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Spread and performance of European earthworms invading North America as indicated by molecular markers and climate chamber experiments

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SUMMARY

European lumbricid earthworms were introduced into northern North America by European settlers about 400 years ago. They are invasive across the continent and cause notable changes in native forest ecosystems. Human-mediated introductions and dispersal significantly contributed to the spread of European species in North America, which commonly are used as fishing bait and are often disposed deliberately in the field. During their range expansion they encountered harsher climatic conditions as compared to their native range in Europe. Variance of abiotic factors and genetic identity or diversity can be of particular importance for successful invasions, because environmental filtering and ecological tolerance of genotypes determines performance, establishment and spread of invasive earthworm species. However, it is unclear if climate or geographic dispersal barriers shape genetic structure of earthworm populations, and whether the successful establishment of populations is based on adaptation or selection. Genetic diversity or identity of invasive earthworms in North America was never analysed in combination with climate conditions or the impact on soil properties and microbial functions.

In my PhD project I investigated the drivers for dispersal of invasive European earthworms in northern North America by using molecular markers and a climate chamber transplantation experiment. Earthworms were collected on continental, regional and local scale, i.e. > 500 km, about 100 km and within 25 km distance, respectively, and analysed using molecular markers for phylogeographic, population genetic and experimental analyses. Each chapter of this thesis investigated specific aspects of earthworm invasion in northern North America, and combined results provide new insights into drivers of earthworm invasion, i.e. (i) connectivity among populations at continental and regional scale, and the importance of dispersal barriers (**Chapter 2**), (ii) connectivity among populations at local scale and genetic structure of a recent invasion (**Chapter 3**), and (iii) role of climate conditions and origin on genetic identity/diversity, and on performance as well as soil and microbial functions (**Chapter 4**).

In the first part (**Chapter 2**) I investigated two invasive earthworm species (*Lumbricus terrestris* and *L. rubellus*) that co-occur in the same habitats but differ in ecology and use as fishing baits. For both species I tested if dispersal barriers, climatic selection, or anthropogenic activities, such as fishing bait disposal, shape the dispersal of free-living earthworm populations and if dispersal mechanisms are the same for both species or if they differ between species. Both species were sampled in five transects ranging from the east coast to the west coast of northern North America, and the sampling design including two major dispersal barriers, three different climate zones. Additionally, the same species

were purchased from bait shops near sampling locations to account for local introductions by bait disposal. Genetic diversity and structure within and among populations and bait shop individuals was assessed using four markers (COI, 16S rDNA, 12S rDNA, H3). Populations of both species are genetically diverse with little geographic structure, which was more pronounced in *L. terrestris* than in *L. rubellus*. Common haplotypes were present in all regions, but locally restricted haplotypes also occurred. Further, two distinct genetic clades of *L. terrestris* co-occurred only in two transects (Alberta and Minnesota). Genotypes identical to bait individuals were omnipresent in field populations of *L. terrestris*. Genetic diversity was high in both species, and invasive populations represented a genetic subset of European earthworms. Geographic and climatic dispersal barriers affected the less mobile species, resulting in differences in genetic structure between the two species. Results indicate common long-distance dispersal vectors that are valid for both species and specific vectors affecting only *L. terrestris*.

The second study (**Chapter 3**) investigated the genetic structure of *L. 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.

Lumbricus terrestris had been introduced into North America from different source populations in Europe for several hundred years and initiated severe changes in the invaded ecosystems. As a first step to disentangle the relative importance of genetic and environmental factors on earthworm invasions I investigated (**Chapter 4**) the performance of earthworm populations from climatically dissimilar locations in different environmental contexts as well as their impact on soil properties and the microbiome. I conducted a yearlong full-factorial transplantation climate chamber experiment with 180 individuals of *L. terrestris,* which were collected from three North American sites with distinct climate conditions, altitude, and history of European settlement. Four combinations of warm and cold temperature conditions, and wet and dry moisture conditions were simulated in a climate chamber, and genetic diversity and identity was determined of surviving individuals and offspring. The results indicated that seasonality of temperature and precipitation was the main determinant for earthworm

biomass gain, offspring number, and activity. Further, results showed significant effects of earthworms on soil moisture and microbial functions that were differently influenced by burrowing and litter burying/feeding activity, respectively. Genetic diversity and identity did not show a clear correlation with earthworm performance and ecosystem functions under the different climate conditions.

By combining genetic diversity of species and population genetic data at different geographic scales with climatic, geographic and historical factors, this thesis exemplifies the utility of molecular markers to address general questions in invasion biology, ecological adaptation and population structure. It further shows the potential but also limitations and caveats of transplantation climate chamber experiments that take genetic information into account. This study addressed several aspects of earthworm invasion in northern North America, i.e. intraspecific variance and climatic adaptation, and provides important insights into dispersal and genetic structure and diversity for future research. In order to understand drivers of earthworm invasion, on continental, regional and local scale, main results of this project were (1) genetic diversity of the two species is reduced, but similar to its native range, (2) passive transport of earthworms is important at all scales but differs among geographic scales, (3) climate and disturbance were identified as additional factors that affect the genetic structure of earthworm populations, and (4) genetic diversity increases in vicinity to human agglomerations. Overall, genetic structure and diversity as well as the importance of dispersal vectors vary among species, ecological groups and the geographic scale, and have to be considered in future research.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Preface

Earthworms are one of the most important functional groups of soil biota in terms of soil formation and maintenance of soil structure, and fertility in natural and anthropogenic ecosystems (Edwards, 2004). The positive effects of earthworms turn into detrimental effects in natural ecosystems that developed without their presence (Hale et al., 2005; Eisenhauer et al., 2007; Hendrix et al., 2008). This phenomenon has become apparent in northern North America that was free of earthworms since the Last Glacial Maximum (LGM) about 20.000 years ago, and was invaded by European earthworms rather recently, about 400 years ago (Gates, 1976; Reynolds, 1994). European earthworms that invade North American forests reduce the thickness of the litter layer, and change density and structure of the understory plant community and mineralisation and mixture of the soil. Their rapid range expansion across North America and strong effects on natural ecosystems raised concerns for biodiversity and nature conservation (James, 2004; Eisenhauer et al., 2010; Cameron et al., 2008; Hendrix et al., 2008; Wardle et al., 2011; for more details please refer to chapter 1.4 “Lumbricid earthworms and their role as ecosystem engineers in North America”).

In this study I investigated the genetic structure of two European earthworm species invading North America to assess historic and recent invasions and identify main drivers of long-distance earthworm dispersal. For this I used molecular markers for phylogeography and population genetics to infer common ancestors at different scales in space and time. The two investigated, congeneric species, *Lumbricus rubellus* and *L. terrestris* (Oligochaeta, Lumbricidae), are common in forests but exhibit different life-styles, i.e. epi-endogeic and anecic, respectively, so that general drivers of earthworm dispersal (relevant for both species) as well as specific drivers (relevant to a single species) can be identified.

Earthworms already have been described by Aristotle about 2,400 years ago but the beneficial aspects of earthworms, i.e. their importance for soil formation processes was described rather recently by Charles Darwin (Darwin, 1881). He recognised the positive aspects at a time when earthworms were generally viewed as pests in agriculture and gardening, and therefore set the standard and course for research in the following two centuries (Brown et al., 2004; Edwards, 2004).

Earthworm research today critically considers both their positive and negative effects as well as reciprocity of the above- and belowground systems. Comprehension of negative effects of earthworms

on ecosystems is closely associated with investigations of the impact of non-native invasive earthworm species on natural and anthropogenic ecosystems (James & Hendrix, 2004). In North America, earthworms affect native ecosystems more drastically than in other invaded regions such as Australia and New Zealand (James & Hendrix, 2004). There, European lumbricid earthworms are only dominant in disturbed habitats, e.g. agroecosystems, due to the presence of a competitive native earthworm fauna, which is absent in northern North America (James & Hendrix, 2004).

1.2 The importance of genetic diversity and identity for invasive species

Genetic diversity and identity affects the performance of species via changes in behaviour, fitness components and response to disturbances. Experimental studies demonstrated that genetic effects are of similar significance as the impact of species diversity on ecosystem functions (Crutsinger et al., 2006; Crutsinger et al., 2007; Crawford et al., 2007; Hughes et al., 2008). Further, genetic diversity and identity may directly affect the invasive potential of species and their impact on native ecosystems (Scheu & Drossel, 2007; Hughes et al., 2008). Therefore, investigating and assessing genetic diversity of invasive earthworms is essential for understanding and managing consequences for native ecosystems.

Only few studies used molecular markers to investigate ecological questions in earthworm ecology and, in particular, the invasion into North American forest ecosystems (Hansen et al., 2005; Cameron et al., 2007; Cameron & Bayne, 2009; Gailing et al., 2012; Fernandez et al., 2015). There is little information on the genetic structure of non-native lumbricid earthworm species in North America. I decided to investigate the two earthworm species *L. terrestris* and *L. rubellus* because variance in molecular markers can be compared in a straightforward way in closely related, i.e. congeneric, species. Further, polyploidy and parthenogenesis, which make inferences by molecular studies difficult, are rare or absent in both species.

In this study I used a combination of molecular markers suitable to address the spatial and temporal scales of my study. These markers were developed in various studies but were not applied to *L. rubellus* and *L. terrestris,* or were not tested on North American populations. These markers comprised nuclear and mitochondrial genes (Histone 3 and 12S, 16S, COI) that are appropriate for phylogeography and fine resolution markers (microsatellites) for population genetic studies. Notably, the mitochondrial genes were also applicable as barcoding markers so that juveniles could be included in this study and misidentifications in the field could be excluded after laboratory work. Developing and adapting laboratory protocols as well as evaluating their informative value for my specific questions and the two earthworm species were essential first steps in this project.

Previous studies that investigated invasive earthworms in North America using molecular markers were restricted to local or regional scales and few individuals, or species, or both (Hansen et al., 2005; Holdsworth et al., 2007; Cameron et al., 2007; Cameron & Bayne, 2009; Gailing et al., 2012). Here, I applied a large scale sampling scheme of forest habitats along the former glaciation line in North America spanning from the east to the west coast and compared genetic diversity and structure of the American populations.

1.3 Earthworm dispersal

Earthworms move actively over short distances, i.e. few meters, or they disperse over larger distances from few hundred meters to several kilometres passively as individuals or cocoons transported by other animals (e.g., vertebrates or birds), by heavy rain, flooding of rivers, and by human activities (e.g., cars, walkers, fishing, horticulture, forestry and agriculture). On the regional (~100 km) or trans-regional (>100 km) scale, passive transport by humans is the most likely mode, bridging large distances, including commercial distribution of fishing baits and agriculture. To infer the relative roles of regional and trans-regional earthworm dispersal I sampled earthworms at regional and trans-regional scale and used molecular markers for phylogeographic and population genetic analyses. Regional sampling included a 100 km transect in southern Alberta (CAN), the trans-regional sampling comprised five transects, each 700-1500 km apart, distributed along the Wisconsian glaciation line in northern North America.

In addition to active and passive dispersal, I tested if two major dispersal barriers in North America, the Great Plains and the Rocky Mountains, affect the distribution and thereby genetic linkage among populations. Further, I investigated if abiotic factors also affect the establishment of populations after dispersal, thereby shaping genetic structure among populations (Eckert et al., 2008). Earthworms are exposed to different climatic regions across their North American distribution range, varying from Atlantic climate at the east coast, to Pacific and Mediterranean climate at the west coast, and to moderate and cold continental climate in the heartland, representing significant differences in temperature and moisture conditions and seasonality. The continental and Pacific climate in North America is more extreme in seasonal moisture and temperature conditions and differs from western and central European climate regions, where invasive earthworms originated. Earthworms invaded and established populations in these three North American climate zones, and I established an experiment that tested if populations from distinct climatic regions adjusted to their regional climate conditions or if climate conditions selected for specific earthworm lineages. For this, I tested in a climate chamber experiment if North American populations of *L. terrestris* show preferences to climate

conditions and if preferences correlate with earthworm origin and if preferences to climate conditions are reflected by genetic identity.

1.4 Lumbricid earthworms and their role as ecosystem engineers in North America

Lumbricid earthworms (Lumbricidae) are soil-living Oligochaeta, a group of Annelida with homogenous segments covered with small bristles that move peristaltically by contractions of longitudinal and circular muscles. Lumbricids are a group of predominantly euro-asiatic terrestrial oligochaets with about 700 described species. Today, they occur worldwide due to recent anthropogenic dispersal, except for areas with very dry soils or stagnant moisture (Gerard, 1967; Phillipson et al., 1976). Lumbricids endure temperatures between 0 and 35°C, though the optimum range for most species is between 10 and 20°C (Lee, 1983; Edwards & Bohlen, 1996). They evade extreme weather conditions like occasional drought or frost by migrating into deeper soil layers, with some species entering dormancy (Lee, 1985). Among the large terrestrial oligochaets ("megadrile" earthworms), lumbricids are the most common and abundant taxon reaching densities from 100 individuals per square meter in agroecosystems to 400 or more individuals per square meter in grasslands and forests (Edwards & Bohlen, 1996; Edwards, 2004). Due to their large body size and high abundance they typically contribute substantially to total soil animal biomass (Edwards, 2004). Invasive earthworms often become dominant over endemic species once introduced in areas with some degree of disturbance, while the earthworm fauna in undisturbed natural ecosystems usually is more resistant resulting in co-existence of native and invasive species (Kalisz & Dotson, 1989; Dalby et al., 1998; Callaham & Blair, 1999; Hendrix & Bohlen, 2002).

The two congeneric European earthworm species *L. rubellus* and *L. terrestris* have similar mating cycles, and cocoon production is determined by seasonal changes in temperate climate regions. It usually starts in spring or early summer followed by a second mating cycle in autumn, resulting in one to 20 cocoons per reproductive cycle (Edwards, 2004). Both species are commonly used as fishing bait, however, *L. rubellus* is less common as bait in Canada (E. Cameron, pers. communication), but frequently sold as bait in the Midwest of the United States (N. Eisenhauer, pers. communication). They differ in morphology, life history and ecology. The soil-dwelling (anecic) earthworm species *L. terrestris* is among the largest lumbricid earthworms with a length of 90-350 mm and 6-10 mm in diameter, and has a brownish to purplish-red dorsal colour with a yellow-orange ventral side (Michaelsen, 1900; Gerard, 1964; Sims, 1973). The posterior region is depressed and paddle shaped. This species is obligatory biparental, copulates on the soil surface and produces spherical cocoons. It lives in permanent vertical burrows up to 1-2 m deep with a terminal chamber, and forages leave material around its burrow entrance, a behaviour that results in a rather stationary life style and low active

dispersal (Edwards & Bohlen, 1996; Sims & Gerard, 1999). *Lumbricus terrestris* is most numerous in grasslands and orchards, but also occurs in woodlands, arable soils and on riverbanks; generally, it prefers soils with a near neutral pH of 6 - 10 (Addison, 2009). This invasive species is of Palaearctic origin but was dispersed within the past 400 years by human activities to Asia, America, Australia, New Zeeland and the Polynesian Islands, and today can be considered as cosmopolitan species (Reynolds, 2004).

In contrast, *L. rubellus* is an epi-endogeic species with a length of 60-130 mm and 3-4 mm in diameter that lives in the humus and upper soil layers, and feeds on surface litter (Michaelsen, 1900; Gerard, 1964). It is similar in colour to *L. terrestris* but the red dorsal area extends more towards the tail, which is not paddle shaped. *Lumbricus rubellus* colonizes a wide range of habitats, usually moist areas with high organic matter; it tolerates low pH values of 3.5-8.4 (Sims & Gerard, 1999) and is frost tolerant (Tiunov et al., 2006). Like *L. terrestris*, this species is obligatory sexual, copulates in the upper soil or litter layer and produces spherical opaque cocoons of olive brown colour (Gates, 1978). Similar to *L. terrestris*, it originally is of Palaearctic distribution, but due to spreading by humans today it is of cosmopolitan distribution.

Through bioturbation both earthworm species facilitate soil formation and maintenance of soil structure and fertility (Lee, 1985; Edwards & Bohlen, 1996; Eisenhauer et al., 2007, 2010) and their effects are generally regarded as positive for plant growth (Scheu, 2003) and plant diversity (Eisenhauer et al., 2008). However, in North America forests developed without the presence of earthworms since the Last Glacial Maximum (Scheu & Parkinson, 1994; Bohlen et al., 2004; Hale et al., 2005; Eisenhauer et al., 2007; Hendrix et al., 2008), resulting in ecosystems with thick organic layers and low nutrient availability (Scheu & Parkinson, 1994; Suárez et al., 2003; Fisk et al., 2004; Hale et al., 2005; Eisenhauer et al., 2007; Hendrix et al., 2008). Here, activities of invading earthworms significantly increase nutrient cycling, plant growth, plant community composition, tree seedling density and understory plant diversity. These effects also resulted in the retreat of endemic North American plant species and even in endangering the fern *Botrychium mormo* (Gundale, 2002; Hale et al., 2005, 2006; McLean et al., 2006; Migge-Kleian et al., 2006; Holdsworth et al., 2007; Eisenhauer et al., 2007, 2009; Nuzzo et al., 2009; Straube et al., 2009). Thus, in non-native ecosystems, European earthworms place a novel selective filter for the performance and competitiveness of tree and herbaceous plant species, altering the course of plant regeneration (Frelich et al., 2012).

Presence, abundance and invasive potential of European earthworms and their impact on North American forest ecosystems depends on species specific behavioural traits and on their ecological group, i.e. anecic, epi- or endogeic earthworms (James & Hendrix, 2004). Their invasive potential will be addressed in Chapter 1.6 “Invasiveness”.

1.5 Distribution in North America and the origin of genetic diversity

The distribution and composition of lumbricid earthworm communities in the northern hemisphere is shaped by historical glaciation cycles as well as current climate conditions. The Last Glacial Maximum (LGM) 25 to 13 thousand years ago, which is called Wisconsinan glaciation in North America and Weichselian glaciation in Europe, almost completely eradicated temperate terrestrial fauna and flora in the northern parts of the American and European continent (Holmstrup, 2003). In North America, native earthworm species went extinct during glaciation in the northern parts (Bohlen et al., 2004; Hewitt & Ibrahim, 2004) and were restricted to few refugial areas in the warmer southern and coastal regions of the continent (Callaham et al., 2006) but they have not recolonized northern habitats yet (Gates, 1982; Reynolds, 1994). About 400 years ago, European earthworms were first introduced into North America at coastal areas by European immigrants, both accidentally and intentionally, and introductions continued, especially during the early periods of European trade and immigration in the 18th and 19th century (Scheu & Parkinson, 1994; Bohlen et al., 2004; Hendrix et al., 2008). Thick organic soil layers and absence of competitive native earthworm species facilitated the successful establishment of non-native earthworm species in forest habitats (Addison, 2009). Today European lumbricid earthworms are common in the eastern and middle-eastern parts of the USA, the Rocky Mountains in Canada, and some populations exist west of the Rocky Mountains on the Pacific coast where they occur in both natural and anthropogenic habitats and ecosystems (Reynolds, 1977, 1994; Scheu & Parkinson, 1994).

Quaternary climate changes also shaped the diversity and distribution of earthworms in Europe. Here, the earthworm distribution was restricted to southern refugia in Iberia, Italy, the Balkans, Turkey and southwestern central Asia and, in contrast to North America, European earthworms recolonized central and northern Europe within the past 10 thousand years (Sechi, 2013). However, present day species diversity still is low as compared to southern regions, decreasing from 180 in France and mainland Europe to 26 in the northern European British Isles (Sims & Gerard, 1999). Sechi (2013) analysed the evolutionary history of European populations of *L. rubellus* by correlating genetic and bioclimatic information and simulating past climate conditions in Europe during the last inter-glacial (LIG) and last glacial (LGM) periods. This study revealed that climate, opposed to soil conditions, is the major limiting factor for earthworm distribution. Further, divergence times showed that most lineages were already established before the LIG and LGM, and survived mostly in southern refuge areas, although other lineage distributions and demographics supported survival in northern refugia.

As North American earthworms directly derive from European populations, it is likely that invasive specimens express different climate preferences and genetic identities due to glacial and postglacial divergences and adaptations of populations in southern and central European refugia.

1.6 Invasiveness

Reynolds (2004) emphasised the importance of investigating the process of earthworm species invasion into North America to understand species distribution, migration and ecological aspects. He stressed to focus on the ecological factors that make the existence of a species possible including adaptations and limitations, i.e. structural, physiological, behavioural, and population dynamics that enable it to establish a population in new areas.

In earthworms, ecological function often determines dispersal abilities (Bouché, 1977) and consequently invasion potential. Although it seems obvious that litter dwelling epigeic species are more likely to be passively transported, followed by endogeic earthworms inhabiting the upper soil layers, and at last anecic species that live in deep vertical burrows, reality seems to be far more complex and related to ecological traits of the species (James & Hendrix, 2004; Caro et al., 2013; Chatelain & Mathieu, 2017). For instance, epigeic and anecic species typically do have more specific habitat requirements than endogeic species restricting their invasiveness, and consequently, endogeic species are more commonly invasive (James & Hendrix, 2004).

Earthworm invasion is most prominent in agroecosystems, particularly in North America, and the absence of native earthworm species suggests their incapability to tolerate frequent soil disturbances (Edwards & Bohlen, 1996; Lavelle et al., 1999). In agricultural fields, species diversity is low due to limited disturbance resistance and invasive potential of most earthworm species. It has been proposed that in future two or three major earthworm taxa will be dominant in agroecosystems across temperate, subtropical and tropical climate zones (James & Hendrix, 2004). In grassland and forest habitats species diversity usually is higher than in arable systems (Scheu & Parkinson, 1994; Callaham & Blair, 1999; Callaham et al., 2001) and earthworm invasion occurs in waves with epigeic species entering the new area first followed by polyhumic earthworms (i.e., endogeic species feeding on mineral soil with high organic matter content) and then the slow-moving anecic species (James & Hendrix, 2004).

1.7 Dispersal and human influence

Active dispersal is considered to be of little importance for the dispersal of lumbricid earthworms because of their soil-living stationary way of living (Edwards, 2004). Earthworms occasionally leave the soil and crawl on the soil-surface in particular after heavy rain or during the mating season. Their effective natural dispersal rate is species specific varying from 1 to 20 meter per year (Martinissen & van den Bosch, 1992). Therefore, the long-distance dispersal of peregrine earthworm species depends on passive transport by animals or humans. Human activities known to facilitate transport earthworms

into distant areas are human migration, horticulture, agriculture and trading of soil or soil-born goods (Suarez et al., 2006; Holdsworth et al., 2007; Keller et al., 2007; Cameron et al., 2007; Cameron & Bayne, 2009).

Today, European earthworms are present worldwide with only few areas occupied by humans that are free of introduced exotic earthworms due to unfavourable soil and/or climate conditions, e.g. the arctic and desserts (James & Hendrix, 2004). Passive transport of earthworms likely changed during historic times due to advancement of human transportation technology, resulting in "old" (i.e., postglacial) and "new" (i.e., recent global dispersal in the past 400 years) distribution patterns in invaded areas. The "old" postglacial distribution and genetic structure of earthworms in Europe was influenced by (I) surviving populations north of the Alps that spread from their refugia to the surrounding areas, and (II) the historic human migrations from south-eastern Europe and the Arabian Peninsula to the north and the simultaneous Neolithic Revolution, i.e. the transition from hunter-gatherer to agricultural societies (Zohary, 1996, 1999; Baker, 2009; Bocquet-Appel, 2011; Sechi, 2013). Contrasting to Europe, in North America only the "new" dispersal vectors formed distribution patterns and, thus, genetic structure likely differs from that in Europe. The "new" dispersal vectors likely affected today's patterns in Europe far less, and comparing both continents genetic structure can reveal the relative importance of historic and recent dispersal.

