005*	*0.061 ± 0.004*	*33.68% ± 2.39%*

American populations the northeastern population from Maine ME ($UH_e = 0.054$, $I = 0.082$) and the population from Northern Michigan MI-Huron ($UH_e = 0.054$, $I = 0.102$) showed the lowest genetic variation.

3.2. Genetic differentiation among populations

Most of the genetic variation was distributed within populations (92.8%), while 2.0% ($\Phi_{ptr} = 0.020$, $p = 0.028$) was distributed among the geographic regions Europe and North America and 5.2% ($\Phi_{ptp} = 0.052$, $p = 0.004$) among populations. The differentiation among North American populations was slightly higher ($\Phi_{ptp} = 0.061$, $p = 0.022$) than among European populations ($\Phi_{ptp} = 0.047$, $p = 0.026$; Table 4).

The highest genetic distance between populations was observed for the French population FRA-Bru and the North American population ME (Maine, United States) (Table 3). Very low genetic distance (Nei's unbiased distance = 0.001) was observed for the European population pair FIN/GER-Tim and for the North American population pairs MI-Houg/MD, MI-Huron/MD and MD/ME. Pairwise Nei's unbiased genetic distances ranged from 0.001 to 0.003 (mean distance = 0.0017) for North American populations, from 0.001 to 0.011 (mean distance = 0.00571, $n = 21$) for European populations and from 0.002 to 0.014 (mean distance = 0.00653, $n = 28$) for interregional comparisons. European populations from the Balkan region (BOS) and from Finland (FIN) showed the lowest genetic distance to North American populations (Table 3). In the UPGMA dendrogram the North American populations grouped together within the European populations as sister group to GER-Bay/GER-Tim/BOS/FIN (Fig. 1a). In the NJ tree, North American populations grouped together, but their relationship to European populations was not resolved (Fig. 1b). Within geographic regions the two Scandinavian populations FIN and SWE grouped together with the two German populations GER-Tim and GER-Bay as sister group to the two French populations FRA-Bru and FRA-Béd. In the North American cluster the eastern populations MD and ME formed one cluster. UPGMA and NJ dendrograms calculated with PAUP as well as the principal component analysis (PCA) based on pairwise character differences between individuals showed no geographic pattern. Thus the PCA revealed four distinct groups, each group containing individuals from different populations and geographic regions, and dendrograms showed no groupings according to geographic origin (data not shown). Likewise the program Structure revealed no population structure for $K = 2$ to $K = 15$ with each individual showing similar likelihoods to belong to each putative cluster K (data not shown).

Table 3
Nei's unbiased genetic distance between *Lumbricus terrestris* populations.

Populations	BOS	SWE	FIN	GER-Tim	GER-Bay	FRA-Bru	FRA-Béd	MI-Houg	MI-Huron	MD	ME
BOS											
SWE	0.008										
FIN	0.002	0.004									
GER-Tim	0.003	0.008	0.001								
GER-Bay	0.003	0.006	0.002	0.003							
FRA-Bru	0.009	0.008	0.005	0.010	0.007						
FRA-Béd	0.005	0.011	0.006	0.008	0.006	0.006					
MI-Houg	**0.003**	**0.009**	**0.005**	**0.006**	**0.006**	**0.010**	**0.006**				
MI-Huron	**0.003**	**0.009**	**0.004**	**0.004**	**0.005**	**0.008**	**0.004**	0.002			
MD	**0.002**	**0.009**	**0.004**	**0.005**	**0.006**	**0.010**	**0.005**	0.001	0.001		
ME	**0.004**	**0.012**	**0.006**	**0.007**	**0.006**	**0.014**	**0.010**	0.002	0.003	0.001	