Apparently, the distribution, frequency and distance of translocation events of earthworms increased with advancing transportation technology, (transcontinental) trade-routes and road networks (Michaelsen, 1900; Crumsey et al., 2014), results in global homogenisation of earthworm communities, in particular in urban areas (Edwards, 2004).

1.8 Molecular markers

For this study, I used a set of molecular markers to investigate phylogeographic structure of the two globally distributed earthworm species *L. rubellus* and *L. terrestris,* and population genetics and migration on local and regional scale. The genetic markers had to (1) identify the target species (barcoding) including juvenile individuals, which are difficult to differentiate using morphological characters, (2) discriminate genetic differences within and among populations to link genetic and geographic structure, (3) detect mitochondrial and nuclear genetic relationships among populations, (4) include protein-coding and non-coding regions, and (5) provide fine-scale resolution to investigate population genetics, migration and ancestry/parentage.

1.8.1 Mitochondrial and nuclear markers

The selected markers included the mitochondrial protein-coding region cytochrome-c-oxidase subunit I (COI) as well as the two non-coding regions of 16S rDNA and 12S rDNA. They were used to infer phylogenetic diversity and lineage identity of the two investigated species. These three mitochondrial markers provide phylogenetic resolution at various phylogenetic depths and intraspecific genetic variance, which is necessary to evaluate and solidify the different parts of the phylogenetic tree (i.e., backbone, intermediate nodes, and terminal splits). Variable molecular makers allow to infer migration routes by tracing relationships of mitochondrial lineages (Cox & Hebert, 2001; Hebert et al., 2004; Chang & James, 2010; Novo et al., 2011). As an essential component of the respiratory chain (Tsukihara et al., 1995), COI retains some degree of conservation across species and was selected as barcoding gene for species identification (Hebert et al., 2003). In earthworms, COI presents intraspecific variations allowing discrimination between populations (Klarica et al., 2012; Decaens et al., 2013). As part of the ribosomes that are essential in the protein biosynthesis 16S rDNA and 12S rDNA differ in their degree of conservation from COI. The ribosomal 12S rDNA represents a rather conserved mitochondrial gene enabling to infer deeper relationships among mitochondrial lineages (Simon et al., 1994). Additionally, the 16S rDNA gene includes a useful barcoding region, which allows unambiguous species identification within the genus *Lumbricus* (Bienert et al., 2012).

Due to the maternal inheritance of mitochondrial markers, it is important to reconstruct population histories of maternal lineages and to compare mitochondrial genealogies with nuclear genetic lineages. Nuclear genes are subject to outcrossing, therefore combine the information of evolutionary processes of different individuals and represent an independent marker to mitochondrial genes. The selected nuclear protein coding region Histone 3 (H3) is one of five main histone proteins involved in the chromatin formation, with special importance for gene regulation (Cox et al., 2005). Therefore, histone proteins are highly conserved with low mutation rates in the DNA sequence providing a deeper phylogenetic level than mitochondrial markers.

All four genetic markers were reasonably well characterised in previous studies on other earthworm species (Folmer et al., 1994; Simon et al., 1994; Colgan et al., 1998; Pèrez-Losada et al., 2009). Their mutation rate decreases from the most variable COI to 16S rDNA to 12S rDNA to H3. The use of four genes with different substitution rates was important, as complex phylogeographic patterns were expected due to jump dispersal and multiple introductions. I expected that these four markers represent a powerful toolbox for investigating genetic diversity, invasion and dispersal of *L. rubellus* and *L. terrestris*.

1.8.2 Microsatellite markers

Microsatellites are sequence regions of repetitive DNA in which specific sequence motifs with a length of 1-6 basepairs are repeated between 5-50 times (Richard et al., 2008; Gulcher, 2012). They can occur at various locations within the genome and are referred to as short tandem repeats (STRs) or simple sequence repeats (SSRs). They have higher mutation rates than other parts of the DNA, especially within the nuclear genome, and therefore represent powerful markers to investigate genetic diversity of closely related individuals or populations (Jarne & Lagoda, 1996). Generally, these highly polymorphic molecular marker have several alleles per locus and typically are inherited co-dominantly (Jarne & Lagoda, 1996). Microsatellites occur in both coding and non-coding regions, although they are more frequent in the latter. They are without function and evolve neutrally without selective pressure, allowing to accumulate mutations quickly, and the resulting variability can be used for DNA fingerprinting (Chistiakov et al., 2006). High mutation rates result from the repetitive nature of microsatellite motifs, adding or losing one or several entire repeat units during the replication process is easy and is called slippage replication. Therefore, their mutation rates are higher than that of most mutation types, such as deletions, insertions or single nucleotide substitutions (Tautz & Schlötterer, 1994). Microsatellites are amplified with fluorescence-labelled primers in a polymerase-chain-reaction (PCR) and genotyped afterwards by capillary electrophoresis to assess the repeat length of the amplicons. Their neutral evolutionary history allows investigating processes, such as mate choice, habitat fragmentation, and historical processes (e.g., bottlenecks, local adaptation, dispersal, and invasion) as well as population size and gene flow (Avise, 1994, 1995; Field et al., 2007). Microsatellites are a common tool in population genetic studies that investigate relatedness among subspecies, populations, groups and individuals.

Of the ten available microsatellite loci that were highly polymorphic and informative for population genetic parameters in southern Germany (Velavan et al., 2007), eight were suitable to investigate North American populations of *L. terrestris* in my study (LTM 128, LTM 163, LTM 165, LTM 187, LTM 193, LTM 252, LTM 278, and LTM 026; Velavan et al., 2007).

In conclusion, the chosen microsatellite markers have the degree of polymorphism and reliability that is required for population genetics, i.e. genetic variance, extent of gene flow, and adaptive potential of genetic lineages (Sakai et al., 2001; Allendorf & Lundquist, 2003; Lawson Handley et al., 2011).

1.9 Objectives and chapter outline

The ongoing earthworm invasion into North American forest ecosystems provides the opportunity to study belowground invasion and the influence of soil-living species on natural ecosystems. I focused my thesis on the drivers of the invasion process itself, i.e. dispersal, gene flow, structure, and abiotic preferences of earthworm populations. The objectives of my study were to assess and compare the genetic structure and diversity of non-native *L. rubellus* and *L. terrestris* in North America and to test if the genetic diversity and structure related to geographical dispersal barriers, climate differences and human activities. Accordingly, the design of this study included different geographic scales with distinct climate conditions and natural dispersal barriers. I used four molecular markers to infer genetic diversity and population structure on different time scales. In a climate chamber transplantation experiment I investigated ecological differentiation among populations from different climate regions and if ecological differences correlated with genetic identity.

The following hypotheses were tested:

1) Populations that established at the east and west coast of North America are genetically distinct, due to environmental filtering by regional climate conditions, i.e. temperature and precipitation. Two major dispersal barriers (Rocky Mountains and Great Plains) maintain the separation between populations. **Chapter 2**
2) Human-mediated dispersal of earthworms counteracts local selection and negates dispersal barriers, resulting in diverse earthworm populations and genotypes that occur in all regions. **Chapter 2**
3) The proximity to human infrastructure (urban areas, road networks, fishing bait disposal) affects genetic diversity and genetically connects earthworm populations. **Chapters 2 and 3**
4) North American earthworms (*L. terrestris*) perform better in temperature and precipitation treatments most similar to conditions at their collection sites. **Chapter 4**
5) Due to environmental filtering by climate conditions genetic identity of earthworms differs at collection sites. Earthworm origin and genetic identity correlate positively with activity (litter consumption) and ecosystem effects (soil water content and microbial functions), with climate conditions of their sampling sites, i.e. they are higher in temperature treatments most similar to conditions at the collection site but lower in the other treatments. **Chapter 4**

Chapter 2:

Invasive lumbricid earthworms in North America – different life-styles, common dispersal?

Lumbricid earthworms initially were introduced to North America by European settlers about 400 years ago from genetically diverse source populations in Europe. Today, they are distributed across most parts of northern North America encountering different climate conditions. Accordingly, I expect that different genotypes dominate in the distinct climate regions due to environmental filtering. Further, I expect that geographic dispersal barriers and anthropogenic activities influence the genetic diversity and structure of earthworms in different regions in North America, i.e. distinct lineages on either side of the barrier, and higher diversity at the coasts and close to human agglomerations. I sampled earthworms from five transects of ~150 to 300 km length (north-south orientation) in three climate regions in Canada and the USA: the warm and moist region of British Columbia, Canada, the cold and dry regions of Alberta, Canada and Minnesota, USA, and the cold and moderately moist regions of Michigan, USA and New York State, USA. To account for human-mediated dispersal by dumping of fishing baits, earthworms were purchased from bait shops near sampling locations in all transect regions to test if bait genotypes contribute to free-living populations, thereby increasing local diversity.

Chapter 3:

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

Forests in the Canadian province Alberta likely have not been invaded by *L. terrestris* for much more than 20 years. This new invasion provides a unique opportunity to investigate the genetic structure of invading earthworm populations. I collected *L. terrestris* within 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, the dataset included fishing baits from three different bait shops in Calgary.

Chapter 4:

Adaptability of non-native *Lumbricus terrestris* to seasonal environmental climate conditions in a climate chamber transplantation experiment.

It is not clear whether successful invasion events were caused by selection processes or inherent ability of the introduced earthworms to adapt. To disentangle the relative importance of genetic and environmental factors for earthworm invasions I studied the performance (biomass gain, offspring number and mortality) of earthworm populations from climatically distinct locations and their impact on soil properties and microorganisms. I conducted a yearlong climate chamber transplantation experiment investigating the performance of *L. terrestris* under seasonal fluctuations of temperature and precipitation. I sampled *L. terrestris* from three North American sites of distinct climate conditions, altitude, and history of human settlement: (i) near Vancouver (British Columbia, Canada; West), (ii) Minneapolis (Minnesota, USA; Centre), and (iii) Newcomb (New York, USA; East), which are expected to be genetically distinct and adapted to local climate conditions.

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CHAPTER 2

INVASIVE LUMBRICID EARTHWORMS IN NORTH AMERICA – DIFFERENT LIFE-STYLES, COMMON DISPERSAL?

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Abstract

Lumbricid earthworms are invasive across northern North America, causing notable changes in forest ecosystems. During their range expansion they encountered harsher climatic conditions compared to their native range in evolutionary short time. This study investigated if (1) dispersal barriers, (2) climatic selection, or (3) anthropogenic activities, such as fishing bait disposal, structure the dispersal of free-living earthworm populations. *Lumbricus terrestris* and *L. rubellus* co-occur in the same habitats but differ in ecology and use as fishing baits. Both species were sampled in five transects ranging from the east to the west coast of northern North America, including major dispersal barriers, three different climate zones, and bait shops near sampling locations. Genetic diversity and structure was compared between the two species, and the presence of free-living bait shop genotypes was assessed using four markers (COI, 16S rDNA, 12S rDNA, H3).

Populations of both species were genetically diverse with some geographic structure, which was more pronounced in *L. terrestris* than in *L. rubellus*. Common haplotypes were present in all regions, but locally restricted haplotypes also occurred. Further, two distinct genetic clades of *L. terrestris* co-occurred only in the two most distant transects (Alberta and Minnesota). Genotypes identical to bait individuals were omnipresent in field populations of *L. terrestris*. Genetic diversity was high in both species, and invasive populations represented a genetic subset of European earthworms. Geographic and climatic dispersal barriers affected the less mobile species, *L. terrestris*, resulting in differences in genetic structure between the two species. Our results indicate common long-distance dispersal vectors and specific vectors affecting only *L. terrestris*. The roles of climate and anthropogenic activities are discussed, providing additional explanations of dispersal and new insights into establishment of invasive earthworms.

Keywords

biological invasion; colonization; genetic clades; agriculture; climate; dispersal barriers

2.1 Introduction

European lumbricid earthworms are among the most successful invasive species in North America (James and Hendrix, 2004). European settlers at the east coast introduced them about 400 years ago, both accidentally and intentionally (Gates, 1976). Similar to many invasive species living above the ground, earthworms substantially alter the functioning of invaded ecosystems (Scheu & Parkinson, 1994; Mooney and Hobbs, 2000; Bohlen et al., 2004; Eisenhauer et al., 2007; Hendrix et al., 2008). They change physical and biotic properties of the soil, which affects the density of other soil invertebrates, plant community composition, and aboveground food webs (Lee, 1985; Edwards & Bohlen, 1996; Eisenhauer et al., 2007, 2010a; Craven et al., 2017; Ferlian et al., 2018). In general, presence of earthworms beneficially affects plant growth (Scheu, 2003) and plant competition (Eisenhauer & Scheu, 2008) where they are native, but can exert contrasting effects on ecosystems that developed without their presence (Bohlen et al., 2004; Hale et al., 2005; Craven et al., 2017).

As successful invaders, earthworms possess high tolerance for a wide range of environmental conditions, though they prefer clay soils with near neutral pH that restricts their distribution (Laverack, 1961; Curry, 2004; Fisichelli et al., 2013). Due to their ability to tolerate disturbances, they also occur in agricultural fields and meadows, with varying frequencies and abundances (Hendrix et al., 1992). However, earthworms are susceptible to prolonged freezing periods, drought and geographic barriers like mountain ranges and large waterbodies, which usually restrict their natural dispersal range (Reynolds, 1994; Eggleton et al., 2009). Active dispersal of earthworms is slow, but they accomplished to spread across northern North America within a few hundred years by passive dispersal or repeated introductions, and today they are present in large areas from the east coast to the mid-west east of the Rocky Mountains in Canada, and the Pacific coast (Reynolds, 1977, 1994; Scheu & Parkinson, 1994). The pronounced ecological consequences of earthworm invasions in North America are well documented, making earthworms one of the best-studied invasive animal species living below the ground (Wardle, 2011) and thus, a unique model system for biological invasion and accompanying effects (Hendrix et al., 2008).

Earlier studies using molecular markers demonstrated that genetic diversity of European earthworm populations in eastern North America is similar or slightly reduced compared to European populations (Hansen et al., 2005; Gailing et al., 2012; Fernandez et al., 2015). Multiple introductions and human-mediated dispersal presumably contributed to this high genetic diversity (Keller et al., 2007; Holdsworth et al., 2007; Cameron et al., 2007; Cameron & Bayne, 2009). However, genetic diversity and structure of invading earthworm species in North America so far have only been studied at local or regional scales. The identified genetic diversity and human-mediated dispersal patterns likely also apply at larger scales, but it is unclear if one common invasion event or several independent

local invasions are responsible for the fast spread of European earthworms in northern North America. In general, the following dispersal scenario of earthworms in North America is likely: European earthworms spread from east to west, following the European migrations and transport of goods, suggesting that earthworm populations are genetically related in large areas east of the Great Plains. This invasive front likely stopped at the North American Midwest, an area of dry grassland and intensive agriculture, and continental climate of severe frost in winter and dry summers. The origin and dispersal of earthworm populations west of the Great Plains remains unclear but may be based on three scenarios. First, populations introduced at the west coast expanded to the east, crossing the Rocky Mountains. Second, multiple independent introductions occurred west and east of the Rocky Mountains. Third, earthworms dispersed from the east of the Rocky Mountains to the west or from east of the Great Plains to the west by long-distance dispersal.

During their expansion across northern North America, European earthworms established in distinct climate zones that differ in the amount and distribution of precipitation across the year, as well as frost intensity and duration, two abiotic factors that are known to drive earthworm distribution (Holmstrup, 2003; Curry, 2004; Uvarov et al., 2011; Fisichelli et al., 2013). At the west coast, precipitation is high (1200 mm y^{-1}), mild frost occurs sporadically and lasts for only few weeks between December and January. By contrast, in the central plains of North America, precipitation is low (400-600 mm y^{-1}), and strong frost conditions typically persist between November and March, with occasional night frost already starting in late August and extending into early June. In the east, precipitation is intermediate (800-1000 mm y^{-1}), and frost conditions typically last from December to February. Given this great range in climatic conditions, and the fast and wide ranging colonization of North America by European earthworms, knowledge on genetic diversity and relationships of populations across North America is needed for understanding dispersal mechanisms and population establishment.

We investigated the genetic structure of *Lumbricus rubellus* and *L. terrestris*, two exotic earthworm species that are widespread and common across northern North America. Both feed on litter but have distinct ecological preferences and life histories (Sims & Gerard, 1999). *Lumbricus rubellus* lives in horizontal burrows near the soil surface, moves freely within the litter layer for foraging, prefers neutral to slightly acidic soils and generally has a higher pH and frost tolerance than *L. terrestris* (Tiunov et al., 2006; Addison, 2009). In contrast, *L. terrestris* prefers neutral to slightly alkaline soils, lives in permanent, vertical burrows of up to 2 m depth, and collects litter in the vicinity of its burrow entrance (Sims & Gerard, 1999; Tiunov et al., 2006; Addison, 2009). Active dispersal rates of the two earthworm species range between 2-4 m y^{-1} for *L. terrestris* and 10-14 m y^{-1} for *L. rubellus* (Marinissen & van den Bosch, 1992). *Lumbricus terrestris* is commonly used as fishing bait and sold in bait shops which likely

facilitates its dispersal. By contrast, *L. rubellus* is rarely sold in bait shops (A. Klein, pers. obs.). Disposal of fishing baits contributes substantially to the introduction and establishment of earthworm populations in recreational and fishing areas (Holdsworth et al., 2007; Keller et al., 2007), but the long-term establishment of these populations and further dispersal in the field remain unclear.

We sampled earthworms from five transects of ~150 to 300 km length (north-south orientation) in three climatic regions in two states in Canada and three states in the USA: the warm and moist region of British Columbia, Canada (BC), the cold and dry regions of Alberta, Canada (AL) and Minnesota, USA (MN), and the cold and moderately moist regions of Michigan, USA (MI) and New York State, USA (NY), respectively. This is the first study investigating the invasion of detritivorous soil animals on continental scale, including two different dispersal barriers and distinct climate zones in its sampling design.

We tested three hypotheses to understand if climate (H1), dispersal barriers (H2), and/or human migrations and transport (H3) predominantly structured the distribution and establishment of European earthworm species in northern North America:

(H1) From a genetically diverse source population, distinct genetic clades established in the different climate zones. By environmental filtering, individuals that were better adapted to regional drought or cold conditions survived, resulting in monophyletic clades in the different regions.

(H2) Earthworms were introduced independently in areas that are separated by major dispersal barriers (the Rocky Mountains and the Great Plains), resulting in distinct genetic clades in the west (BC, AL). In contrast, east of the Great Plains (MN, MI, NY) geographic dispersal barriers are less extreme and therefore, genetic structure is less pronounced or absent.

(H3) Human-mediated dispersal of earthworms counteracts local selection and disregards dispersal barriers, resulting in diverse earthworm populations and genotypes that are represented in all regions without any local clades occurring.

To account for human-mediated dispersal by dumping of fishing baits, which is a severe problem in northern North America (Holdsworth et al., 2007; Hale, 2008; Seidl & Klepeis, 2011), we purchased earthworms from bait shops near sampling locations in all transects to test if bait genotypes contribute to free-living populations, thereby increasing local diversity.

2.2 Material and methods

2.2.1 Sampling design – dispersal barriers and climate

Between May and July 2014 and in June 2015, we collected *L. terrestris* and *L. rubellus* along five transects (regions) spanning from east to west of the northern North American continent, ranging in the USA from New York State (Adirondack Mountains, transect NY), to the Midwest, i.e. Michigan (upper peninsula, transect MI) and Minnesota (near Minneapolis/St. Paul, transect MN; **Table 1**). In Canada, we collected earthworms east and west of the Rocky Mountains in Alberta (south of Calgary, transect AL) and British Columbia (near Vancouver, transect BC). Distances among transects ranged between 700-1600 km, and within transects earthworms were collected at five sampling locations with north-south orientation that were 20-80 km apart. The two major dispersal barriers for plants and animals are the extensive dry grassland areas of the Great Plains extending between transects Minnesota (USA) and Alberta (Canada), and the Rocky Mountains, which separate the two Canadian transects Alberta and British Columbia. Climate in east and central northern North America is similar to continental climate in Europe, but seasonality in North America is harsher with hotter and drier summers, and longer and colder winters, which is most extreme in Alberta and Minnesota. Climate in British Columbia differs from that in Europe as three different climate zones (Mediterranean, Continental and Oceanic) co-occur in the Greater Vancouver area.

Table 1: Overview of sampling area, abbreviations of sampling locations and climatic characteristics of each transect. AMP, annual mean temperature; AMT, annual mean temperature. See Appendix Table S4 for GPS coordinates.

sampling region	climate zone	climate characteristics	sampling location	soil pH	sequence code	GPS coordinates [decimal grade]	county/ regional district	human pop. density [population/km²]	human pop. density/ sampling region [population/km²]
British Columbia (BC)	mixed Mediterranean, oceanic and continental	warm and moist AMP: ~1200 mm/year AMT: 6-16°C	Cypress Provincial Park	5.7	BC_I	49.359167, -123.2098	Greater Vancouver	854.8	577.27
			Golden Ears Provincial Park	4.83	BC_II	49.293133, -122.491467	Greater Vancouver	854.8	
			Cultus Lake	4.64	BC_III	49.048333, -121.97435	Fraser Valley	22.2	
Alberta (AL)	cold continental	cold and dry AMP: 400-750 mm/year AMT: -2-9°C	Crandell Lake	6.61	AL_I	49.088417, -113.968483	Southern Alberta	3.6	138.05
			Waterton Springs	5.83	AL_II	49.1335, -113.848533	Southern Alberta	3.6	
			Maycroft	6.82	AL_III	49.922167, -114.323167	Southern Alberta	3.6	
			Eden Valley	6.73	AL_IV	50.4, -114.5	Calgary Region	272.5	
			Fish Creek Park, Calgary	7.68	AL_V	50.898167, -114.012	Calgary Region	272.5	
			Nose Hill Park, Calgary	n/a	AL_VI	51.127667, -114.119914	Calgary Region	272.5	
Minnesota (MN)	cold continental	cold and dry AMP: 400-750 mm/year AMT: -2-9°C	Nerstrand	7.26	MN_I	44.34665, -93.16717	Rice County	50	364.67
			Wood-Rill SNA	n/a	MN_II	44.98497, -93.54187	Hennepin County	804	
			Wolsfeld Wood SNA	n/a	MN_III	45.000733, -93.572533	Hennepin County	804	
			Warner Nature Center	n/a	MN_IV	45.172017, -92.825533	Washington County	240	
			Pine Needles Preserve	n/a	MN_V	45.21008, -92.76455	Washington County	240	
			Rush City	5.18	MN_VI	45.687547, -92.877519	Chisago County	50	
Michigan (MI)	moderate continental	cold and moderately moist AMP: 800-1000 mm/year AMT: 0-9°C	Turner	6.57	MI_I	44.148567, -83.585617	Arenac County	17	19.5
			Tawas City	6.43	MI_II	44.3203, -83.587817	Iosco County	18	
			Alpena	3.94	MI_III	45.046983, -83.60005	Alpena County	25	
			Gaylord	3.51	MI_IV	45.006967, -84.727967	Otsego County	18	
New York (NY)	moderate continental	cold and moderately moist AMP: 800-1000 mm/year AMT: 0-9°C	Hamilton	n/a	NY_I	42.814568, -75.527465	Madison County	43	67.17
			Norwich	n/a	NY_II	42.523065, -75.532291	Chenango County	22	
			Newcomb	4.64	NY_III	43.9718, -74.223833	Essex County	8	
			Lower Saranac Lake	5.88	NY_IV	44.300367, -74.1559	Essex County	8	
			Lake Placid	3.93	NY_V	44.3044, -73.986067	Essex County	8	
			Portland Waterfront	4	NY_VI	40.9478, -75.11935	Northampton County	314	

Earthworms were collected in forests by turning over logs, hand sorting of litter, digging or applying mustard solution to extract earthworms from soil. We measured soil pH from sampling locations; seven locations in our study were sampled by cooperation partners and were not available for pH measurements. Additionally, we purchased earthworms sold as fishing baits in bait shops close to sampling locations; all bait shops exclusively sold *L. terrestris*, restricting the bait shop dataset to a single species. Earthworms were washed, stored in 75% ethanol in the field and later transferred in the laboratory into 95% ethanol and stored at 16°C. One centimetre of tail tissue of each individual was cut and shipped to the University of Göttingen (Germany) for molecular analyses; remaining body parts are stored as voucher specimens at the University of Minnesota (Minneapolis-St. Paul, MN) and the University of British Columbia (Vancouver, BC).