Genetic distance between populations from different regions (Europe, North America) are printed in bold.
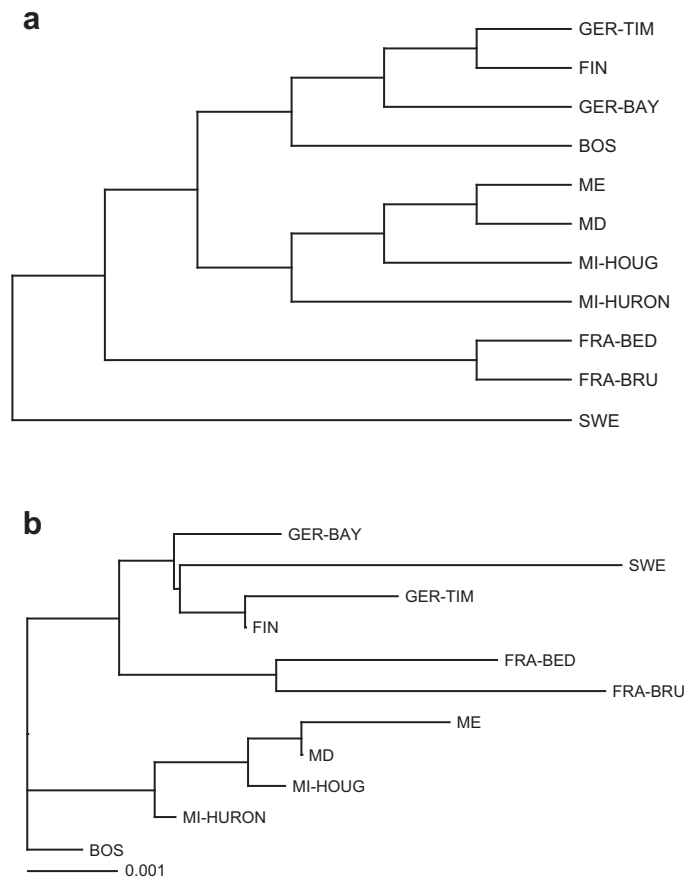
Fig. 1. UPGMA (a) and Neighbor-Joining (b) dendrograms based on Nei's unbiased genetic distances.

Spatial autocorrelation analysis revealed no significant fine scale genetic structure in North American earthworm populations (data not shown) suggesting a random distribution of genotypes within populations. No spatial data for individual samples were available for European populations.

4. Discussion

4.1. Genetic variation within European and North American populations (Hypothesis 1)

Nei' unbiased genetic diversity and Shannon's Information index (Shannon entropy) were chosen to compare levels of genetic variation between North American and European populations, since they include both variety (number of different variants) and balance (frequency of the variants) information about genetic diversity with Shannon's index being more sensitive to variety (Sherwin, 2010; Sherwin et al., 2006).

Similar levels of genetic variation in terms of Nei's unbiased genetic diversity and Shannon' Information index in North American and European populations suggested the absence of strong reductions in population size (genetic bottlenecks) during population establishment and expansion. Thus, the establishment of new *L. terrestris* populations in formerly earthworm-free habitats was most likely not the result of long-distance dispersal of only a few adult individuals or cocoons. The relatively high genetic variation of North American earthworm populations from different geographic regions could be the result of human-mediated dispersal of genetically diverse groups of individuals for example by soil movements or other vectors especially in more urbanized regions. Only a slightly lower genetic variation of *L. terrestris* populations was found in more isolated forest sites MI-Huron (Huron Mountain Reserve) and ME (Maine, Hemlock sugar maple forest) as compared to sampling sites close to cities (MI-Houg, MD). The assessment of genetic variation of isolated populations at the species' distribution edge and from urbanized areas is necessary to confirm such differences in genetic variation. Earlier analyses in Northern Russia and on the Faroe islands showed strong evidence that population establishment of previously earthworm-free habitats was associated with human disturbance (e.g. passive transport of earthworms by soil) (Enckell et al., 1986; Tiunov et al., 2006). The relatively

low active dispersal rate of earthworms (about 10 m per year, Mather and Christensen, 1988) suggests that active human-mediated dispersal was also the main agent for the rapid population expansion of *L. terrestris* in North America.

Among European populations similar levels of genetic variation were observed in the present study. Similar to North America, during the last glacial period the permafrost soils of Central and Northern Europe were devoid of *L. terrestris* (14,000–22,000 years ago) and population expansion by natural dispersal and human-mediated transport resulted in the re-colonization of regions as far North as northern Scandinavia and northern Russia (Tiunov et al., 2006). The lowest genetic variation was observed in the population from Northern Europe (Finland) for which a more recent postglacial re-colonization was expected as compared to more southern populations in France, Bosnia Herzegovina or Germany. However, similar to North American populations, there was no evidence for a severe genetic bottleneck during the species' re-colonization history. In accordance with our results genetic variation analyses at AFLP markers of 15 European *L. terrestris* populations (seven of which were included in the present study) from Scandinavia, Central Europe, Western France and the Balkan region (Bosnia Herzegovina) revealed only minor differences in genetic variation for the different populations (Richter, 2009).