2.2.2 Genetic analyses

Genomic DNA was extracted with the Genaxxon DNA Tissue Mini Prep Kit (Genaxxon; Ulm, Germany) following the manufacturer's protocol. Four molecular markers were amplified: the mitochondrial genes COI (~600 bp; Folmer et al., 1994), 16S rDNA (~750 bp; Pèrez-Losada et al., 2009), and 12S rDNA (~400 bp; Simon et al., 1994), and the nuclear gene Histone 3 (~350 bp; Colgan et al., 1998). The PCR cycling conditions had 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 and were sequenced at the Göttingen Genome Sequencing Laboratory (Georg August University Göttingen) and SeqLab Göttingen (Microsynth; Balgach, Switzerland). These sequence data for the GenBank databases are to be submitted (GenBank www.ncbi.nlm.nih.gov/genbank). Sequences were checked with Sequencher 4.9 (Gene Codes Corporation, USA), and ambiguous positions were coded as wobble bases. Consensus sequences of the individual genes were assembled in BioEdit 7.0.1 (Hall, 1999) and aligned with ClustalW. Genes were analysed individually and in a combined matrix of 2,150 bp; all positions with wobble bases were deleted for further analyses. Sequence alignments (single genes and combined) were collapsed into haplotype alignments using FaBox 1.41 (Villesen, 2007). The best-fit models of sequence evolution were estimated with TOPALi v2.5 (Milne et al., 2004) using the Akaike information criterion (AIC; Akaike, 1973). Trees were constructed using MrBayes 3.2. (Ronquist et al., 2012), partitioning the combined alignment to the following lset parameters for *L. rubellus* (COI: nst=2, rates=invgamma; 16S rDNA: nst=2, rates=invgamma; 12S rDNA: nst=6, rates=invgamma; H3: nst=1, rates=invgamma) and *L. terrestris* (COI: nst=6, rates=gamma; 16S rDNA: nst=6, rates=invgamma; 12S rDNA: nst=2, rates=invgamma; H3: nst=1, rates=equal). A mcmc run of 4 million generations with default settings was performed. We compared the North American haplotype identities with European earthworms

with Bayesian phylogenetic trees of the COI and H3 datasets including sequences available from NCBI. A list of the data sources is found in Appendix 1 Table S6 a) and b). Parameter settings were nst=6, rates=invgamma and default settings for the mcmc run.

2.2.3 Phylogeography and genetic differentiation across putative dispersal barriers

Spatial distribution of genetic clades was analysed with haplotype networks and constructed for 16S rDNA, which provided the most informative resolution. Median-joining (MJ) networks (Bandelt et al., 1999) were constructed with PopART (University of Otago, Dunedin, New Zealand) and edited using Inkscape (Software Freedom Conservancy, USA). Parameters were set to equal weights for all mutations and the epsilon parameter to zero to restrict the choice of possible links in the final network.

To test hypotheses about climatic and geographic dispersal barriers, we used analyses of molecular variance (AMOVA) and analysed genetic differentiation among populations using the distance method of Tajima & Nei, pairwise differences without Gamma correction, and pairwise genetic distances using Arlequin 3.5.2.2 (Excoffier, 2015). AMOVAs were calculated with COI, the most variable gene regarding nucleotide diversity (**Table S3**), and earthworm populations were assigned *a priori* according to our first hypotheses (H1) into climate zones separating populations from British Columbia (mixed climate), Alberta and Minnesota (cold continental climate), Michigan and New York (moderate continental climate). To test for the relevance of geographic barriers (H2), populations were analysed in three different combinations: Great Plains as main dispersal barrier (BC, AL vs. MN, MI, NY), Rocky Mountains as main dispersal barrier (BC vs. AL, MN, MI, NY), and Rocky Mountains and Great Plains as main dispersal barriers (BC vs. AL vs. MN, MI, NY). Human influence on reducing the effect of dispersal barriers was tested by comparing genetic variance among transects (BC vs. AL vs. MN vs. MI vs. NY). If human transport plays a significant role for earthworms across large geographic distances (H3), genetic variance should be similar among regions.

2.2.4 Climate data

The response of genetically diverse earthworms to ecological factors was inspected by using a multiple regression matrix (MRM). Bioclimatic data were retrieved from WorldClim v2 bioclimatic variables database (Fick & Hijmans, 2017) and had a spatial resolution of ~5 km^2. The response matrix compared genetic pairwise differences of the COI sequence data and was calculated with the Analysis of Phylogenetics and Evolution (ape) package (Paradis et al., 2004). Tested factors were (1) environmental abiotic parameters, i.e. annual mean temperature (BIO01), maximum temperature of the warmest month (BIO05), minimum temperature of the coldest month (BIO06), mean temperature

of the wettest quarter (BIO08), mean temperature of the driest quarter (BIO09), annual precipitation (BIO12), precipitation of the driest month (BIO14), precipitation seasonality (BIO15), and (2) the geographical parameter spatial distance and elevation (**Table S4**). Data were transformed into scaled explanatory distance matrices for standardisation. The spatial distance between each pair of samples was calculated using the Geographic Distance Generator v1.2.3 (Ersts, 2014) with the World Geodetic System (1984) setting for the reference spheroid and then normalised by dividing the values by the maximum distance value, thus measuring the absolute but normalized distances. The MRM function was executed with the R package ecodist (Goslee & Urban, 2007).

2.2.5 Linear regression analyses

We analysed correlations between sampling success as proxy for earthworm abundance and genetic diversity (nucleotide diversity and number of genetic clades in transects) with environmental factors potentially affecting earthworm distribution and abundance (i.e., sampling location soil pH and human population density; **Table 1**) performing simple linear regression analyses in Microsoft Excel 2013. The number of genetic clades per transect referred to the clades of the COI phylogenetic trees (**Fig. 1a, 2a**). Human population densities for each sampling region (BC, AL, MN, MI, and NY) were calculated based on data obtained from the US Census Bureau (https://www.census.gov/) and Statistics Canada (http://www.statcan.gc.ca/) using the mean of counties (USA) or regional districts (Canada) for each sampling location.

2.3 Results

2.3.1 Sampling and genetic diversity

In total, 120 *L. rubellus* (LR) and 122 *L. terrestris* (LT) individuals were sampled from the 25 locations. The number of individuals per transect varied from 12 to 48 for *L. terrestris* and from 12 to 37 for *L. rubellus* (**Fig. S1, Table S5**). Abundances of both species were similar in British Columbia and Michigan, but differed in the other transects with higher numbers of *L. rubellus* in Minnesota and New York, and *L. terrestris* being more common in Alberta (**Fig. S1, Table S5**). Nucleotide (NUD) and haplotype diversity (HTD) was greater in *L. rubellus* and decreased in both species from COI to 16S rDNA to 12S rDNA to H3 (**Table S3**). Overall, nucleotide diversity of COI was two or three times higher in *L. rubellus* than in *L. terrestris* (**Table 2**) and varied among transects. Transect New York was most diverse in *L. rubellus*, followed by Alberta, while transects British Columbia, Michigan and Minnesota had similar diversity. In contrast, nucleotide diversity of COI in *L. terrestris* was highest in Minnesota and similar in transects British Columbia and Alberta; diversity was low in transects New York and Michigan. The

combination of transects showed that a considerable fraction of polymorphism was overlapping among transects, except for the two rather distinct populations of *L. rubellus* from New York and *L. terrestris* from Minnesota. Genetic variance and diversity in bait (n=104) and field populations of *L. terrestris* were very similar (**Table S2**).

Table 2: Nucleotide diversity of *Lumbricus rubellus* and *L. terrestris* in each transect and in different combinations of transects to assess complementary diversity across the sampling area. Mean percentages [%] with standard deviation (SD); *, nucleotide diversity in transect NY without sampling point E, which differed significantly in nucleotide composition.

	L. rubellus	***L. terrestris***
transect	**nucleotide diversity ± SD [%]**	
BC	4.25 ± 2.19	2.62 ± 1.37
AL	5.13 ± 2.72	2.24 ± 1.14
MN	4.43 ± 2.21	2.82 ± 1.45
MI	4.14 ± 2.14	1.55 ± 0.83
NY	7.10 ± 3.51	1.40 ± 0.78
NY*	5.65 ± 2.87	/
combination		
BC, AL	4.59 ± 2.30	2.67 ± 1.34
BC, MN	4.63 ± 2.29	3.00 ± 1.50
BC, MI	4.15 ± 2.07	2.32 ± 1.19
BC, NY	6.46 ± 3.16	2.61 ± 1.33
AL, MN	4.86 ± 2.40	2.47 ± 1.24
AL, MI	4.46 ± 2.24	2.43 ± 1.22
AL, NY	6.79 ± 3.33	2.47 ± 1.24
MN, MI	4.65 ± 2.30	2.64 ± 1.34
MN, NY	6.43 ± 3.14	2.76 ± 1.40
MI, NY	6.49 ± 3.18	1.55 ± 0.82
MN, MI, NY	6.11 ± 2.98	2.52 ± 1.27

2.3.2 Relatedness and spatial distribution

In both species, earthworms were closely related resulting in phylogenetic trees with a weakly supported backbone and clades with mixed geographic origin. Accordingly, phylogenetic and geographic structure was generally weak, in particular in *L. rubellus*. However, in both species, some populations formed well-supported clades (posterior probabilities: 0.95-1; **Fig. 1a**) that were also recovered by haplotype network analyses. In *L. rubellus*, two clades comprised closely related individuals from all transects (mixed clades 1 and 4 with 37 and 60 individuals, respectively). However, five individuals from Minnesota (clade 2, green) and 18 individuals from New York (clade 3, blue) were distinct and did not occur in other transects (**Fig. 1b**). All North American COI haplotypes of *L. rubellus* could be assigned to lineages from Europe (Sechi, 2013; Giska et al., 2015). Haplotypes of clade 4

correspond to the widespread European lineages A1-A3. Haplotypes in clade 1 and 2 clustered with European lineages C and D from eastern Europe (Poland, Hungary, Balkans), and haplotypes in clade 3 clustered with lineage H, which is restricted to Germany and Austria. We compared COI lineages with the H3 dataset to check if mitochondrial and nuclear markers correspond. The North American haplotypes of the COI clades 1, 2 and 4 carried the same H3 lineage that is also common in Europe (Martinsson & Erséus, 2017). Three individuals from Michigan (clade 4) carried a different H3 lineage, which is undescribed in Europe. Clade 3 comprised several H3 haplotypes, one known from Europe (Martinsson & Erséus, 2017) and one also present as widespread H3 lineage in the common COI clade 4.

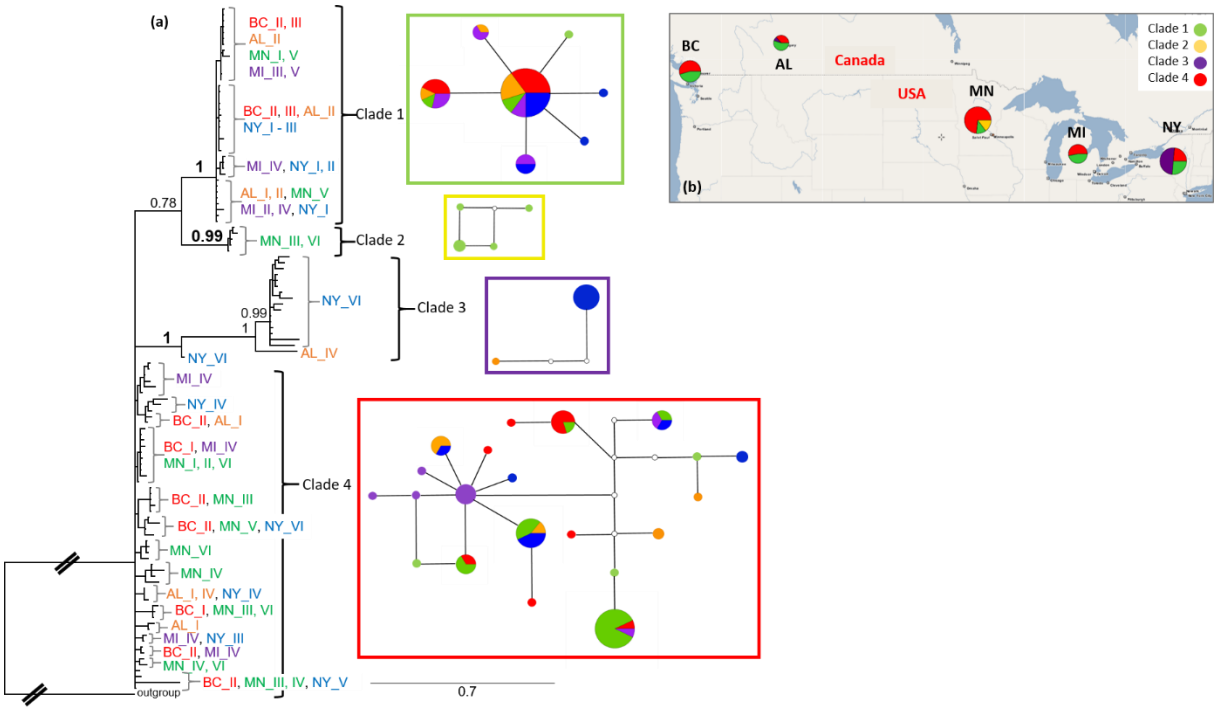

Figure 1: Bayesian phylogenetic tree based on a supermatrix of four genes (COI, 16S, 12S, H3) of 120 individuals of *Lumbricus rubellus* (a), and distribution and abundance of the four genetic clades in the five transects across northern North America (b). The corresponding clades of the haplotype network analysis based on 16S are provided next to each clade, the area of each circle is proportional to the numbers of individuals for each haplotype, the colour code refers to the five transects British Columbia (BC, red), Alberta (AL, orange), Minnesota (MN, green), Michigan (MI, violet), and New York (NY, blue). For abbreviations of sampling locations see Table 1, posterior probabilities of well-supported clades are highlighted in bold.

Genetic distances among populations of *L. terrestris* were less distinct but had more haplotypes separating into more clades than *L. rubellus* (**Fig. 2a, b**). The largest clade of *L. terrestris* (clade 2, 52 individuals) included haplotypes from all transects. The second largest clade (clade 1, 32 individuals) consisted of a haplotype predominantly found in Alberta (orange) and Minnesota (green) and in one individual from New York (blue). Further, haplotypes from Alberta also occurred in separate clades together with Minnesota (clade 4, 7 individuals), British Columbia (red, single individual) and Michigan

(violet, clade 5, 16 individuals). Notably, Minnesota and British Columbia also had distinct haplotypes that formed isolated monophyletic clades (clades 6, 5 individuals and 3, 5 individuals).

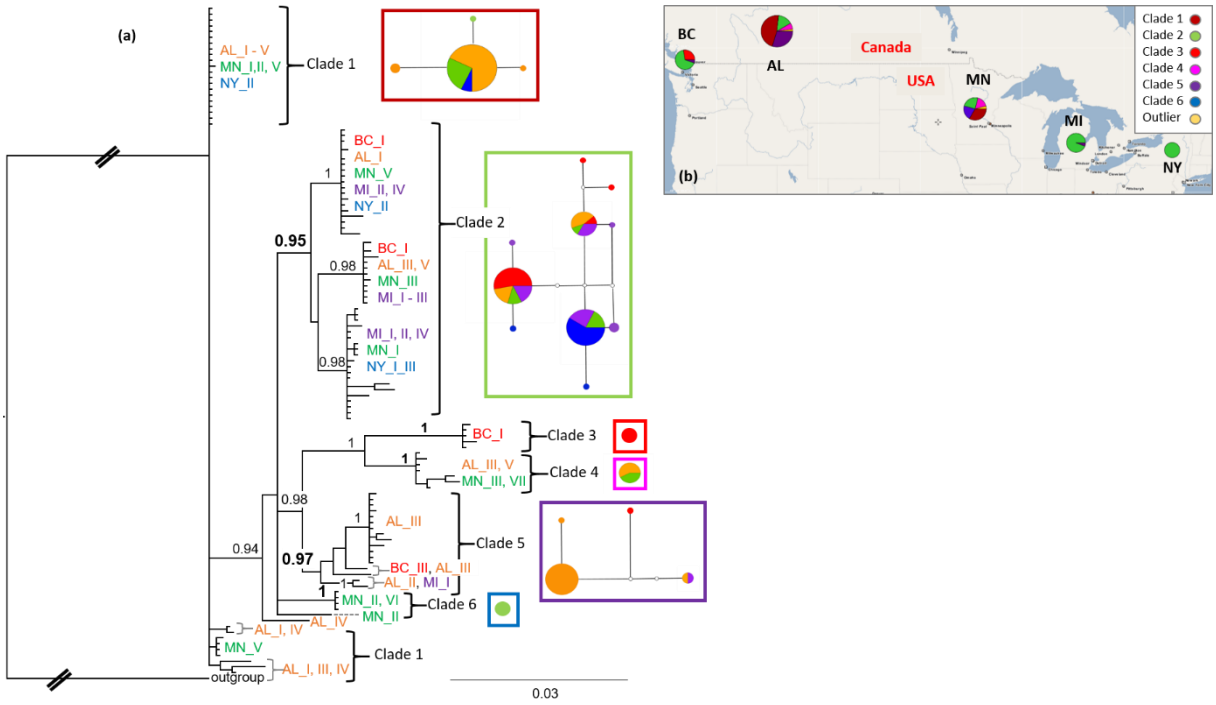

Figure 2: Bayesian phylogenetic tree based on a supermatrix of four genes (COI, 16S, 12S, H3) of 122 individuals of *Lumbricus terrestris* (a), and distribution and abundance of the seven genetic clades in the five transects across northern North America (b). The corresponding clades of the haplotype network analysis based on 16S are provided next to each clade, the area of each circle is proportional to the numbers of individuals for each haplotype, the colour code refers to the five transects as in Fig. 1. For abbreviations of sampling locations see Table 1, posterior probabilities of well-supported clades are highlighted in bold.

Most haplotypes of *L. terrestris* from bait shops were identical to common and widespread haplotypes from field populations (**Fig. 3**). Only few haplotypes formed separate clades (mainly AL and BC) or were related to rare field haplotypes (BC) from the same sampling region. The North American COI and H3 haplotypes of *L. terrestris* were closely related or identical to haplotypes described from Europe or North America in previous studies (**Fig. S2, S3**; Brown et al., 1999; King et al., 2008, 2010; James et al., 2010; Richard et al., 2010; Novo et al., 2011; Klarica et al., 2012; Souleman et al., 2016; Martinsson & Erséus, 2017).

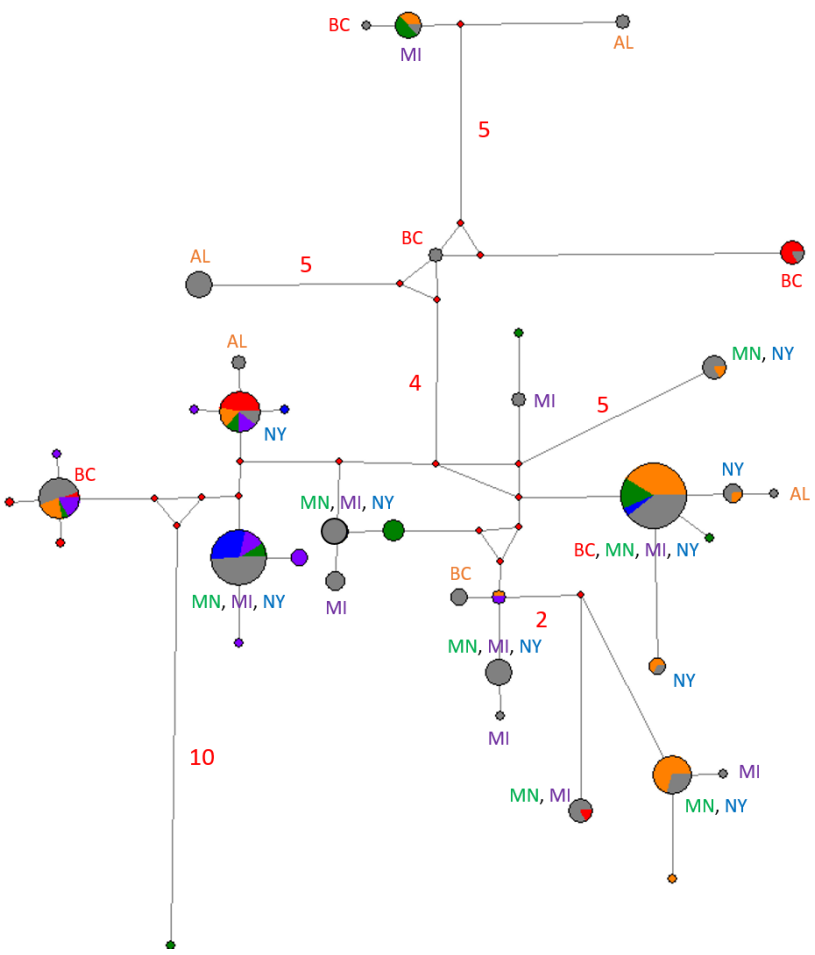

Figure 3: Haplotype network based on 16S of 122 individuals of *Lumbricus terrestris* sampled in 25 field locations and of 104 individuals purchased in nearby bait shops. Bait shop individuals are grey and labelled with transects of their origin. Colour codes of transects and field individuals correspond to transects in Figs. 1 and 2.

2.3.3 Genetic differentiation across putative barriers

Analysis of molecular variance (AMOVA) across all four genes showed that most of the molecular variance was at local scale (within sampling points = populations, **Table S1**), with ~92-94% of variance in *L. rubellus* and ~70-73% in *L. terrestris* in the most variable gene (COI, **Table 3**).

Table 3: Analyses of molecular variance (AMOVA) of *Lumbricus rubellus* and *L. terrestris* assigned a priori into populations separated by climate regions, geographic barriers (Great Plains and Rocky Mountains) or by geographic distance between transects. Molecular variance is given in percent.

	L. rubellus								*L. terrestris*							
tested barriers	**within sp**	**d.f.**	**within gg**	**d.f.**	**among gg**	**d.f.**	**FCT**	**p-value**	**within sp**	**d.f.**	**within gg**	**d.f.**	**among gg**	**d.f.**	**FCT**	**p-value**
Climate																
(BC vs AL,MN vs MI,NY)	93.32	104	4.92	17	1.76	2	0.02	0.022	70.58	102	18.20	16	11.22	2	0.11	0.001
Great Plains & Rocky Mountains																
(BC vs AL vs MN,MI,NY)	92.47	104	4.81	17	2.71	2	0.03	0.011	71.96	102	20.00	16	8.04	2	0.08	0.015
Great Plains																
(BC,AL vs MN,MI,NY)	93.48	104	5.81	18	0.71	1	0.01	0.157	72.04	102	22.44	17	5.51	1	0.06	0.036
Rocky Mountains																
(BC vs AL,MN,MI,NY)	92.52	104	5.43	18	2.06	1	0.02	0.057	72.47	102	24.84	17	2.69	1	0.03	0.224
Transect																
(BC vs AL vs MN vs MI vs NY)	93.39	104	3.75	15	2.86	4	0.03	0.001	72.62	102	17.92	14	9.46	4	0.09	0.006

In both species, molecular variance predominantly resided at population level but was much clearer in *L. rubellus* with only 3.75% of variance among populations compared to *L. terrestris* with 17.92% (**Table 3**). Analyses based on *a priori* assigned populations to test for effects of climate (H1: transects BC vs. AL, MN vs. MI, NY), geographic barriers (H2: Great Plains = transects BC, AL vs. MN, MI, NY; Rocky Mountains = transects BC vs. AL, MN, MI, NY; Great Plains and Rocky Mountains = transects BC vs. AL vs. MN, MI, NY), and distance (transects BC vs. AL vs. MN vs. MI vs. NY) on population structure also showed very little variance for *L. rubellus* within (3.75%-5.81%) and among geographic populations (0.71%-2.86%), thereby rejecting all hypotheses for this species. However, *L. terrestris* generally showed a higher genetic structure with 11.22% variance among climate regions (H1) followed by distance among regions (9.46%).

2.3.4 Importance of bioclimatic factors

The MRM showed contrasting results for the two earthworm species; the permutation test (R^2) indicated that 23% and 5% of the variance were explained by climatic variables for *L. rubellus* and *L. terrestris*, respectively (**Table S4**). *Lumbricus rubellus* correlated significantly ($p<0.002$) with all tested bioclimatic factors except for the minimum temperature in the coldest month (BIO06; $p=0.755$) and precipitation seasonality (BIO15; $p=0.084$). In *L. terrestris*, correlations generally were not significant ($p>0.130$), except for the minimum temperature of the coldest month (BIO06; $p=0.022$). To estimate the importance of climatic factors for the local clades of *L. rubellus* and widespread clades of *L. terrestris*, we repeated the analysis with reduced datasets containing only the widespread clades 1 and 4 of *L. rubellus* and clade 2 of *L. terrestris.*

Table 4: Genetic variance of *Lumbricus rubellus* and *L. terrestris* explained by bioclimatic factors, for local and widespread genetic clades. *P > 0.05, **0.001 < P < 0.01, ***P < 0.001; n/a not available due to small sample size; r^2 standardized coefficient of a regression analysis indicating the influence of the bioclimatic factors (independent variable) on genetic variance (dependent variable).

	local		**widespread**	
bioclimatic factor	***L. rubellus***	***L. terrestris***	***L. rubellus***	***L. terrestris***
annual mean temp.	n/a	0.278	0.391	0.490
max. temp. warmest month	n/a	0.855	0.004**	0.099
min. temp. coldest month	n/a	0.027*	0.001***	0.297
mean temp. wettest month	n/a	0.003**	0.011*	0.580
mean temp. driest month	n/a	0.844	0.001***	0.107
annual precipitation	n/a	0.57	0.868	0.848
precipitation driest month	n/a	0.001***	0.192	0.319
precipitation seasonality	n/a	0.007**	0.017*	0.575
	r^2=n/a, p=n/a	r^2=0.23, p=0.001	r^2=0.04, p=0.001	r^2=0.11, p=0.001

In the reduced dataset, the variance explained by climatic factors decreased strongly for *L. rubellus,* but increased for *L. terrestris* (**Table S4**). Temperature and seasonal precipitation explained 4% of the genetic variance of clades 1 and 4 of *L. rubellus*, and temperature of the coldest and wettest month and precipitation explained 23% of the variance of clade 2 of *L. terrestris*.

2.3.5 Other environmental factors

The abundance of earthworms tended to correlate with soil pH with contrasting results for the two species. *Lumbricus rubellus* occurred more often in acidic soils (pH≤5.5, R^2=0.715; **Fig. S4**), and *L. terrestris* was more abundant in neutral soils (pH>6, R^2=0.865; **Fig. S4**). Human population density was positively correlated with nucleotide diversity in *L. terrestris* (R^2=0.701; **Fig. 4**), i.e. transects in British Columbia, Alberta and Minnesota had the highest nucleotide diversities (≥2.25%) and densities of >100 population/km^2. Transects in Michigan and New York had the lowest nucleotide diversities (≤1.5 %) and densities of <70 population/km^2. Nucleotide diversity of *L. rubellus* was weakly negatively correlated with human population density (R^2=0.197; **Fig. 4**); however, the negative correlation likely was due to a single sampling location in New York (clade 3; **Fig. 1a, b**) that included distinct haplotypes (**Table 2**).

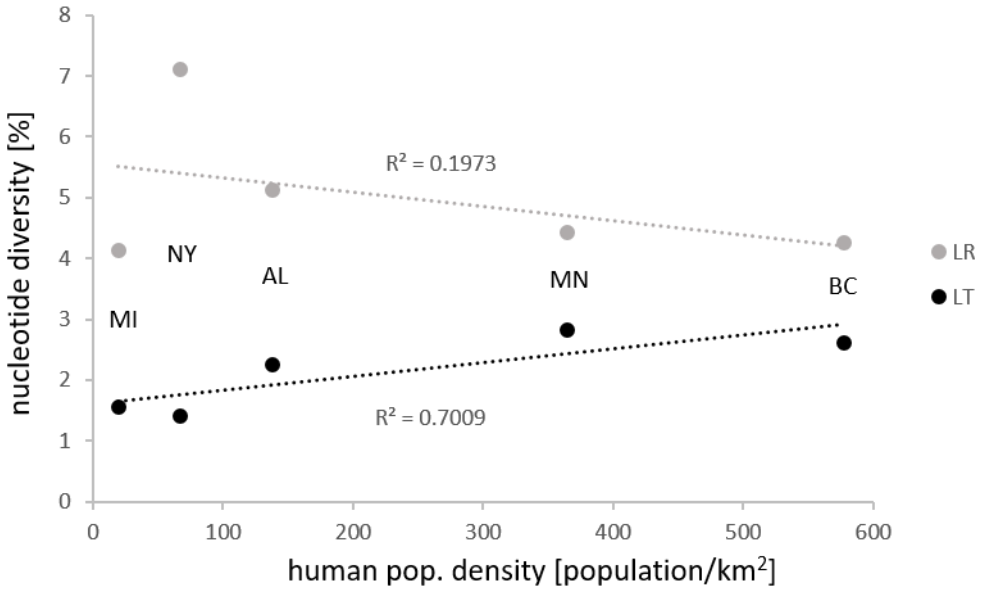

Figure 3: Correlation of nucleotide diversity (in percent) of *Lumbricus rubellus* and *L. terresris* with human population density in the five transects.

2.4 Discussion

2.4.1 Genetic diversity

This study shows that northern North American populations of the two earthworm species *L. rubellus* and *L. terrestris* share the same genetic lineages with populations of their native range in Europe. However, genetic diversity is lower in North America than in Europe, which is typical for invasive species (Sakai et al., 2001; Allendorf & Lundquist, 2003; King et al., 2008; Donnelly et al., 2013; Donnelly et al., 2014; Giska et al., 2015). Consistent with studies in Europe, genetic diversity in *L. rubellus* was higher than in *L. terrestris* (King et al., 2008; Martinsson & Erséus, 2017), and intraspecific genetic distances of COI were comparable with those reported from Europe (King et al., 2008; James et al., 2010; Klarica et al., 2012).

In North America, common and widespread haplotypes dominated in both species, but genetic and geographic structure differed. Among populations of *L. rubellus,* two genetic lineages predominantly occurred in each of the studied sampling regions, except in New York. Interestingly, haplotypes belonged to common and widespread European lineages (Sechi, 2013), indicating that North American *L. rubellus* populations represent a genetic subset of Europe's diversity. The distribution and abundance of genetic lineages of *L. rubellus* in Europe was potentially shaped by survival of cold tolerant populations during the Last Glacial Maximum (~25,000-13,000 years ago) in northern and central Europe (Sechi, 2013). Further, clades with deeply divergent mitochondrial lineages were considered to be descendants from pre-glacial refuge populations that adapted to local climate conditions (Sechi, 2013; Giska et al., 2015). Similar to *L. rubellus,* all mitochondrial and nuclear lineages of northern North American *L. terrestris* corresponded with haplotypes described from Europe.

The co-occurring pattern of omnipresent lineages of *L. rubellus* and *L. terrestris* across northern North America suggests a common origin and mode of dispersal for both species. In particular road constructions, traffic, logging, fishing and agriculture have been identified as main drivers of earthworm range expansion in North America (Marinissen & van den Bosh, 1992, Dymond et al., 1997; Casson et al., 2002; Holdsworth et al., 2007; Gundale et al., 2005; Cameron et al., 2008; Cameron and Bayne, 2009) and certainly also apply here. Human-mediated long-range dispersal by passive transport is more likely for *L. rubellus,* which lives in leaf litter near or on the soil surface than for soil-dwelling endogeic or anecic species (Terhivuo & Saura, 1997). However, the presence of locally occurring lineages in *L. rubellus* and the distinct genetic assembly of *L. terrestris* in Alberta and Minnesota indicate that additional factors affected the dispersal and introduction of these two earthworm species.

The relevance of bait abandonment for the distribution of *L. rubellus* is difficult to assess. This species has been commonly used as fishing bait (Reynolds, 1977), but was not sold in any bait shops we purchased earthworms from. However, dispersal via bait abandonment in the past cannot be excluded. In contrast, *L. terrestris* is the most commonly sold live fishing bait in northern North America today, and a large fraction of individuals from bait shops and the field shared identical or closely related haplotypes, indicating that bait abandonment contributes significantly to the spread of *L. terrestris*. Historically, earthworms sold as fishing baits were collected from fields and sold locally, but establishment of refrigerated warehouses by large distributors selling pre-packed baits nationwide might additionally contribute to long-distance spread of genetic diversity. This assumption is supported by the study of fine resolution markers at local scale in Calgary, Alberta (Klein et al., 2017). Here, at large scale, bait haplotypes from Alberta and British Columbia in part did not match the haplotypes of nearby field populations, but rather field populations of far distant transects. However, bait cannot be the only source and vector for dispersal of *L. terrestris*, since the two most distant transects of Alberta and Minnesota contained three genetic clades that occurred nowhere else, indicating the existence of a distinct dispersal vector that connects these two transects.

2.4.2 Climate and dispersal barriers

Genetic variation among regions was very low for *L. rubellus,* and bioclimatic factors or dispersal barriers did not explain the distribution of common lineages, which agrees with its higher tolerance to frost (Sims & Gerard, 1999; Tiunov et al., 2006; Fisichelli et al., 2013). The ability of epigeic earthworms to quickly adapt to cold and fluctuating temperatures through behavioural and physiological changes (Holmstrup, 2003), and their persistence to perturbations, such as heavy metal pollution by fertilizers and intoxication by pesticides, are well known (Kruse & Barrett, 1985; Levine et al., 1989; Edwards & Bohlen, 1996). Although consecutive summer droughts can have strong effects on epigeic earthworms (Eggleton et al., 2009), drought resistance of cocoons allows persistence through drought periods (Holmstrup & Loeschcke, 2003).

In contrast to *L. rubellus*, genetic variance in the common lineages of *L. terrestris* in part was related to climate factors, in particular frost, drought, and seasonality. These results correspond to findings that anecic earthworm species are negatively affected by prolonged drought periods, high frequency of freeze-thaw cycles and low soil moisture during their prime reproductive periods in spring and autumn (Sims & Gerard, 1999; Curry, 2004; Addison, 2009). Conform to these findings, the distinct genetic composition of populations in Alberta and Minnesota correlated with the continental climate in both transects. However, if the more severe frost and drought periods in these regions facilitated genetic diversity by continuous extinctions and reintroductions, or if only climatically pre-adapted

lineages were able to establish viable populations in these areas needs to be investigated under controlled experimental conditions (Holmstrup, 2003).

2.4.3 Correlations with other factors affecting population structure

In our restricted dataset of other environmental factors, soil pH affected the abundance of both earthworm species and was consistent with their contrasting ecological preferences. However, *L. rubellus* and *L. terrestris* occurred at sites with high and low pH, indicating that pH did not directly affect earthworm distribution but rather nucleotide diversity, as larger populations likely contain more genetic variance, and therefore pH potentially influences the genetic structure of earthworm populations. However, effects of pH on earthworms are generally difficult to explain, as individuals can withstand soil pH values outside their optimal range, and earthworm activity may also alter soil pH (Drouin et al., 2016).

Human population density as proxy for human activities and anthropogenic dispersal of earthworms increased genetic diversity of *L. terrestris* but not that of *L. rubellus*. This apparently contrasting pattern can be explained by the nearly ubiquitous occurrence of haplotypes in *L. rubellus,* undermining the detection of any correlation. However, *L. terrestris* seems to be closer associated with human activity and transport. Transects closest to urban centres, such as Vancouver, Calgary, and Minneapolis-St. Paul, and agricultural land (Alberta and Minnesota) were genetically more diverse and appeared in the latter case to be connected by a common source population or a common distribution mode. Our continental data are in accordance with previous findings at the regional scale, which showed decreasing genetic variance outside the urban area of Calgary (Klein et al., 2017). Further, agricultural land often holds high population densities of earthworms and even acted as source of non-native earthworm introduction in New York (Suarez et al., 2006). In Alberta, earthworms occur more often in the south-western region with high agricultural activity (Cameron & Bayne, 2009). High densities and resilience of *L. terrestris* to mechanical disturbance increases the probability of cocoons being transported on tires of trucks or agricultural machinery or in potted plants (Marinissen & van den Bosh, 1992; Suarez et al., 2006).

2.4.4 Conclusions

Genetic diversity and structure of the two invasive earthworm species *L. rubellus* and *L. terrestris* was homogenous across all regions indicating a dominant common dispersal vector and the ability to adjust to most environmental conditions in northern North America. However, *L. terrestris* was genetically more structured, and here its genetic variance positively correlated with harsh climatic

conditions in central North America as well as with human activities, such as traffic and land use. In contrast to *L. rubellus*, this species is common in arable fields with frequent disturbances, and distinctness of genetic lineages occurring predominantly in transects of Alberta and Minnesota could be explained by their position at the edges of the North American corn-belt. Overall, we did not find any support for a continuous invasive front spreading from the east to the west coast. Genetic patterns indicate that both species have common long-distance distribution vector(s) or even a common source population. For *L. terrestris,* nation-wide bait distributors potentially play a major role as dispersal agent of field populations. In the past two decades, the globalisation of economy has changed infrastructure, intensity and range of traffic including commercial distribution of soil-related goods, and potentially will increase dispersal of *L. rubellus* and *L. terrestris.*

Our present study exemplifies how earthworms as belowground invaders with substantial differences in life history traits can be used to test broad questions in invasion ecology, such as the genetic underpinnings of successful invasion events, geographic and climatic dispersal barriers, as well as the human role in ecologically relevant invasions.

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Supplementary material

Table S.1: Analysis of molecular variance (AMOVA) of the four genes used in this study for *Lumbricus rubellus* and *L. terrestris*. d.f. = degrees of freedom.

	L. rubellus						*L. terrestris*					
gene	within populations	d.f.	among populations	d.f.	FST	p-value	within populations	d.f.	among populations	d.f.	FST	p-value
COI	93.87	104	6.13	19	0.06	0.001	73.90	102	26.10	18	0.26	0.001
16S	70.45	99	29.55	20	0.30	0.001	72.00	101	28.00	20	0.28	0.001
12S	59.87	98	40.13	18	0.40	0.001	64.55	98	35.45	20	0.36	0.001
H3	77.55	97	22.45	19	0.23	0.001	101.33	97	-1.33	20	-0.01	0.505
mean	75.44		24.56				77.95		22.05			

Table S.2: Diversity indices with standard deviation and sampling size of the field, bait and combined population datasets of *Lumbricus terrestris*.

COI	***L. terrestris* (field)**	***L. terrestris* (bait)**	***L. terrestris* (combined)**
No. of individuals	124	106	230
Nucleotide Diversity	0.027 ± 0.014	0.029 ± 0.014	0.031 ± 0.015
Haplotype Diversity	0.90 ± 0.01	0.92 ± 0.02	0.92 ± 0.01
Mean no. pairwise diff	16.12 ± 7.24	17.14 ± 7.69	18.21 0.02

Table S.3: Haplotype and nucleotide diversity indices for all four genes per sampling transects and in total of *Lumbricus rubellus* and *L. terrestris*.

Code	Location	COI				16S			
		LR		LT		LR		LT	
		HTD	NUD	HTD	NUD	HTD	NUD	HTD	NUD
BC	British Columbia	0.91	4.3	0.73	2.6	0.86	1.6	0.70	1.5
AL	Alberta	0.89	5.1	0.79	2.2	0.88	1.7	0.77	1.2
MN	Minnesota	0.95	4.4	0.90	2.8	0.88	1.9	0.86	1.3
MI	Michigan	0.95	4.1	0.78	1.5	0.95	2.0	0.89	0.7
MN	New York State	0.98	7.1	0.58	1.4	0.75	1.9	0.50	0.7
Total	**North America**	**0.98**	**6.00%**	**0.9**	**2.70%**	**0.93**	**2.10%**	**0.89**	**1.40%**

Table S3 continued.

Code	Location	12S				H3			
		LR		LT		LR		LT	
		HTD	NUD	HTD	NUD	HTD	NUD	HTD	NUD
BC	British Columbia	0.73	0.9	0.86	0.7	0	0	0.45	0.2
AL	Alberta	0.71	1.1	0.71	0.8	0	0	0.16	0.2
MN	Minnesota	0.82	1.1	0.84	1.2	0.12	0.09	0	0
MI	Michigan	0.84	1.0	0.71	0.4	0.40	0.1	0	0
MN	New York State	0.72	1.5	0.68	0.4	0.74	0.5	0.15	0.2
Total	**North America**	**0.87**	**1.40%**	**0.86**	**0.90%**	**0.36**	**0.20%**	**0.16**	**0.10%**

Table S.4: Genetic variance of *Lumbricus rubellus* and *L. terrestris* explained by bioclimatic factors, for all genetic clades. *P > 0.05, **0.001 < P < 0.01, ***P < 0.001; r^2 standardized coefficient of a regression analysis indicating the influence of the bioclimatic factors (independent variable) on genetic variance (dependent variable).

	complete dataset	
Bioclimatic factor	***L. rubellus***	***L. terrestris***
annual mean temp.	0.001***	0.769
max. temp. warmest month	0.001***	0.98
min. temp. coldest month	0.755	0.022*
mean temp. wettest month	0.002**	0.597
mean temp. driest month	0.001***	0.368
annual precipitation	0.001***	0.153
precipitation driest month	0.001***	0.815
precipitation seasonality	0.084	0.134
	r^2=0.23, p=0.001	r^2=0.05, p=0.002

Table S.5: Individual and haplotype overview for each gene and sampling location of *Lumbricus rubellus* and *L. terrestris*.

		# Individuals/Haplotypes							
		COI		16S		12S		H3	
Code	Location	LR	LT	LR	LT	LR	LT	LR	LT
A_I	Cypress Provincial Park	3 / 3	17 / 4	3 / 3	17 / 5	3 / 1	17 / 3	2 / 1	17 / 3
A_II	Golden Ears Provincial Park	14 / 8	0	14 / 8	0	14 / 5	0	14 / 1	0
A_III	Cultus Lake	4 / 2	1 / 1	4 / 2	1 / 1	4 / 1	1 / 1	4 / 1	1 / 1
Transect BC	**British Columbia**	**21 / 11**	**18 / 5**	**21 / 10**	**18 / 6**	**21 / 7**	**18 / 4**	**20 / 1**	**18 / 3**
B_I	Waterton Park, Crandell Lake	5 / 5	9 / 2	5 / 4	9 / 3	5 / 3	9 / 2	5 / 1	9 / 2
B_II	Waterton Springs Campground	5 / 3	8 / 4	5 / 2	9 / 3	5 / 1	8 / 3	5 / 1	8 / 1
B_III	Maycroft	0	22 / 8	0	21 / 5	0	21 / 3	0	21 / 2
B_IV	Eden Valley	2 / 2	7 / 4	2 / 2	7 / 3	2 / 2	7 / 3	2 / 1	7 / 2
B_V	Calgary, Fish Creek Park	0	2 / 2	0	2 / 2	0	2 / 2	0	2 / 1
B_VI	Calgary, Nose Hill Park	0	0	0	0	0	0	0	0
Transect AL	**Alberta**	**12 / 8**	**48 / 14**	**12 / 7**	**48 / 10**	**12 / 4**	**47 / 7**	**12 / 1**	**47 / 4**
C_I	Nerstrand	5 / 3	5 / 2	5 / 4	5 / 2	5 / 3	5 / 2	5 / 1	5 / 1
C_II	Wood-Rill SNA	4 / 3	5 / 3	4 / 1	5 / 3	4 / 2	5 / 2	4 / 1	4 / 1
C_III	Pine Needles Preserve	5 / 4	5 / 5	5 / 5	4 / 2	5 / 2	4 / 2	5 / 1	4 / 1
C_IV	Warner Nature Center	4 / 4	8 / 3	4 / 4	8 / 4	3 / 2	7 / 2	3 / 1	7 / 1
C_V	Wolsfeld SNA	11 / 11	3 / 2	10 / 7	3 / 2	10 / 5	3 / 2	10 / 1	3 / 1
C_VII	Rush City	8 / 6	0	7 / 3	0	7 / 2	0	7 / 2	0
Transect MN	**Minnesota**	**37 / 25**	**26 / 11**	**35 / 19**	**25 / 9**	**34 / 9**	**24 / 8**	**34 / 3**	**23 / 1**
D_II_A	Turner	0	9 / 3	0	9 / 6	0	9 / 3	0	9 / 1
D_III_A	Tawas City	5 / 4	3 / 3	4 / 3	4 / 3	4 / 2	3 / 2	4 / 1	3 / 1
D_IV_A	Alpena	0	1 / 1	1 / 1	1 / 1	0	1 / 1	0	1 / 1
D_IV_C	Gaylord	13 / 9	4 / 3	13 / 9	4 / 3	13 / 6	4 / 2	13 / 3	4 / 1
Transect MI	**Michigan**	**18 / 12**	**17 / 6**	**18 / 11**	**18 / 8**	**17 / 7**	**17 / 4**	**17 / 3**	**17 / 1**
E_I_A	Hamilton	6 / 5	1 / 1	5 / 3	1 / 1	5 / 2	1 / 1	5 / 2	1 / 1
E_I_B	Norwich	6 / 5	5 / 4	3 / 3	5 / 3	3 / 1	5 / 3	3 / 1	5 / 2
E_III	Newcomb	3 / 3	6 / 1	2 / 2	5 / 1	2 / 2	5 / 2	2 / 1	5 / 1
E_IV	Lower Saranac Lake	6 / 6	0	6 / 3	1 / 1	6 / 3	1 / 1	6 / 4	1 / 1
E_V	Lake Placid	0	0	1 / 1	1 / 1	0	1 / 1	1 / 1	1 / 1
E_VI	Portland Waterfront Park	13 / 12	0	17 / 1	0	17 / 2	0	17 / 6	0
Transect NY	**New York State**	**34 / 31**	**12 / 5**	**34 / 11**	**13 / 3**	**33 / 7**	**13 / 4**	**34 / 9**	**13 / 2**
Total	**North America**	**120 / 75**	**122 / 30**	**118 / 37**	**119 / 23**	**118 / 18**	**119 / 14**	**117 / 11**	**118 / 5**