In summary, invasions of *L. terrestris* into earthworm-free habitats in Northern Europe and North America were not associated with a reduction in genetic variation suggesting effective human-mediated transport and random mating within populations of this obligatory outcrossing species. The absence of family structures among individuals within North American populations as shown by the random distribution of genotypes in the spatial autocorrelation analysis suggests random mating within populations and/or current human-mediated admixture as the result of extensive transport.

4.2. Genetic variation among populations (Hypothesis 2)

The overall low genetic distance among *L. terrestris* populations and the clustering of individual samples independent from their geographic origin (data not shown) reflects the recent population expansion in Europe (after last glacial maximum of Quaternary glaciations about 14,000 years ago) and in North America (since the 18th century). The lower average genetic distances between North American populations (mean distance = 0.0017, $n = 6$) compared to European populations (0.00571, $n = 21$) and interregional comparisons (mean distance = 0.00653, $n = 28$) are in accordance with the much shorter divergence time since earthworms have been introduced to North America. Considering the relatively high within population genetic variation for North American populations, the higher similarity among North American populations suggests a restricted geographic origin or multiple geographic origins and population admixture before population expansion. Multiple and recent earthworm introductions from different geographic regions to different parts of Eastern North America are less likely for the studied samples assuming similar dispersal rates in North America and Europe.

The North American populations grouped together in the UPGMA and Neighbor-Joining trees based on genetic distances between populations and showed the lowest genetic distance to the European populations from the Balkan region (BOS) and Finland (FIN). Interestingly these two European geographically distant populations showed a high genetic similarity in the present study (see Table 3) and at AFLP markers (Richter, 2009) in accordance with preferential long-distance dispersal pathways that were also observed for other plant and animal species in Europe (Taberlet et al., 1998). Due to the limited number of European populations we are not able to narrow down the geographic origins of North American *L. terrestris* populations. The British Isles, not sampled here, are a possible source of North American populations because of intense early trade with North America and the practice of using soil ballast during this period (Brown, 1940; Lindroth, 1957). Due to the use of *L. terrestris* as fishing bait, to digest waste organic material and to improve garden soils, a large number of different introduction events to North America over time are likely (Gates, 1976). The ongoing transport and mixing of populations will hamper the identification of the geographic origins of North American *L. terrestris* populations.

The low percentage of genetic variation that was distributed among both European and North American populations in the present study and the absence of population structure are typical for outcrossing species with effective means of dispersal and a continuous distributions range corroborating the absence of drastic decreases in population size during population expansion.

However, our sampling of earthworm populations was restricted to the Northeastern part of the United States and did not cover the whole distribution range in Europe. Thus, higher genetic differentiation might be present between populations from other geographic regions. For example, a relatively high genetic differentiation of 15.17% at nuclear microsatellites was

Table 4
Summary of analysis of molecular variance (AMOVA) for marker phenotypes.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	Percentage of variation (%)	Φ-statistics	p-value
Between regions	1	46.949	46.949	2.0	0.020	0.028
Among populations	9	205.080	22.787	5.2	0.052	0.004
Within populations	148	1897.034	12.818	92.8		
North America						
Among populations	3	80.388	26.796	6.1	0.061	0.022
Within populations	65	836.004	12.862	93.9		
Europe						
Among populations	6	124.692	20.782	4.7	0.047	0.026
Within populations	83	1061.030	12.783	95.3		

observed between a Canadian sample set of 32 individuals (exact origin not known) and sample sites in southwest Germany (Tübingen, Baden-Württemberg) (Velavan et al., 2009) suggesting that the geographic origin of this Canadian population was not included in our sample.

Sequencing of the cytochrome oxidase I gene in samples classified as *L. terrestris* L. revealed the presence of the cryptic species *L. herculeus* in some European, but not in North American specimen (James et al., 2010). Sequences obtained for the European samples included in the present study showed only sequence variants of *L. terrestris* (Richter et al. in prep). The absence of significant population structure and the low differentiation between European and North American populations in the present study suggest the absence of *L. herculeus* from our sample.

4.3. Conclusions

The observation of similar levels of genetic variation in North American and European populations and the comparatively high similarity of North American *L. terrestris* populations suggests their establishment from genetically diverse founder populations and rapid human-mediated population expansion. The analysis of additional European and North American *L. terrestris* populations and more discriminative genetic markers might help to narrow down the geographic origins of North American populations.

Acknowledgments

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