Table S.6: NCBI accession numbers and references of COI and H3 sequences used in this study for a) *Lumbricus rubellus* and b) *L. terrestris*.

a)	NCBI accession number	Publication
	COI	
	KP642090 - KP642108	Sechi 2013
	KT731474 - KT731500	Giska et al. 2015
	H3	
	FJ214242, FJ214257	Lund et al. 2008 (unpubl.)
	KX790528 - KX790694	Martinsson and Erséus 2017

b)	NCBI accession number	Publication
	COI	
	FJ214211, FJ214212, FJ214230	Lund et al. 2008 (unpubl.)
	AM774289	King et al. 2008
	FN658823 - FN658826	King et al. 2010
	HQ024537 - HQ024671	James et al. 2010
	HE611677 - HE611681	Bienert et al. 2012 (unpubl.)
	JN869933 - JN869947	Klarica et al. 2012
	GU206213 - GU206226, GU206238, HM388349 FJ937295, FJ937305 - FJ937311, FJ937319 - FJ937327	Porco et al. 2012
	KU888473 - KU888617	Souleman et al. 2016
	KX790424 - KX790505	Martinsson and Erséus 2017
	H3	
	AF185262	Brown et al. 1999
	FJ214240, FJ214241, FJ214260	Lund et al. 2008 (unpubl.)
	HQ691227	Novo et al. 2011
	HE611679	Bienert et al. 2012 (unpubl.)
	KX790551 - KX790696	Martinsson and Erséus 2017

Table S.7: Overview of sampling regions, including transects, climate zones and climatic characteristics, abbreviations of sampling locations, soil pH, and human population densities retrieved at county or regional district level from the US Census Bureau (https://www.census.gov/) and Statistics Canada (http://www.statcan.gc.ca/).

sampling transect	climate zone	climate characteristics	sampling location		soil pH	GPS coordinates [decimal grade]	county/ regional district	human pop. density [population/km²]	human pop. density/sampling region [population/km²]
British Columbia (BC)	mixed Mediterranean, oceanic and continental	warm and moist AMP: ~1200 mm/year AMT: 6-16°C	Cypress Provincial Park	BC_I	5.7	49.359167, -123.2098	Greater Vancouver	854.8	577.27
			Golden Ears Provincial Park	BC_II	4.83	49.293133, -122.491467	Greater Vancouver	854.8	
			Cultus Lake	BC_III	4.64	49.048333, -121.97435	Fraser Valley	22.2	
Alberta (AL)	cold continental	cold and dry AMP: 400-750 mm/year AMT: -2-9°C	Crandell Lake	AL_I	6.61	49.088417, -113.968483	Southern Alberta	3.6	138.05
			Waterton Springs	AL_II	5.83	49.1335, -113.848533	Southern Alberta	3.6	
			Maycroft	AL_III	6.82	49.922167, -114.323167	Southern Alberta	3.6	
			Eden Valley	AL_IV	6.73	50.4, -114.5	Calgary Region	272.5	
			Fish Creek Park, Calgary	AL_V	7.68	50.898167, -114.012	Calgary Region	272.5	
			Nose Hill Park, Calgary	AL_VI	n/a	51.127667, -114.119914	Calgary Region	272.5	
Minnesota (MN)	cold continental	cold and dry AMP: 400-750 mm/year AMT: -2-9°C	Nerstrand	MN_I	7.26	44.34665, -93.16717	Rice County	50	364.67
			Wood-Rill SNA	MN_II	n/a	44.98497, -93.54187	Hennepin County	804	
			Wolsfeld Wood SNA	MN_III	n/a	45.000733, -93.572533	Hennepin County	804	
			Warner Nature Center	MN_IV	n/a	45.172017, -92.825533	Washington County	240	
			Pine Needles Preserve	MN_V	n/a	45.21008, -92.76455	Washington County	240	
			Rush City	MN_VI	5.18	45.687547, -92.877519	Chisago County	50	
Michigan (MI)	moderate continental	cold and moderately moist AMP: 800-1000 mm/year AMT: 0-9°C	Turner	MI_I	6.57	44.148567, -83.585617	Arenac County	17	19.5
			Tawas City	MI_II	6.43	44.3203, -83.587817	Iosco County	18	
			Alpena	MI_III	3.94	45.046983, -83.60005	Alpena County	25	
			Gaylord	MI_IV	3.51	45.006967, -84.727967	Otsego County	18	
New York (NY)	moderate continental	cold and moderately moist AMP: 800-1000 mm/year AMT: 0-9°C	Hamilton	NY_I	n/a	42.814568, -75.527465	Madison County	43	67.17
			Norwich	NY_II	n/a	42.523065, -75.532291	Chenango County	22	
			Newcomb	NY_III	4.64	43.9718, -74.223833	Essex County	8	
			Lower Saranac Lake	NY_IV	5.88	44.300367, -74.1559	Essex County	8	
			Lake Placid	NY_V	3.93	44.3044, -73.986067	Essex County	8	
			Portland Waterfront	NY_VI	4	40.9478, -75.11935	Northampton County	314	

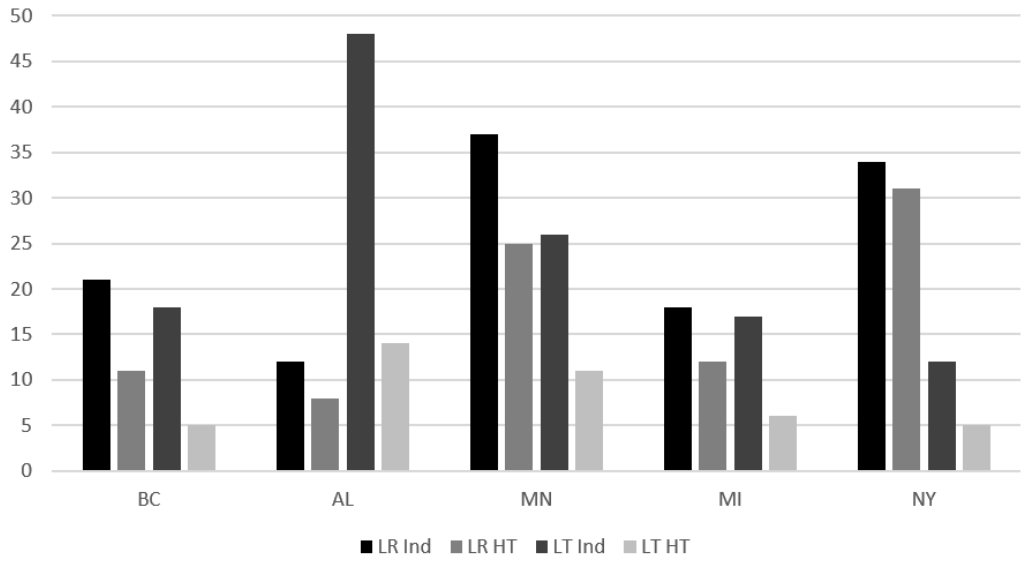

Figure S.1: Total numbers of individuals and COI haplotypes of *Lumbricus rubellus* (LR) and *L. terrestris* (LT) sampled at 25 locations in five transects across northern North America. BC = British Columbia, AL = Alberta, MN = Minnesota, MI = Michigan, NY = New York.

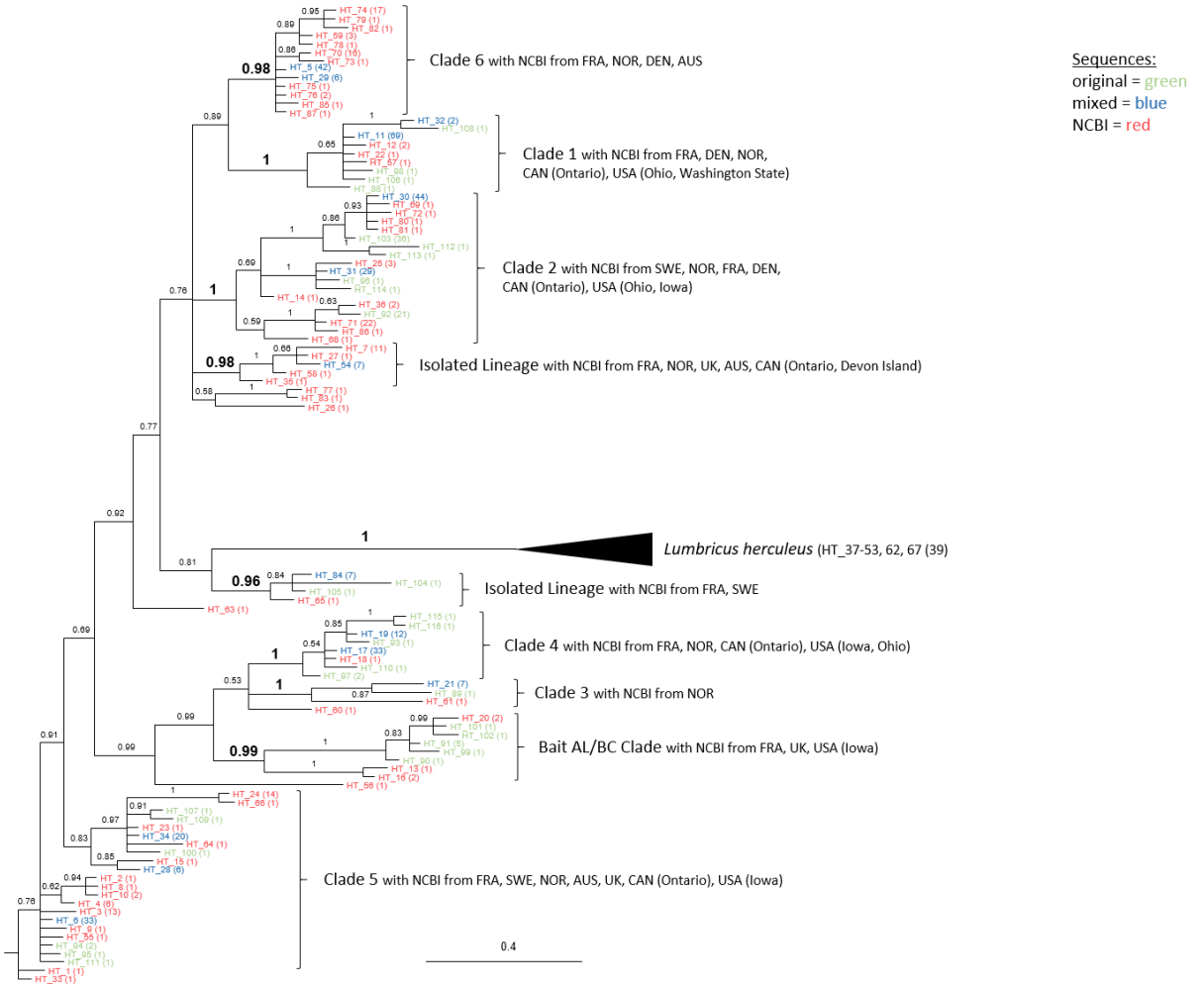

Figure S.2: Bayesian phylogenetic tree based on H3 of 120 individuals of *Lumbricus terrestris* from this study (five transects across northern North America) and 64 individuals from NCBI. Number of individuals are given in brackets behind the haplotype number. Geographic origin of NCBI sequences within a marked genetic clade is indicated for each clade.

Figure S.3: Bayesian phylogenetic tree based on COI of 122 individuals of *Lumbricus terrestris* from this study (five transects across northern North America) and 367 individuals from NCBI.

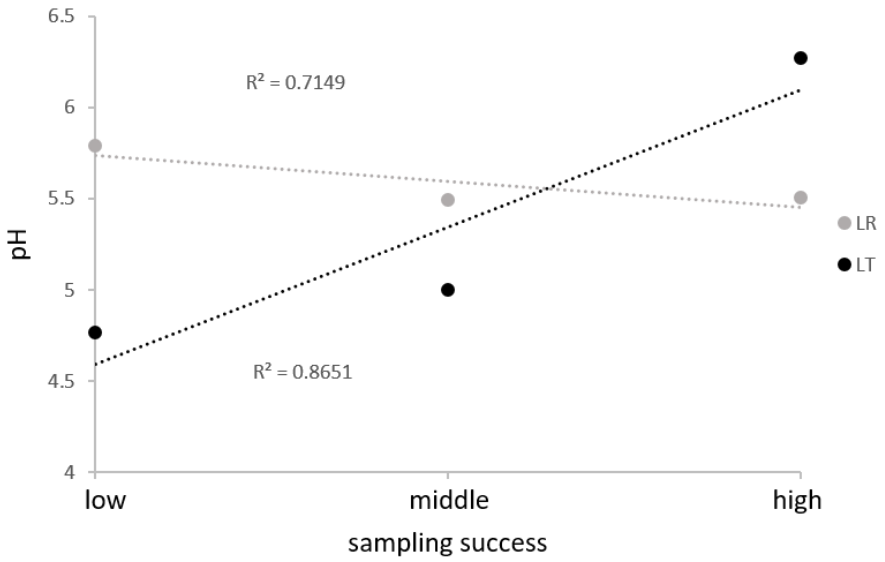

Figure S.4: Correlation of pH and sampling success of *Lumbricus rubellus* (LR) and *L. terresris* (LT) at each sampling location in northern North America. Sampling success was categorized in low (n=0), middle (n<5) and high (n≥5).

CHAPTER 3

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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

Andreas Klein, Erin K. Cameron, Bastian Heimburger, Nico Eisenhauer, Stefan Scheu, Ina Schaefer

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.

Keywords

microsatellites; exotic earthworms; invasion; gene flow; dispersal; population structure; soil

3.1 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 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.

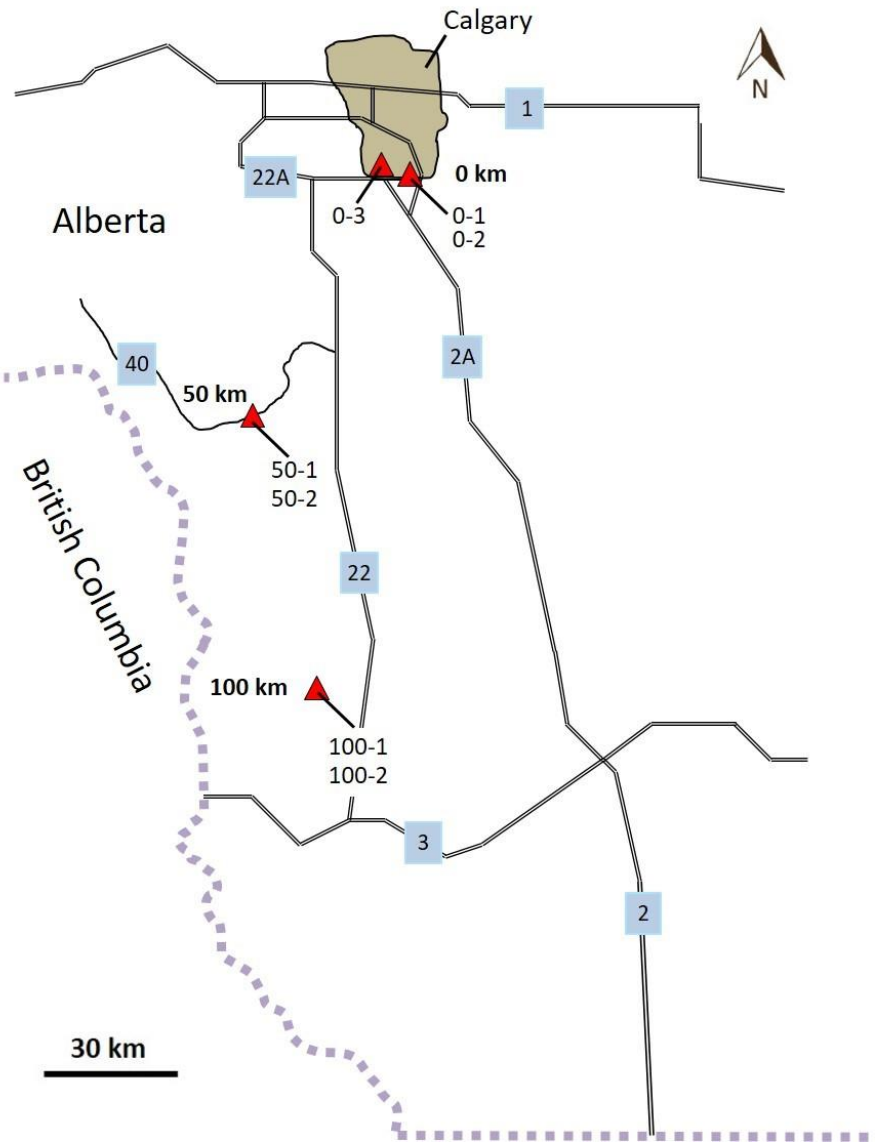

Figure 1: Sampling locations of *Lumbricus terrestris* in southern Alberta, Canada. Individuals were sampled in Calgary (shaded area) and 50 km and 100 km south of Calgary. Subpopulations (triangles) 0–1 and 0–2, 50-1 and 50-2, 100-1 and 100-2 are 5m apart; subpopulation 0–3 is 5 km apart from subpopulations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

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 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.

3.2 Material and methods

3.2.1 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 5,000 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 *Lumbricus 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 *L. herculeus* individuals from the study of James et al. (2010).

Table 5: 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

3.2.2 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 S.1 and S.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).

3.2.3 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).

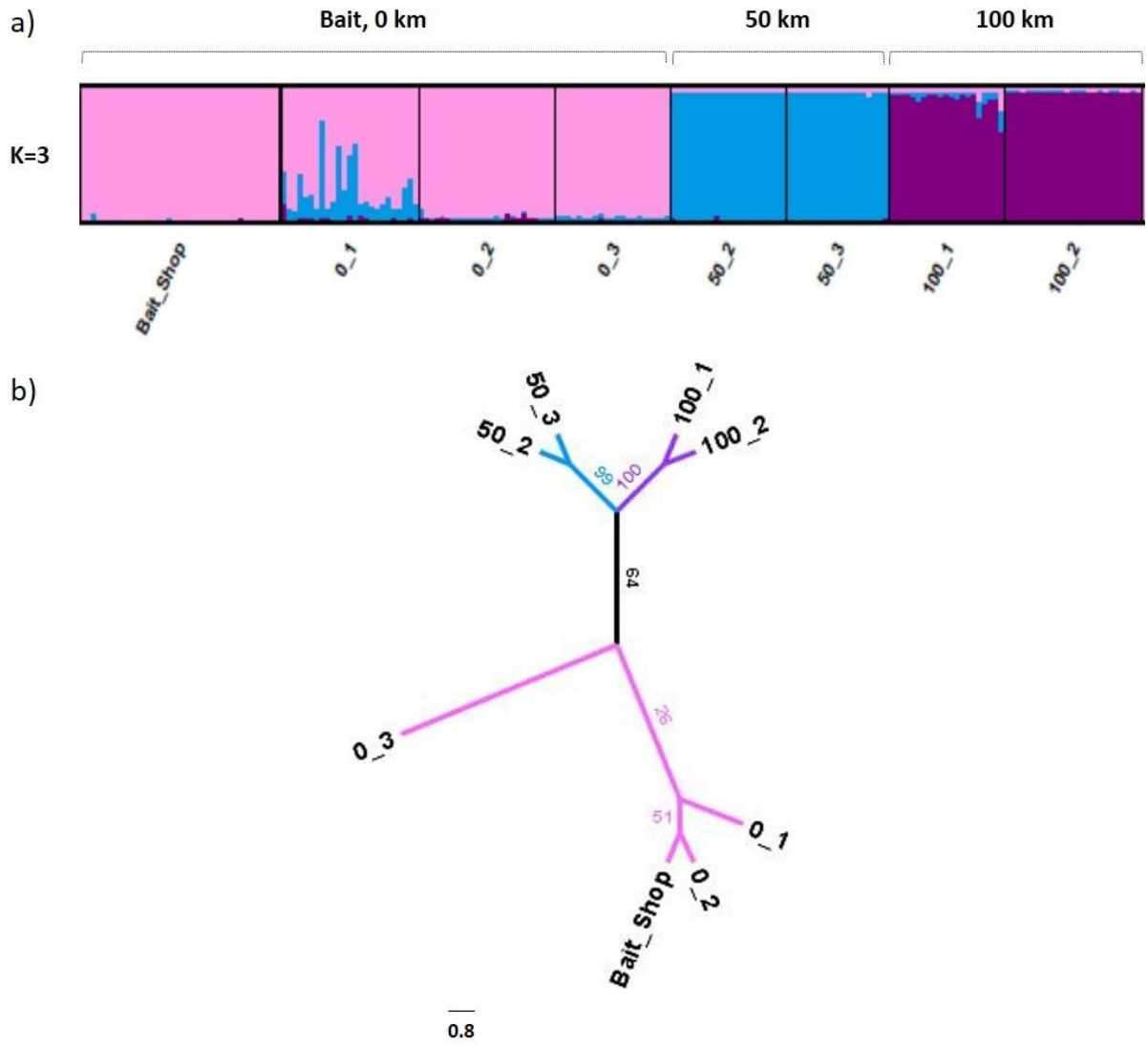

Figure 2: a) Bar plots depicting the assignment of individuals of *Lumbricus terrestris* from three sampling areas to a specified number of clusters (k). Assigned clusters are shown on top, the number of genetic clusters (k) on the left, sampling locations below. Each individual is represented by a thin vertical bar. b) Unrooted population tree based on Nei'sD 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.

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 (N_a) and observed (H_o) and expected (H_e) 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).

3.3 Results

3.3.1. 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*).

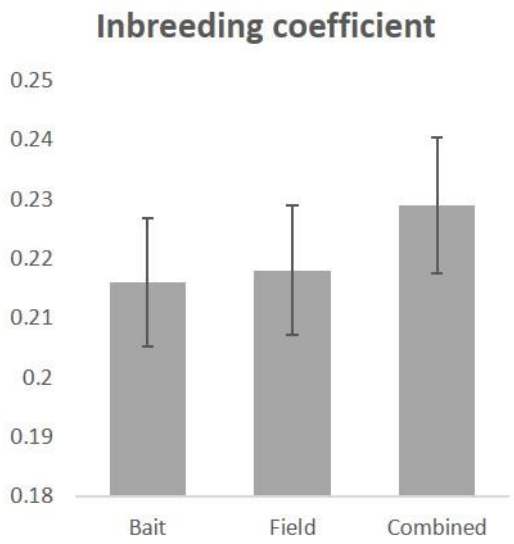

Figure 3: Mean inbreeding coefficients for field, bait shop, and the combined datasets of field and bait shop populations of *Lumbricus terrestris*. Error bars are standard errors.

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. S.1** in Supplementary material). The 190

sequences of 16S rDNA consisted of 26 haplotypes (**Table S.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. S.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.

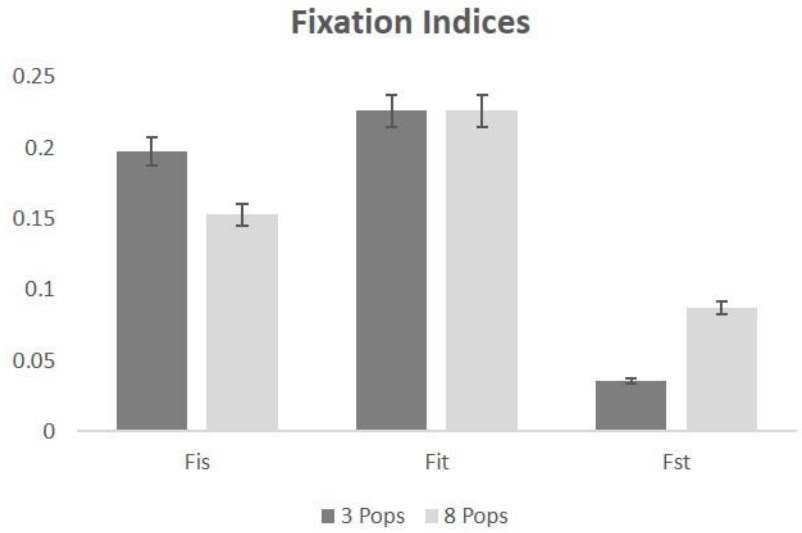

Figure 4: Fixation indices (F_{IS}, F_{IT}, and F_{ST}) for the three populations of *Lumbricus terrestris* assigned by STRUCTURE and eight subpopulations from the seven sampling points as well as the pooled bait shop samples. Error bars are standard errors.

3.3.2 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 (H_o) 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 (H_e) was higher than H_o. 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.

Table 6: 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	0.020	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	0.040	0					
Pop4_50_2	0.040	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	0.070	0.086	0.072	0.070	0.050	0	
Pop8_Bait	0.037	0.029	0.037	0.046	0.056	0.053	0.068	0

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

Table 7: 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%

3.3.3 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. S.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. 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.

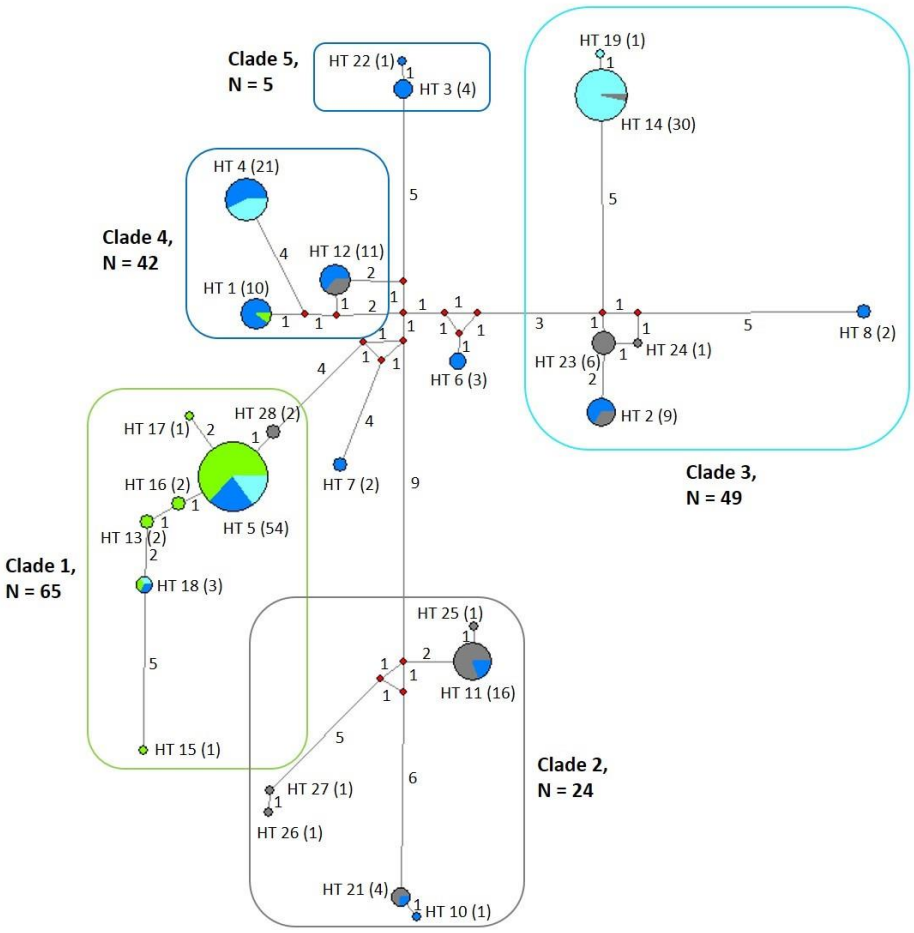

Figure 5: Haplotype network based on 16S rDNA of *Lumbricus terrestris* from sampling locations in Calgary (blue) and 50 km (green) and 100 km (turquoise) south of Calgary as well as from three bait shops (grey). Clades were assigned in accordance with branch/node support by posterior probabilities. 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.

3.4 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 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.

Acknowledgements

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Supplementary material

Table S.2: PCR cycling conditions for the mitochondrial 16S r DNA fragment and seven microsatellite markers for *Lumbricus terrestris* (Velavan et al., 2007, Development and characterisation of novel microsatellite markers for the common earthworm (*Lumbricus terrestris* L.), Molecular Ecology Notes, 7; 1060-1062).

Step	Temperature	Time	Cycles
	16S		
1	95°C	3 min	
2	95°C	30 sec	40x
3	53°C	1 min	
4	72°C	1 min	
5	72°C	10 min	
6	8°C	~	
	LTM 126, 163, 252		
1	95°C	3 min	
2	94°C	1 min	35x
3	58/58/51°C	1 min	
4	72°C	1 min	
5	72°C	10 min	
6	8°C	~	
	LTM 187, 193, 026, 278		
1	95°C	15 min	
2	95°C	1min	35x
3	56/56/54/51°C	1 min	
4	72°C	1 min	
5	72°C	10 min	
6	8°C	~	

Table S.3: Detailed PCR mastermix (in µl) used in this study for the mitochondrial 16S rDNA fragment and seven microsatellite markers for *Lumbricus terrestris* (Velavan et al., 2007, Development and characterisation of novel microsatellite markers for the common earthworm (*Lumbricus terrestris* L.), Molecular Ecology Notes, 7; 1060-1062).

	16S (*Taq* polymerase, Thermo Scientific)	**LTM126, 163, 278** (*Taq* polymerase, Thermo Scientific)	**LTM278** (HotStarTaq Mastermix, Genaxxon)	**LTM187, 193, 026** (HotStarTaq Mastermix, Genaxxon)
H_2O	11.75	5.75	4.5	5.5
BSA (~4%)	1.25	1.25	1.0	1.0
Buffer (KCl)	2.5	2.5	-	-
dNTPs (10 mM)	1.0	2.5	-	-
$MgCl_2$ (25 mM)	3.5	3.75	1.0	1.5
Primer 1 (10 mM)	1.0	2.5	1.5	1.0
Primer 2 (10 mM)	1.0	2.5	1.5	1.0
Taq (5 U/µl)	0.5	0.5	12.5	12.5
Template DNA	2.5	3.75	3.0	2.5
Total volume	25	25	25	25

Table S.3: 16S Haplotype list summarizing the name of haplotypes (# Haplotype), the number of individuals assigned to each haplotype (# Individuals) and the respective names of individuals and NCBI accession numbers for each haplotype.

# Haplotype	# Individuals	Individual code	Accession number
1	10	0_1_8	KM986898.1
		0_1_12	KM986901.1
		0_1_10	KM986899.1
		0_1_19	KY499487
		0_2_3	KM986909.1
		0_2_5	KM986911.1
		0_2_12	KM986918.1
		50_2_8	KM986957.1
		0_1_3	KM986894.1
		0_1_15	KM986904.1
2	9	0_1_13	KM986902.1
		0_2_6	KM986912.1
		0_2_20	KY499489
		0_2_13	KM986919.1
		0_2_29	KY499498
		0_1_17	KM986905.1
		Bait_II_8	KY499553
		Bait_II_11	KY499549
		Bait_II_12	KY499550
3	4	0_1_14	KM986903.1
		0_1_2	KM986893.1
		0_2_23	KY499492
		0_1_18	KM986906.1
4	21	0_1_1	KM986892.1
		0_1_5	KM986896.1
		0_2_1	KM986907.1
		0_2_7	KM986913.1
		0_2_8	KM986914.1
		0_2_25	KY499494
		0_2_26	KY499495
		0_2_27	KY499496
		0_2_14	KM986920.1
		0_2_15	KM986921.1
		100_1_19	KY499510
		100_1_20	KY499511
		100_2_8	KM986987.1
		100_2_11	KM986990.1
		100_1_1	KM986965.1
		100_1_9	KM986973.1
		100_1_10	KM986974.1
		100_1_14	KM986978.1
		0_1_4	KM986895.1
		0_1_6	KM986897.1
		100_1_17	KY499505
5	54	0_2_2	KM986908.1
		0_2_19	KY499488

Table S.3: 16S Haplotype list continued.

# Haplotype	# Individuals	Individual code	Accession number
5	54	0_2_9	KM986915.1
		0_3_19	KY499499
		0_3_20	KY499500
		0_3_21	KY499501
		0_3_22	KY499502
		0_3_23	KY499503
		50_1_1	KM986935.1
		50_1_2	KM986936.1
		50_1_3	KM986937.1
		50_1_4	KM986938.1
		50_1_12	KM986946.1
		50_1_13	KM986947.1
		50_1_14	KM986948.1
		50_1_19	KY499528
		50_2_2	KM986951.1
		50_2_5	KM986954.1
		50_2_10	KM986959.1
		50_2_20	KY499531
		50_2_21	KY499532
		50_2_22	KY499533
		100_1_13	KM986977.1
		100_2_19	KY499514
		100_2_20	KY499515
		100_2_21	KY499516
		100_2_23	KY499518
		50_1_5	KM986939.1
		50_1_6	KM986940.1
		50_1_7	KM986941.1
		50_1_9	KM986943.1
		50_1_10	KM986944.1
		50_1_11	KM986945.1
		50_2_1	KM986950.1
		50_2_4	KM986953.1
		50_2_6	KM986955.1
		50_2_9	KM986958.1
		50_2_12	KM986961.1
		50_2_13	KM986962.1
		50_2_14	KM986963.1
		50_2_15	KM986964.1
		100_2_14	KM986993.1
		0_3_7	KM986926.1
		0_3_15	KM986934.1
		0_3_16	KY499484
		0_3_17	KY499485
		50_1_16	KY499522
		50_1_17	KY499523
		50_1_18	KY499524
		50_2_16	KY499525

Table S.3: 16S Haplotype list continued.

# Haplotype	# Individuals	Individual code	Accession number
5	54	50_2_17	KY499526
		50_2_18	KY499527
		100_1_16	KY499504
		100_2_17	KY499508
6	3	0_2_4	KM986910.1
		0_2_11	KM986917.1
		0_2_24	KY499493
7	2	0_2_21	KY499490
		0_1_16	KY499483
8	2	0_2_22	KY499491
		0_2_10	KM986916.1
10	1	0_2_28	KY499497
11	16	0_3_1	KM986922.1
		0_3_11	KM986930.1
		0_3_10	KM986929.1
		Bait_III_1	KM987005.1
		Bait_III_2	KM987006.1
		Bait_III_5	KM987009.1
		Bait_III_7	KY499538
		Bait_III_10	KY499534
		Bait_III_11	KY499535
		Bait_I_1	KM986995.1
		Bait_I_2	KM986996.1
		Bait_I_3	KM986997.1
		Bait_I_6	KY499544
		Bait_I_9	KY499547
		Bait_I_10	KY499541
		Bait_I_11	KY499542
12	11	0_3_2	KM986923.1
		0_3_5	KM986924.1
		0_3_6	KM986925.1
		0_3_8	KM986927.1
		0_3_9	KM986928.1
		0_3_12	KM986931.1
		0_3_13	KM986932.1
		Bait_II_1	KM987000.1
		Bait_II_2	KM987001.1
		Bait_II_6	KY499551
		Bait_II_7	KY499552
13	2	50_1_20	KY499529
		50_2_19	KY499530
14	30	100_1_2	KM986966.1
		100_1_3	KM986967.1
		100_1_6	KM986970.1
		100_1_11	KM986975.1
		100_1_12	KM986976.1
		100_1_21	KY499512
		100_1_22	KY499513

Table S.3: 16S Haplotype list continued.

# Haplotype	# Individuals	Individual code	Accession number
14	30	100_2_4	KM986983.1
		100_2_5	KM986984.1
		100_2_9	KM986988.1
		100_2_10	KM986989.1
		100_2_12	KM986991.1
		100_2_13	KM986992.1
		100_2_15	KM986994.1
		100_2_22	KY499517
		100_2_24	KY499519
		100_2_25	KY499520
		100_2_26	KY499521
		100_1_4	KM986968.1
		100_1_5	KM986969.1
		100_1_8	KM986972.1
		100_1_15	KM986979.1
		100_2_1	KM986980.1
		100_2_2	KM986981.1
		100_2_3	KM986982.1
		100_2_7	KM986986.1
		100_1_18	KY499506
		100_2_16	KY499507
		100_2_18	KY499509
		Bait_I_4	KM986998.1
15	1	50_1_8	KM986942.1
16	2	50_1_15	KM986949.1
		50_2_3	KM986952.1
17	1	50_2_7	KM986956.1
18	3	50_2_11	KM986960.1
		100_2_6	KM986985.1
		0_3_14	KM986933.1
19	1	100_1_7	KM986971.1
21	4	0_1_11	KM986900.1
		Bait_II_3	KM987002.1
		Bait_II_5	KM987004.1
		Bait_II_10	KY499548
22	1	0_3_18	KY499486
23	6	Bait_III_3	KM987007.1
		Bait_III_4	KM987008.1
		Bait_III_6	KY499537
		Bait_III_8	KY499539
		Bait_III_9	KY499540
		Bait_I_12	KY499543
24	1	Bait_III_12	KY499536
25	1	Bait_I_5	KM986999.1
26	1	Bait_I_7	KY499545
27	1	Bait_I_8	KY499546
28	2	Bait_II_4	KM987003.1
		Bait_II_9	KY499554

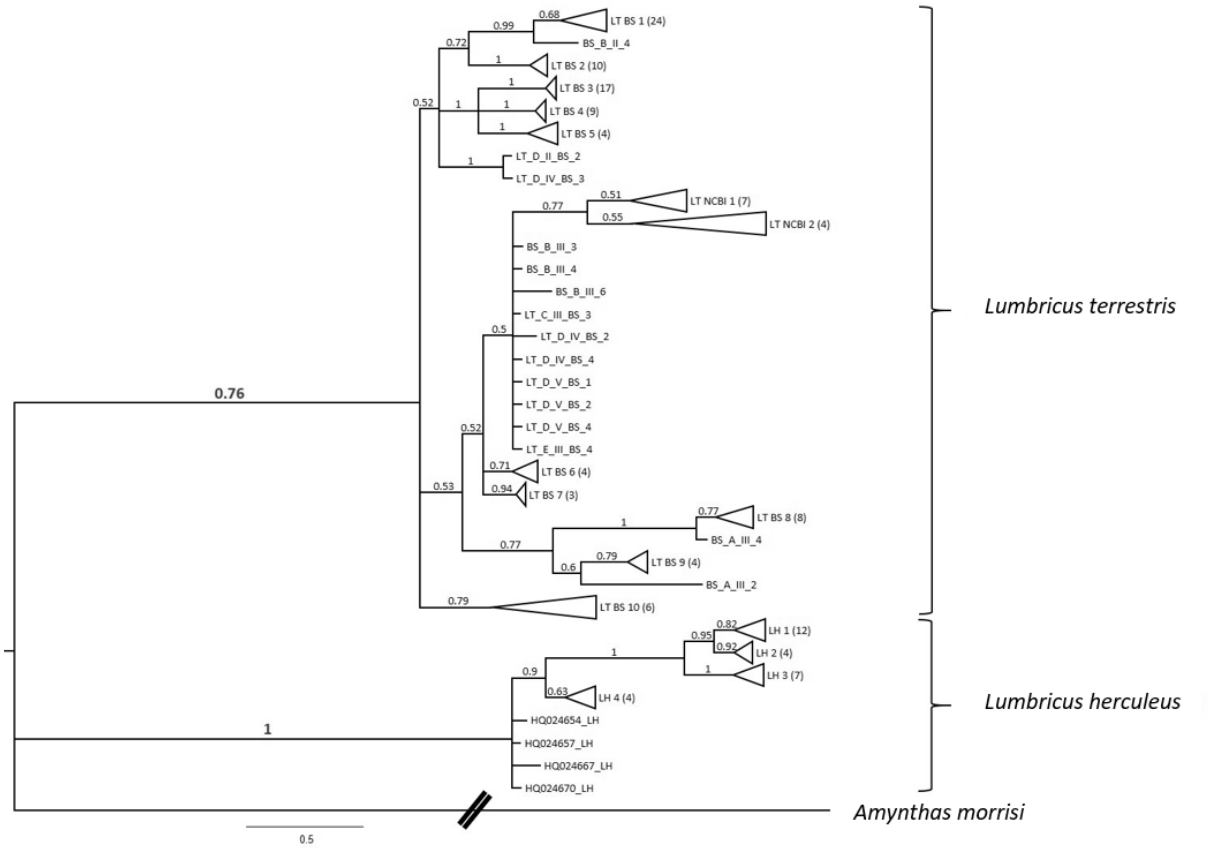

Figure S.4: Phylogenetic tree based on COI DNA of *Lumbricus terrestris* from 21 bait shop samples including the three bait shops from Calgary together with COI sequences from NCBI of *L. terrestris* and it cryptic species *Lumbricus herculeus* from the study of James et al. 2010. The main split between both cryptic species is indicated by posterior probabilities (bold).

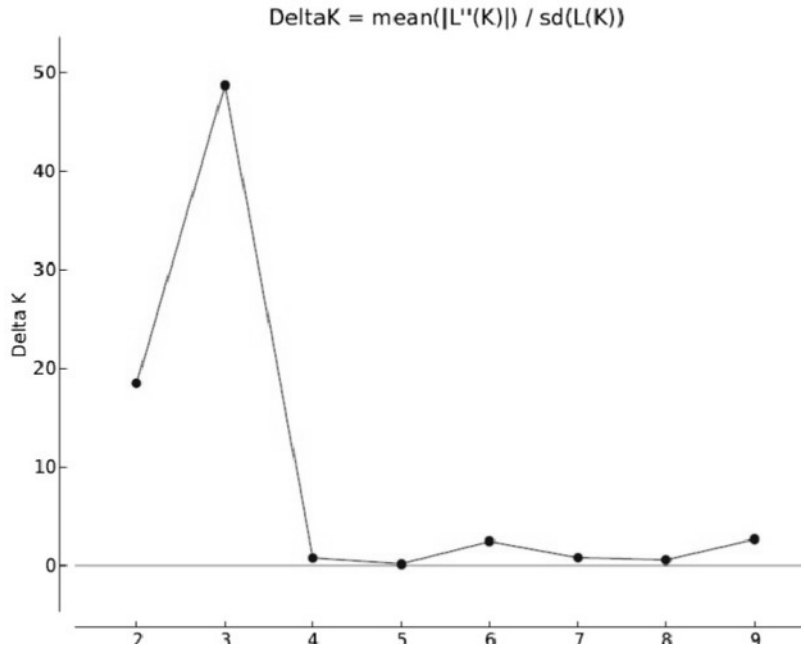

Figure S.5: DeltaK value chart of the STRUCTURE population assignment analysis; highest value indicates the most likely number of populations within the dataset.

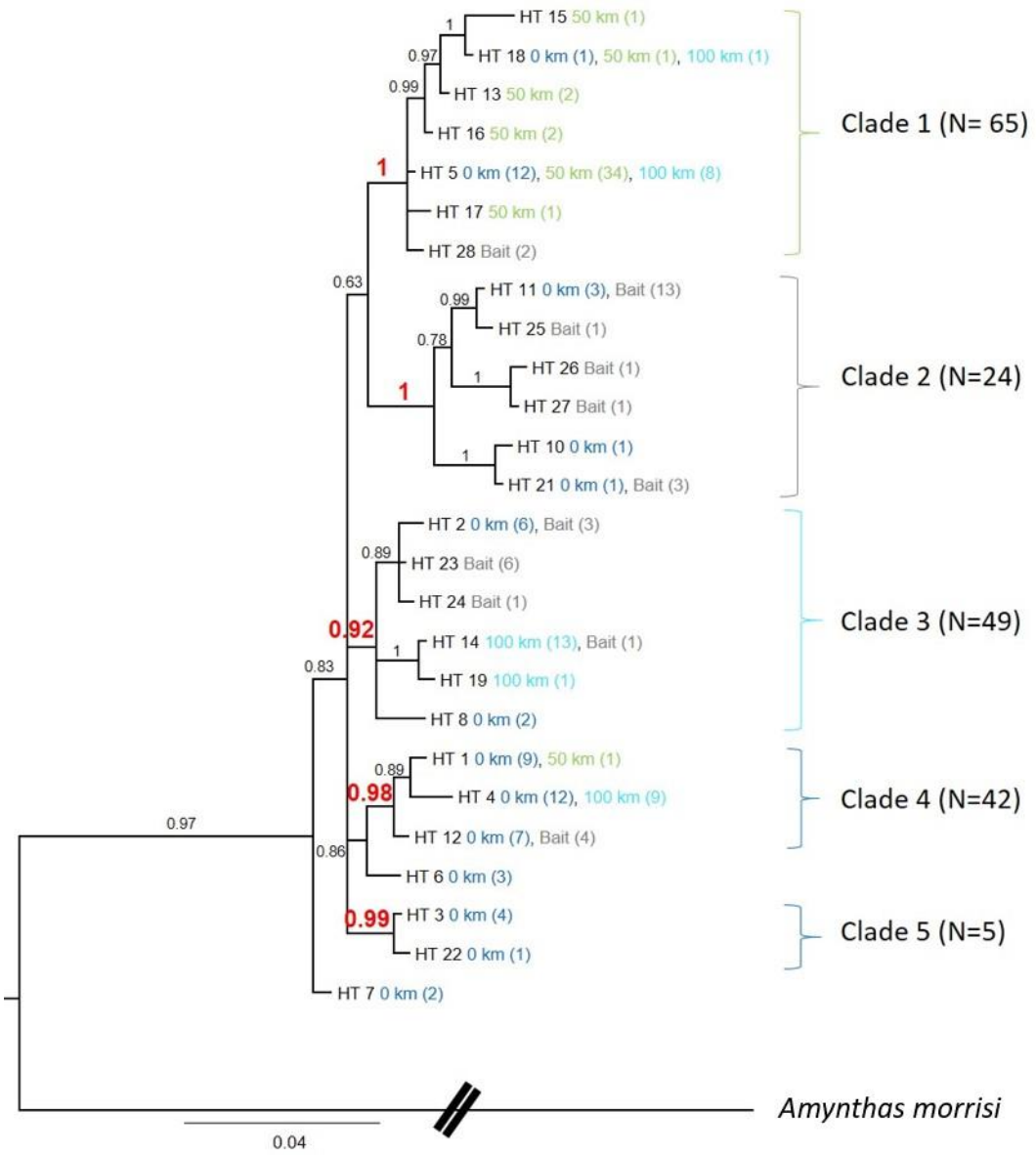

Figure S.6: Phylogenetic haplotype tree based on 16S rDNA of *Lumbricus terrestris* from sampling locations in Calgary (blue) and 50 km (green) and 100 km (turquoise) south of Calgary as well as from three bait shops in Calgary (grey). Clades were assigned in accordance with haplotype network results and branch/node support by posterior probabilities. Sampling locations included are assigned to each clade and numbers of individuals per sampling location are indicated in brackets. Numbers on nodes are posterior probabilities support values.

CHAPTER 4

ADAPTABILITY OF NON-NATIVE *LUMBRICUS TERRESTRIS* TO SEASONAL ENVIRONMENTAL CLIMATE CONDITIONS IN A CLIMATE CHAMBER TRANSPLANTATION EXPERIMENT

Andreas Klein, Laura Holla, Stefan Scheu, Ina Schaefer, Nico Eisenhauer

Abstract

Temperature and precipitation significantly affect the performance of lumbricid earthworms. However, studies investigating the combined effects of temperature and precipitation on earthworm performance are rare, and were never linked to genetic diversity/identity of earthworms and their impact on soil properties or microbial functions. Variation of abiotic factors and genetic identity can be of particular importance for invasive species due to environmental filtering or introduction histories. The earthworm *Lumbricus terrestris* was introduced to North America from different source populations in Europe several hundred years ago and is responsible for severe changes in ecosystems in invaded areas. Here, we conducted a yearlong full-factorial transplantation climate chamber experiment with 180 individuals of *L. terrestris,* which were collected from three North American sites with distinct climate conditions, altitude, and history of European settlement. We simulated four temperature and moisture combinations; and genetic diversity and identity was determined of surviving individuals and offspring.

Our results indicate that seasonality of temperature and precipitation was the main determinant for earthworm biomass gain, offspring number, and activity. Further, we show significant effects of earthworms on soil moisture and microbial functions related to high burrowing and litter burying/feeding activity, respectively. Genetic diversity and identity did not show clear correlation with earthworm performance and ecosystem functions under the different climate conditions. Nevertheless, investigating intraspecific diversity in context of invasion and ecosystem effects of earthworms is promising; caveats and outlooks how to integrate genetic identity in future experimental settings are discussed.

Keywords

North America; invasion biology; genetic diversity and identity; microbial functions; soil properties

4.1 Introduction

Invasions of natural communities by non-indigenous species are a threat to native biodiversity and are currently rated as one of the most important environmental problems on global-scale (Sala et al., 2000; Wardle et al., 2011; Murphy and Romanuk, 2014). Thus, exploring the environmental drivers and filters that determine the spread of invasive species is necessary to understand and predict invasions. For instance, temperature and precipitation are supposed to be two of the main environmental factors determining survival, growth, reproduction, and activity of invasive earthworms (Swift et al., 1979; Holmstrup, 2003; Fisichelli et al., 2013).

Although climate effects on earthworms have been investigated before (Byzova, 1973, 2007; Lee, 1985; Daniels et al., 1996), this has almost exclusively been done in short-term laboratory experiments under constant temperature conditions ignoring natural fluctuations (Butt, 1991; Butt et al., 1992; Edwards and Bohlen, 1996; Berry and Jordan, 2001). More recent studies added diurnal and seasonal changes of temperature in field and laboratory experiments (Holmstrup, 2003; Uvarov et al., 2011) but did not include the combination of the two most important factors driving the invasion of northern North America by European earthworms, i.e. temperature (particularly frost events) and precipitation. Considering temperature and precipitation is important to relate results to real world ecosystems (Cossins and Bowler, 1987) and to identify factors limiting dispersal and distribution of invasive earthworms.

The role of freeze/thaw fluctuations on overwintering success has been studied for cocoons of earthworms (Jensen and Holmstrup, 1997). Furthermore, earthworm species benefit from the absence of diurnal fluctuations, and the frequency and severity of frost events may have contrasting effects on different earthworm species, facilitating the coexistence of species of similar trophic position (Uvarov et al., 2011). Earthworms significantly affect the turnover, drainage, and aeriation of soil layers by mixing of leaf litter with mineral soil via their burrowing activities (Lee, 1985; Edwards & Bohlen, 1996; Hale et al., 2006; Migge-Kleian et al., 2006; Eisenhauer et al., 2007, 2010). Knowledge on the performance of earthworms therefore is important for understanding how climate change will affect the functioning of soils (Uvarov et al., 2011), such as decomposition processes, nutrient cycling, and water infiltration.

Here, we investigated the invasive earthworm species *Lumbricus terrestris* L., 1758, the most common anecic earthworm in regions of northern North America invaded by earthworms (Hendrix et al., 2008). *Lumbricus terrestris* preferentially colonizes neutral to slightly alkaline soils, lives in permanent, vertical burrows up to 2 m deep, and feeds on litter collected on the surface in the vicinity of its burrows (Sims and Gerard, 1999; Tiunov et al., 2006; Addison, 2009). *Lumbricus terrestris* reproduces 1-2 cocoons per month (Butt et al., 1994) and has an estimated active dispersal rate of 4

m y^{-1} (Curry, 1988; Marinissen and van den Bosch, 1992), and its life expectance is up to six years under laboratory conditions (Lee, 1985; Butt et al., 1994). Mating occurs from March to December (Gates, 1961) either with two peaks in spring and autumn or one in early summer depending on seasonal fluctuations in temperate (short winters) or continental climate conditions (long and cold winters; Butt et al., 1994). Despite its low reproductive rate and limited active dispersal, *L. terrestris* is a common invasive species around the world (Hendrix et al., 2008), and has spread from coast to coasts of northern North America across the continent within the past 400 years since introduced by European settlers (Reynolds, 1977, 1994; Scheu and Parkinson, 1994; Bohlen et al., 2004; Hendrix et al., 2008).

In their new habitat, *L. terrestris* encountered climate conditions that, depending on the region, differed substantially from the source locations in Europe, such as more extreme seasonal maximum and minimum temperatures and precipitation in central parts of northern North America (Utescher et al., 2017). It remains unclear whether the successful invasion was based on selection processes or on the inherent ability of the introduced specimens of *L. terrestris* to adapt to varying local conditions. Previous studies suggested multiple introduction events and high genetic diversity at regional scales for *L. terrestris* and other earthworm species (*Dendrobeana octaedra*), and highlighted the importance of genetic diversity and identity for range expansion and adaptation (Cameron et al., 2008; Gailing et al., 2012; Klein et al., 2017). For disentangling the relative importance of genetic and environmental factors for earthworm invasions studies investigating the performance of earthworm populations from climatically dissimilar locations of invaded regions in different environmental contexts are needed.

Genetic diversity and haplotype identity have important implications for the success of invasive species by influencing the performance the ability of the invading species to adapt to local environmental conditions. A genetically diverse population likely occupies a wider range of ecological niches (Scheu and Drossel, 2007; Hughes et al., 2008), and thus has the potential to increase abundance through micro-niche adapted genotypes as well as the likelihood of successful colonisation of previously unoccupied (earthworm-free) areas and habitats.

Consequentially, parameters linked to genetic diversity of earthworm populations, such as biomass, fitness, behavioural changes, resistance to disturbances, intraspecific competition, and community structure can affect ecological functions at the population, community, and ecosystem level (Tsutsui et al., 2003; Gamfeldt et al., 2005; Mattila and Seeley, 2007; Hughes et al., 2008). These effects may include changes in soil structure and moisture, nutrient mineralisation, litter decomposition, and plant seed development (Edwards & Bohlen, 1996; Hughes et al., 2008; Eisenhauer et al., 2010). In turn, this can have feedback effects on species diversity, fitness, and abundance of earthworm populations and their genetic diversity or identity, thus causing a cascade feeding back to the success of earthworm invasions into North American forests (Craven et al., 2017).

Crutsinger et al. (2007) provided experimental evidence that in plants intraspecific diversity and distinct genotypes can act as barriers for invasion of species, and concluded that the loss of intraspecific diversity within a dominant plant species can increase the susceptibility of the ecosystem to invasion. This has several implications for earthworm invasions. First, high intraspecific diversity of an invading species can positively affect invasion success by resisting or replace native "barrier species". Second, it can facilitate successive invasion stages after initial establishment of the invasive earthworm species, by forming a new barrier for other species or populations of lower intraspecific diversity. And third, distinct genotypes of earthworms that established in non-native areas also may operate as an invasion barrier. Moreover, there is evidence that the genetic diversity of plant species affects arthropod diversity and net productivity of ecosystems (Crutsinger et al., 2006), indicating that genetic diversity effects of populations can have consequences for whole ecosystem (Hughes et al., 2008). Accordingly, genetic diversity of invasive earthworm populations may also be relevant for invasion success and the intensity of their effects on invaded ecosystems and changes in soil properties.

Here, we conducted a yearlong transplantation climate chamber experiment investigating the performance (biomass change, offspring number, and mortality) of *L. terrestris* under seasonal temperature and precipitation (moisture) fluctuations as well as subsequent ecosystem effects (litter consumption, soil water content, and microbial functions). Further, we sequenced the COI gene of surviving adult earthworms and offspring to analyse if genetic identity and diversity correlates positively with earthworm performance and intensity of ecosystem effects.

We collected *L. terrestris* from three northern North American sites of distinct climate conditions, altitude, and history of European settlement: (i) near Vancouver (British Columbia, CAN; West), (ii) Minneapolis (Minnesota, USA; Centre), and (iii) Newcomb (New York, USA; East). Based on preliminary molecular screenings, the collected earthworms were expected to be genetically diverse and distinct in each region. We simulated four temperature and moisture combinations. Two combinations, i.e. warm and moist, and cold and dry, represented the climate conditions in the West (British Columbia) and Centre/East (Minnesota and New York) of northern North America, respectively. The other two combinations, i.e. warm and dry, and cold and moist, represented climate conditions from Europe and foreign to their European and North American ranges, respectively. The moisture treatments also differed in seasonality. In the moist (West) treatments, moisture was high during the simulated autumn/winter season, and low in the spring/summer season, while this pattern was reversed in the dry treatments (Centre/East). To avoid bias due to different soil properties and microbial communities at the sampling regions, mesocosms were stocked with forest soil from Germany foreign to all earthworms.

We tested the hypotheses that (1a) generally, earthworm performance will be highest (i.e. maximum biomass increase, minimum mortality, maximum offspring number) in treatments with warm and moist conditions irrespective of their origin. Alternatively, (1b) earthworms may perform better in temperature and precipitation treatments most similar to conditions at their sampling site. (2) North American earthworms originate from a genetically diverse (European) source population, but different lineages established in West, Centre, and East due to environmental filtering, i.e. distinct precipitation and temperature conditions. Accordingly, genetic identity of earthworms correlates with climate conditions of the West, Centre, and East. (3) Origin and genetic identity of earthworms affect their activity (litter consumption) and ecosystem effects (soil water content and microbial functions) in the different treatments. This study is the first attempt to link earthworm responses to climatic conditions and ecosystem effects with the genetic diversity and identity of earthworms.

4.2 Material and methods

4.2.1 Sampling of earthworms, litter, and soil

Earthworms were sampled at three different sites in the USA and Canada in June 2015. The East sampling site in the Adirondack Mountains in New York State was a mixed deciduous-coniferous forest at 500 m altitude, which is dominated by *Acer* and *Betula* with interspersed *Tsuga*, *Picea,* and *Pinus* species. It is located in the northeast of Upstate New York and was historically shaped by forestry and heavy deforestation. It became a forest preserve park in the 20th century and has increasingly been used as a recreational area for tourism (APA, https://apa.ny.gov). Climate in the Adirondacks is humid continental (warm summers and cold winters) with a precipitation of ca. 950 mm y^{-1}; annual mean, minimum, and maximum air temperatures are 4.7 °C, -1.5 °C, and 10.9 °C, respectively (NOAA, www.noaa.gov).

The Centre sampling site was a forest at 300 m altitude close to the Twin Cities Minneapolis-St. Paul in Minnesota. The deciduous forest is dominated by sugar maple, red oak, and basswood. The area was first settled in mid-19th century by European immigrants from Germany, who started cultivating crops around today's remaining forests and used for light forestry and sugar maple production until after the Second World War and became a designated scientific and natural area (DNR, http://www.dnr.state.mn.us/snas) in 1970. Climate in southern Minnesota is humid continental (hot summers and cold winters) with a precipitation of ca. 800 mm y^{-1}; annual mean, minimum, and maximum air temperatures are 7.5 °C, 2.2 °C, and 12.7 °C, respectively (NOAA, www.noaa.gov).

The West sampling location was situated in Vancouver; however, due to drought in May/June 2015 combined with an already unusually dry winter and spring seasons, it was impossible to find any *L.*

terrestris specimens in forest areas. Therefore, we purchased worms from a local bait shop selling individuals originating from within the Greater Vancouver area. The area of Greater Vancouver was settled by Europeans in mid-18th century who started with fur trade which developed into major trading posts, settlements, and later cities. Mining, agriculture, forestry and fishing developed later in mid-19th century and caused rapid population growth. Today, the area is mainly urban with only small patches of trees, meadows, or fields (Davis & Mooney 1986). Climate in Vancouver is moderate oceanic (warm dry summers and mild rainy winters) with a precipitation of ca. 1200 mm y^{-1}; annual mean, minimum, and maximum air temperatures are 10.4 °C, 6.9 °C, and 14.0 °C, respectively (NOAA, www.noaa.gov).

Earthworms were extracted from soil by applying mustard solution (Eisenhauer et al., 2008), followed by hand-sorting. Sampled earthworms were washed with water, stored in soil-filled plastic boxes at 4 °C, shipped via airmail to Göttingen University, Germany, and allowed to acclimatise for one month until the start of the experiment (Fründ et al., 2010). Sick and lethargic individuals were disposed, and in total 180 adult individuals were selected for the experiment. Soil and leaf litter were obtained from a deciduous forest dominated by European beech (*Fagus sylvatica*) in the vicinity of Göttingen (Göttinger Wald), which also is colonized by *L. terrestris*. The forest floor was cleared from litter, and the upper 20 cm of the soil were excavated and sieved twice (first 1 cm then 0.2 cm) to remove earthworms, cocoons, predators as well as stones, wood, and roots. Leaf litter was collected, dried at room temperature and stored until use. Prior to placement into the microcosms it was sorted into tree species, weighed, and mixed for the experiment to ensure uniform food supply. The litter mixture consisted of 11 tree genera: *Fagus* (25.0 %), *Salix* (13.6 %), *Fraxinus* (12.4 %), *Quercus* (10.3 %), *Betula* (8.8 %), *Alnus* (7.9 %), *Acer* (7.6 %), *Prunus* (4.8 %), *Populus* (4.7 %), *Tilia* (2.6 %), and *Ulmus* (2.5 %).

4.2.2 Mesocosms

Mesocosms consisted of cylindrical plastic (PVC) tubes of 21 cm inner diameter and 40 cm height, covered at the top and bottom with air and water permeable gauze (200 µm) attached by tape and cable straps to prevent earthworms from escaping. Mesocosms were filled with ~30 cm (12 kg) soil and topped by 100 g of the deciduous leaf litter mix to resemble natural forest floor conditions. Litter consumption was monitored fortnightly by checking litter ground coverage (Patoine et al., 2017). When litter coverage dropped to 5 - 10%, 10 g of dried leaf litter was added so that the soil again was completely covered; the added amount of litter was recorded to estimate decomposition. During the winter/freezing period, the mesocosms were wrapped in air bubble film and placed on styrofoam sockets for insulation to simulate frost conditions increasing from the top to the bottom.

4.2.3 Climate chamber experiment

The climate data for the climate chamber settings were obtained from the National Oceanic and Atmospheric Administration (NOAA, USA); settings were based on the mean of ten-year temperature and precipitation records for Vancouver and Minneapolis. Due to its altitude, (New York State) temperature and precipitation is similar at the sampling point in the Adirondack Mountains to the one in Minnesota (NOAA, USA). Consequently, these two locations were pooled into one climate setting for the experiment. Temperature and watering schedules were calculated based on a mean ten-year climate record database and transformed to fit the scale of the mesocosms, including upper and lower limits to avoid too dry and too moist soil water conditions or too high mortality by freezing in winter. Previous tests for water uptake and time to freeze-through of the mesocosms allowed to estimate maximum and minimum values for the climate chamber settings: Temperature maximum of 17 °C and a minimum of 0.5 °C, and precipitation maximum of 200 ml and minimum of 50 ml (**Fig. S.1**).

A full-factorial design with two temperature and moisture settings was set up (**Fig. S.2**). Mesocosms were assembled randomly in climate chambers and swapped between both climate chambers every month to avoid potential chamber effects. Three adult individuals of *L. terrestris* from one of the three sampling regions were added to each mesocosm; this resulted in a total of 60 mesocosms (3 earthworm origin treatments x 2 temperature treatments x 2 humidity treatments x 5 replicates; **Fig. S.2**). The number of added earthworms per mesocosm resembled densities in invaded forest areas (Eisenhauer et al., 2007).

A total of 17 response variables were measured at the end of the yearlong experiment, i.e. biomass change (per mesocosm and individual), mortality, offspring number and biomass, number of other earthworms and biomass, weight difference start/end per mesocosm, litter consumption, water content, soil microbial biomass, basal respiration, soil microbial metabolic quotient as well as the genetic variables (haplotype identity, mean pairwise differences, nucleotide and haplotype diversity) separately for surviving adults and juveniles. Soil temperature and moisture were recorded using TinyTag data loggers (Gemini Ltd., Chichester, UK) with five loggers per climate chamber to detect possible temperature differences within the climate chamber. Earthworm activity was tracked indirectly through periodical measurements of litter ground coverage (fortnightly).

4.2.4 Genetic analyses

Earthworms were collected, washed, weighed, and transferred into 95% ethanol and stored for genetic analyses. Genomic DNA was extracted using Genaxxon DNA Tissue Mini Prep Kit (Genaxxon; Ulm, Germany) following the manufacturer's protocol. The mitochondrial gene COI (~600 bp; Folmer

et al., 1994) was amplified in both adult and juvenile individuals. The PCR cycling conditions had 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 and were sequenced at SeqLab Göttingen (Microsynth; Balgach, Switzerland). All sequences are to be submitted (GenBank www.ncbi.nlm.nih.gov/genbank). Sequences were checked with Sequencher 4.9 (Gene Codes Corporation, USA), and ambiguous positions were coded as wobble bases or deleted in the alignments.

Consensus sequences of adults, juveniles, and a combined dataset (adults and juveniles) were aligned with ClustalW. The three sequence alignments were collapsed into haplotype alignments using FaBox 1.41 (Villesen, 2007). The best fit models of sequence evolution were estimated with TOPALi v2.5 (Milne et al., 2004) using the Akaike information criterion (AIC; Akaike, 1973). The best model was HKY+I+G for all three datasets. Phylogenetic trees were constructed with MrBayes 3.2 (Ronquist and Huelsenbeck, 2003) using the following parameters: lset nst=2 rates=propinv and a mcmc run of 8 million generations, a sampling frequency of 2500 and burn-in of 2500. The outgroup *Aporrectodea rosea* was used in the trees. To analyse genetic structure in the context of climate settings, a haplotype network was constructed for the complete *L. terrestris* dataset. The alignment was checked and converted for network analysis using DNA Alignment 1.3.3.1 (Fluxus Technology Ltd., Suffolk, England). The Median-joining (MJ) network (Bandelt et al., 1999) was constructed with PopART (University of Otago, Dunedin, New Zealand) and edited in Inkscape (Software Freedom Conservancy, USA). Arlequin (Excoffier, 2010) was used to calculate the diversity indices (nucleotide diversity, haplotype diversity and mean number of pairwise differences) separating the complete dataset into the following subsets: (1) juvenile, adult, and combined, (2) all earthworms from West, Centre, and East, (3) adults from West, Centre, and East, and (4) all earthworms that separated into the two clades from West, and earthworms from Centre and East were combined.

4.2.5 Soil microbial measurements

Approximately 4.5 g soil (fresh weight) was used to determine basal respiration, soil microbial biomass, and soil microbial metabolic quotient. Using an automated respirometer (Scheu, 1992), microbial respiration (µl O_2 g^{-1} soil dry mass h^{-1}) was measured continuously as mean of the O_2 consumption rates between 14-24 h at 20°C after the start of the measurements. Soil microbial biomass was calculated from the maximum initial respiratory response (MIRR) after addition of D-glucose-monohydrate using the substrate-induced respiration method (SIR) (Anderson and Domsch, 1978; Beck et al., 1997). Catabolic enzymes of soil microorganisms were saturated by adding 40 mg

glucose g^{-1} soil dry mass as aqueous solution. Soil microbial metabolic quotient was calculated by dividing basal respiration by soil microbial biomass.

4.2.6 Statistical analysis

All statistical analyses were conducted with R studio v1.0.136 (RStudio Inc., Boston, USA) using the packages 'car', 'MuMIn', and 'picewiseSEM'. Treatment effects (earthworm origin, temperature, precipitation, and interactions) were analysed using a general linear model (GLM) and a type-II-ANOVA in a multifactorial approach with covariates. In total, four variables were considered covariates, i.e. start biomass, mortality, number of other earthworm species, and soil water content, which were included individually or in combination in the analyses.

Two principal pathway analyses (structural equation modelling, SEM) were performed based on the revealed correlations to test the hypotheses of causal relationships among the tested parameters. The first focused on earthworm sampling origin as well as climate conditions and its influence on genetic diversity, initial biomass, and on the response variables mortality, final biomass, and offspring number. The second analysis considered the genetic identity of earthworms and its influence on initial biomass or response variables as well as the mutual connection between genetic identity and genetic diversity.

4.3 Results

4.3.1 General harvest results

In total 141 adult and 56 juvenile individuals were sampled at the end of the experiment; thus, out of 180 initially introduced adult earthworms, 39 were not retrieved and recorded as dead. Three out of the 60 mesocosms, two from East (New York) and one from Centre (Minnesota), did not contain any surviving adult earthworms. Mortality was lowest in earthworms from Centre and number of offspring was highest in earthworms from West (Vancouver). Genotyping resulted in 29 (adult) and 14 (juvenile) COI haplotypes; the combined dataset consisted of 33 haplotypes.

4.3.2 Earthworm performance

Temperature and earthworm origin significantly affected earthworm performance (biomass gain, offspring number, and offspring biomass; $p<0.05$ for all). Mortality was marginally significantly affected by soil water content ($p=0.067$) with a slight decrease in mortality with increasing soil water content (**Table 1, Fig. S.3**). Initial earthworm biomass differed among the three origin populations decreasing from the West (3.64 g ± 0.16) to Centre (2.99 g ± 0.18) to the East (2.27 g ± 0.05; **Fig. 1a**), and biomass gain decreased with higher initial biomass and increased with mortality (both $p=0.001$; **Fig. S.4**, **S.5**). Thus, initial earthworm biomass was negatively correlated with biomass gain over the course of the experiment (**Table 1, Fig. S.4**).

Table 1: ANOVA results for the response variables mean individual biomass gain (LogRRewWeightInd), offspring number (no. offspring), offspring weight, mortality, number of other earthworms (no. other worms), litter consumption, water content, microbial carbon (Cmic), oxygen consumption (qO2), nucleotide diversity adults (NucDivAd), haplotype diversity adults (HTDivAd), and mean pairwise differences adults (MPDifAd). Treatment variables are temperature (T), precipitation (P), earthworm origin (O), and interactions of all three treatment variables (TxP, TxO, PxO, TxPxO). Initial total biomass, mortality, no. other worms, and water content were used as co-variables. df = degrees of freedom, χ2 = chi-square, p = probability value.

		responses																					
		LogRRew WeightInd		no. offspring		offspring weight		mortality		no. other worms		litter consumption		water content		Cmic		qO2		NucDivAd		HTDivAd		MPDifAd	
co-variables	d.f.	χ2 -value	p -value	χ2 -value	p -value	χ2 -value	p -value	χ2 -value	p -value	χ2 -value	p -value	χ2 -value	p -value	χ2 -value	p -value	χ2 -value	p -value	χ2 -value	p -value	χ2 -value	p -value	χ2 -value	p -value	χ2 -value	p -value
start weight total	1	15.02	<0.001	0.11	0.743	0.17	0.677	0.79	0.374	0.07	0.790	0.93	0.336	0.01	0.906	0.97	0.326	0.07	0.791	0.74	0.388	0.71	0.401	0.74	0.391
mortality	1	25.74	<0.001	1.46	0.227	2.65	0.104	/	/	0.32	0.570	97.66	<0.001	3.35	0.067	1.63	0.202	0.01	0.958	1.18	0.278	0.16	0.693	1.14	0.285
no. other worms	1	1.16	0.281	0.09	0.765	0.14	0.709	0.32	0.570	/	/	1.49	0.223	9.39	0.002	0.26	0.608	0.83	0.363	0.10	0.746	0.29	0.587	0.08	0.771
water content	1	2.43	0.119	0.77	0.381	0.01	0.984	3.35	0.067	9.39	0.002	20.72	<0.001	/	/	16.14	<0.001	1.20	0.274	1.61	0.205	0.99	0.319	1.58	0.209
variables																									
T	1	6.10	0.014	3.91	0.048	5.88	0.015	0.03	0.855	2.85	0.092	15.26	<0.001	11.47	<0.001	14.40	<0.001	12.32	<0.001	4.09	0.043	1.47	0.226	4.09	0.043
P	1	0.38	0.540	0.36	0.550	0.43	0.511	1.55	0.213	1.25	0.263	?	0.992	0.80	0.371	18.31	<0.001	14.69	<0.001	1.95	0.163	1.53	0.216	2.00	0.157
O	2	7.37	0.025	9.70	0.008	6.37	0.041	2.21	0.331	1.80	0.407	3.95	0.138	0.19	0.911	3.78	0.151	1.31	0.519	4.24	0.120	2.75	0.253	4.16	0.125
TxP	1	5.39	0.020	0.25	0.617	0.50	0.478	2.49	0.115	0.04	0.849	0.02	0.888	6.37	0.012	0.85	0.356	0.98	0.323	1.80	0.180	1.03	0.310	1.78	0.183
TxO	2	4.03	0.133	1.12	0.571	1.47	0.481	2.96	0.227	0.26	0.876	6.67	0.036	0.60	0.741	6.20	0.045	1.77	0.412	1.06	0.587	0.45	0.799	1.18	0.554
PxO	2	0.63	0.728	0.86	0.650	0.98	0.612	0.23	0.890	0.83	0.660	1.19	0.552	0.73	0.696	7.52	0.023	7.48	0.024	5.20	0.074	8.56	0.014	5.30	0.071
TxPxO	2	1.71	0.426	1.91	0.384	2.30	0.316	2.39	0.302	2.38	0.305	0.04	0.982	9.98	0.007	7.97	0.019	6.31	0.043	5.81	0.055	5.18	0.075	5.70	0.058

Furthermore, earthworm biomass gain was affected by the experimental treatments, being higher under moist conditions than under dry conditions in the colder temperature treatment, and being higher in the dryer soil in the warmer temperature treatment ($p=0.020$; **Table 1, Fig. 1b**). Offspring number tended to increase in the higher temperature treatment ($p=0.087$; **Table 1, Fig. 1d**). Moreover, earthworms from the West had higher numbers of offspring than the Centre and East populations ($p=0.021$; **Table 1, Fig. 1c**).

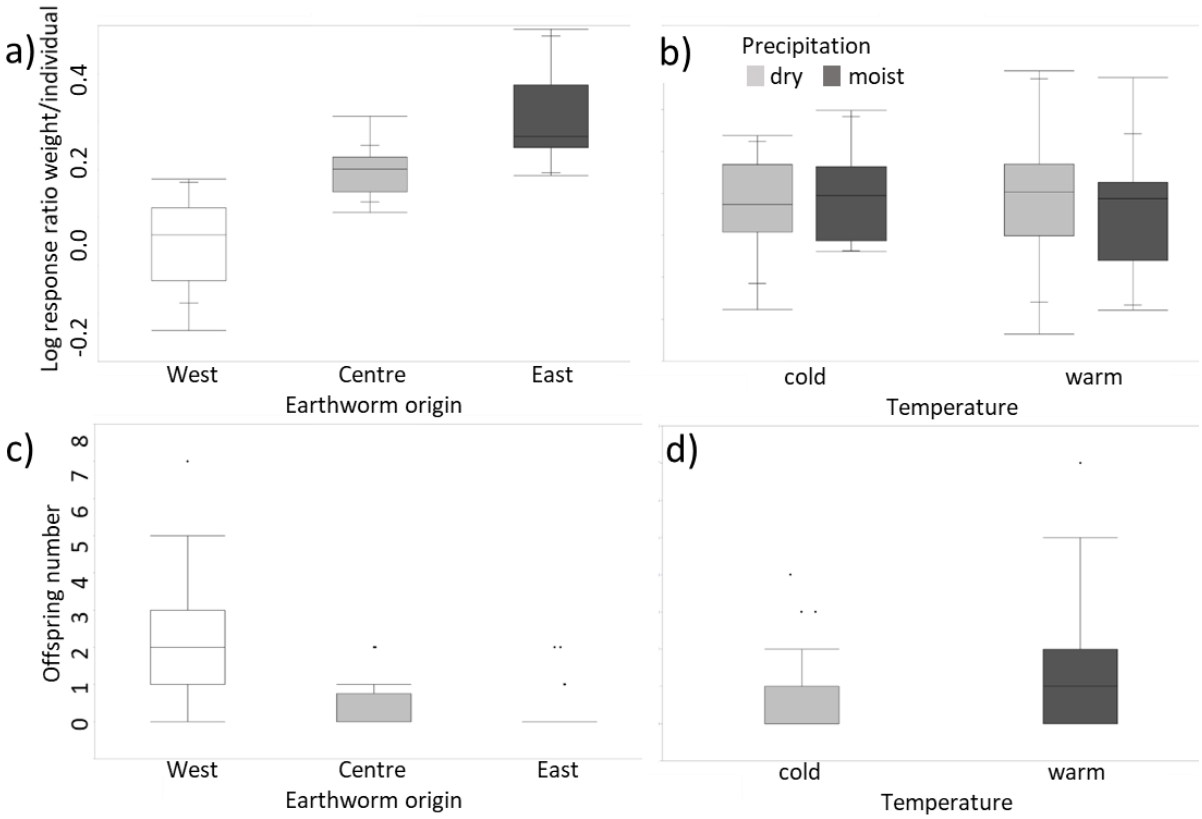

Figure 1: Performance of *Lumbricus terrestris* (box plots); a) weight gain as affected by earthworm origin, b) weight gain as affected by temperature and precipitation, c) offspring number as affected by temperature, and d) offspring number as affected by earthworm origin.

4.3.3 Earthworm genetic diversity

The haplotype network (HTNW) analysis of the COI sequence of all surviving adults and hatched juveniles revealed three distinct genetic groups (=lineages) but did not coincide with their geographic origin. Clades in the HTNW corresponded with well supported clades in the phylogenetic analyses (posterior probabilities 0.85-0.97, **Fig. S.6**). The earthworm population from West separated into distinct genetic lineages (West 1 and West 2) containing 42 and 31 individuals, respectively. West juveniles shared the haplotypes of adult earthworms harvested from the same mesocosms or were at least closely related to them (**Fig. 2a**). The earthworm populations from Centre and East were represented by a single lineage (Centre/East) containing 121 individuals. All juveniles from Centre and East shared haplotypes with at least one adult from the same mesocosm, except for one juvenile individual, which was located within West 1 and was deleted as an outlier. Notably, 13 individuals from West individuals clustered among haplotypes from Centre/East.

The haplotype diversity within populations was highest in the adult and combined (adult and juveniles) datasets, and lowest for juveniles, but nucleotide diversity was very similar among these

three datasets (**Table 2a**). Comparing nucleotide diversity among the three regions of earthworm origin, the population from West had the highest nucleotide diversity but were similar for earthworm populations from Centre and East (**Table 2b**), both with and without juveniles (**Table 2c**). Furthermore, we checked the diversity indices for each genetic lineage as indicated by the HTNW and Bayesian phylogenetic tree. Nucleotide diversity was low in lineages West 1 and West 2, and lineage Centre/East had a significantly higher nucleotide diversity but also more individuals (**Table 2d**).

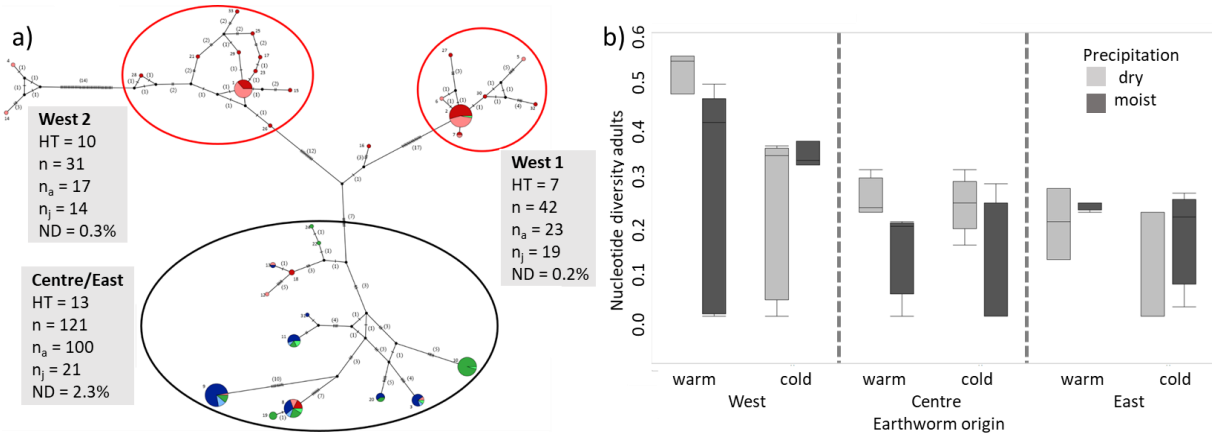

Figure 2: Genetic diversity of *Lumbricus terrestris*; a) unrooted haplotype network of mitochondrial COI sequences of the combined adult and juvenile dataset. Size of the pie charts indicate number of individuals of the respective haplotype. Short orthogonal black lines on the paths connecting the haplotypes represent the number of mutation steps between two haplotypes; numbers of steps >1 indicated in brackets. Numbers next to the haplotypes indicate the haplotype number. Colours represent the origin region of individuals, west = red, central = green and east = blue. Adults marked in dark and juveniles in light colour. Designated genetic lineages are indicated by coloured frames red = western lineage and black = mixed lineage) with the number of individuals and haplotypes given in grey boxes. b) Nucleotide diversity of adult *L. terrestris* as affected by temperature, precipitation and earthworm origin.

Nucleotide diversity (p=0.055), mean pairwise differences (p=0.058), and haplotype diversity (p=0.075) were marginally significantly affected by three-way interaction effects of temperature, precipitation, and earthworm origin. Genetic diversity was highest in earthworms from West under warm and dry conditions and lowest in the earthworm population from the Centre under warm and moist conditions (**Fig. 2b, S.7**).

Table 2: Genetic diversity values and number of individuals for a) adult, juvenile and combined datasets, b) combined adult and juvenile populations of West, Centre and East sampling origin, c) adult populations of West, Centre and East sampling origin, and d) genetic lineage West 1, West 2 and Centre/East. ND = nucleotide diversity, HTD = haplotype diversity, MPD = mean pairwise differences.

	dataset	# individual	ND [%]	HTD	MPD
a)	adult	141	3.4 ± 1.7	0.89 ± 0.01	21.03 ± 9.34
	juvenile	56	4.0 ± 2.0	0.84 ± 0.03	24.36 ± 10.86
	combined	197	3.7 ± 1.8	0.89 ± 0.01	22.57 ± 9.98
b)	West	88	3.4 ± 1.7	0.79 ± 0.03	20.57 ± 9.17
	Centre	60	1.9 ± 0.9	0.69 ± 0.05	11.90 ± 5.46
	East	49	2.1 ± 1.0	0.73 ± 0.06	12.67 ± 5.81
c)	West adult	47	3.1 ± 1.6	0.81 ± 0.05	19.12 ± 8.62
	Centre adult	52	2.0 ± 1.0	0.69 ± 0.06	11.96 ± 5.50
	East adult	42	1.9 ± 1.0	0.68 ± 0.07	11.59 ± 5.36
d)	West 1	42	0.3 ± 0.2	0.50 ± 0.11	1.60 ± 0.98
	West 2	31	0.2 ± 0.1	0.31 ± 0.09	0.98 ± 0.68
	Central/East	121	2.3 ± 1.1	0.80 ± 0.02	13.80 ± 6.24

4.3.4 Earthworm effects on ecosystem functions

The SEMs revealed no significant influence of earthworm genetic diversity on functions like earthworm performance and soil abiotic and biotic properties. Litter consumption increased under warmer conditions (**Table 1**), low earthworm mortality (**Fig. S.8**), and high soil water content (all $p<0.001$; **Fig. S.9**). Moreover, litter consumption varied with earthworm origin but this depended on temperature ($p=0.036$), with lowest consumption rates in treatments with earthworms from East under cold conditions and highest litter consumption in treatments with earthworms from Centre under warm conditions (**Fig. 3a**). Soil water content was significantly affected ($p=0.007$) by the three-way interaction of temperature, precipitation, and origin, with the highest soil water content for treatments with earthworms from East under cold and moist conditions, and lowest values in treatments with earthworms from West under warm and moist conditions (**Fig. 3b**).

Soil water content and the three-way interaction of temperature, origin, and precipitation significantly affected both soil microbial biomass and soil microbial metabolic quotient (**Table 1; Fig. S.10**). Microbial biomass was lowest under cold and moist conditions in treatments with earthworms from West, and highest under dry conditions in treatments with earthworms from Centre and East (**Table 1, Fig. 3c**). The soil microbial metabolic quotient was high under cold and moist conditions in

treatments with earthworms from West, and low under dry conditions in treatments with earthworms from Centre and East (**Fig. 3d**).

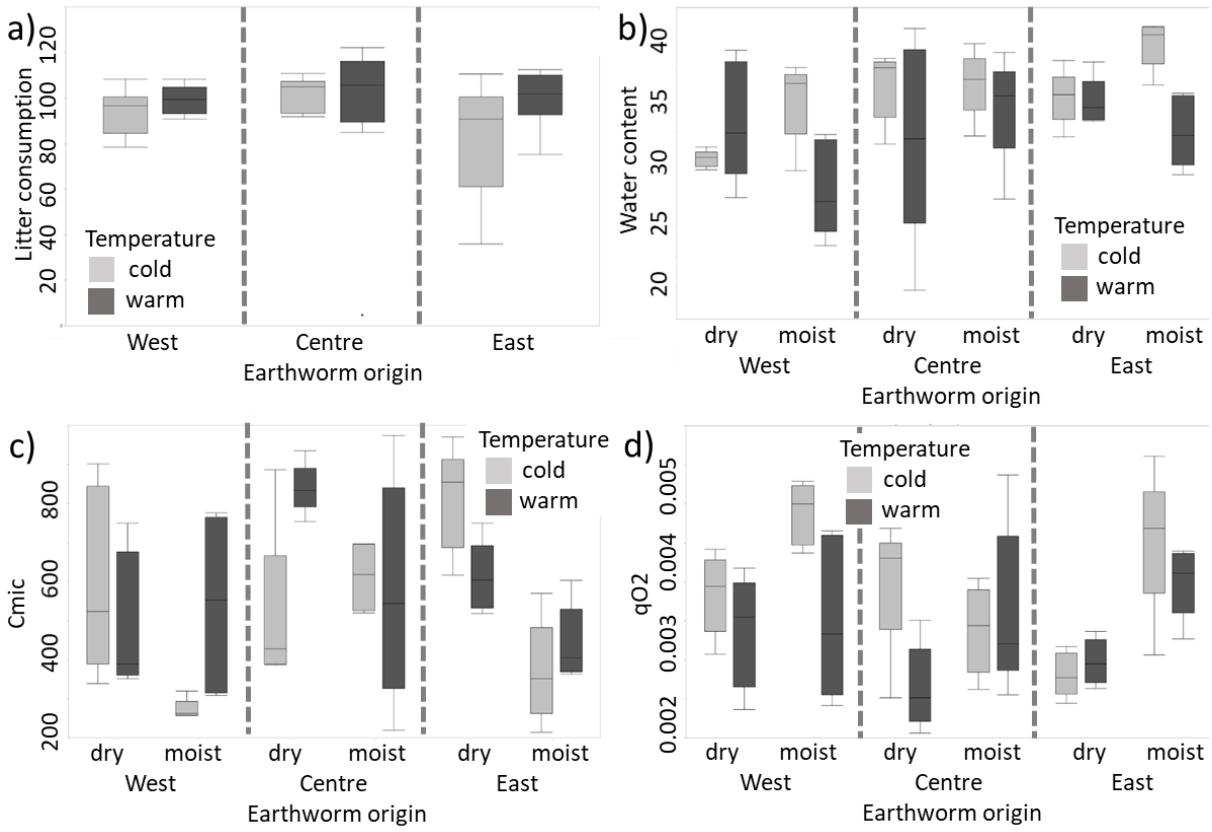

Figure 3: Effects of *Lumbricus terrestris* on mesocosm properties; a) litter consumption as affected by earthworm origin and temperature, b) water content as affected by temperature, precipitation and earthworm origin, c) microbial biomass (Cmic) as affected by temperature, precipitation and earthworm origin, and d) metabolic quotient (qO_2) as affected by temperature, precipitation and earthworm origin.

4.4 Discussion

4.4.1 Earthworm performance

The results of this study showed that earthworm performance depended on their site of origin and climate conditions i.e., the combination of temperature and precipitation. The initial biomass negatively affected biomass gain (the most important direct indicator for performance) during the experimental period, potentially indicating high intraspecific competition, either for food or space. Curry (1998) already stressed that intraspecific competition might be of major importance for structuring earthworm communities, and our results provide further evidence. Due to constant refilling of leaf litter, we speculate that competition for food was of minor importance. However, space was limited by the mesocosms' volume. Individuals originating from the West had high initial biomass (mean 3.6 g) and their final biomass (mean 4.2 g) likely approached the maximum under laboratory

conditions. This is supported by previous laboratory experiments in which *L. terrestris* reached a mean biomass between 4 and 7 g (Butt et al., 1994), with earthworm growth being limited by container size as well as the presence and foraging activity of other individuals (Nuutinen & Butt, 2005; Eriksen-Hamel & Whalen, 2007). Earthworms from Centre and East had lower initial biomasses (~0.5 to ~1.5 g lower), and intraspecific competition as well as size limitations likely did not influence biomass gain during the experimental period. Earthworms from all three origins had a comparable final biomass (mean 4.2 to 4.7 g), supporting that space limited maximal biomass.

The number of offspring differed significantly between West and Centre/East origin indicating fitness differences among the sampled origin sites at the start of the experimental period. However, all introduced earthworms possessed a completely developed clitellum and comparable activity levels (movement) without any visible differences except initial biomass.

The differential performance in biomass gain and offspring number suggests that earthworms adopted different strategies during the initial phase of the experiment. The larger earthworms from the West were more likely to invest in reproduction instead of biomass gain, because they were already close to their biomass limit. Contrastingly, earthworms from Centre and East were more likely to invest in biomass gain instead of reproduction. Similar patterns have been reported for *L. rubellus* and *D. octaedra* with higher offspring production in experimental treatments with higher initial biomass (Uvarov et al., 2011). The high number of offspring in earthworms from the West increased the likelihood of competition within the mesocosms, likely further limiting biomass gain. If mortality was high, the available space per individual increased and enabled higher biomass gain for the remaining individuals confirming previous studies where overall biomass of *L. terrestris* was found to be similar between microcosms with and without mortality (Patoine et al., 2017).

Our results indicate that the combination of temperature and humidity, i.e. seasonality, is an important, but rarely studied, determinant of earthworm performance. Of the four experimentally tested temperature and humidity combinations in this study, two represented the climate at the sampling locations West (i.e., warm and moist) and Centre/East (i.e., cold and dry). In contrast to our hypothesis (1a) suggesting that warm and moist conditions would be the most favourable for earthworm performance, biomass gain was highest under warm but dry conditions, irrespective of the sampling location (1b). However, this pattern likely was due to an inversion of seasonality , i.e. high temperatures in summer and low temperatures in winter, but high precipitation during autumn and winter in the West treatment (warm and moist), and during spring and summer in the East treatment (cold and dry), in the experimental treatments; temperature followed similar patterns in both treatments. Therefore, high cumulative annual moisture is inadequate for explaining the higher performance (biomass gain) as high evaporation and low precipitation coincided in the West, resulting

in regular droughts in spring/summer and likely causing drought stress for earthworms (Plum & Filser, 2005). Further, earthworms presumably were unable to take benefit of the high moisture during winter as they likely were inactive to avoid harm from frost (Plum & Filser, 2005). In contrast, earthworms benefited in treatments of high temperature and low moisture, because high evaporation was complemented by high precipitation during spring/summer favouring food consumption and subsequent biomass gain.

Reproduction success was positively affected (in trend) by warm temperature indicating either more frequent mating activity of adult earthworms resulting in two reproduction periods (autumn and spring), increased survival rates of the hatched juveniles, or a combination of both. Furthermore, the prolonged winter frost in East temperature (cold) treatments less negatively affected growth, reproduction, and mortality as compared to the summer droughts in West temperature (warm) treatments, leading to an overall positive effect and confirming recent publications on soil animal activity patterns. For instance, Thakur et al. (2018) revealed that warming decreased feeding activity of soil detritivores by -14% when combined with precipitation reductions, while warming with ambient precipitation had negligible net effects.

Notably, the most favourable climate conditions in our experiment are non-existent in large parts of northern North America, and are more similar to the small strip of east coast climate influenced by the Atlantic Ocean, or the Atlantic and moderate climate in western and central Europe. Nevertheless, European earthworms were able to also successfully invade and establish at these non-optimal conditions, indicating adaptation or broad ecological tolerance.

4.4.2 Earthworm genetic diversity

The molecular analysis revealed the existence of three major genetic lineages, two in the West, and one common lineage in the Centre and East. This confirms our hypothesis (H2) that genetic identity differs between the regions, and may be related to local climate conditions. The nucleotide and haplotype diversity was higher in the West than in Centre/East, but the difference between these regions was solely based on the existence of two distinct lineages in the West, while only one lineage was present in Centre/East. The earthworms from the West possessed the highest genetic diversity indices across all climate treatment combinations, which coincides with the existence of two distinct genetic lineages in the West. However, the large variation within treatments may indicate that surviving individuals possessed high genetic variation by coincidence rather than being explained by climate selection or adaptation.

Generally, genetic diversity was highest in warm and dry, and lowest in warm and moist climate conditions, irrespective of the site earthworms originated from, suggesting a climate effect on earthworm performance by reducing survival of individuals, and thus decreasing genetic diversity. This assumption is further supported by the absence of any climate-linked origin effects on earthworm performance, suggesting no ecologically relevant genetic difference between the lineages. Overall, our results suggest strong stochastic effects in the combination of genetic identity of earthworms in the mesocosms, and a potential mild environmental filtering between the West and Centre/East sites. These results contradict patterns described in the literature (e.g., Doncaster et al., 2000: Tagg et al., 2005), where less favourable environmental conditions enhance genetic diversity. However, due to the retrospective nature of our genetic analyses, which could only record genetic diversity of surviving earthworms, the total initial genetic diversity and the initial diversity of the individual mesocosm assemblages are unknown. To answer the question whether genetic diversity and identity cause significant differences in earthworm performance in future experiments, the genetic identity and diversity of earthworm communities need to be assessed prior to the experimental start, thus enabling a pre-determined setting of various levels of genetic diversity and identity.

4.4.3 Earthworm effects on ecosystem functions

The higher litter consumption of *L. terrestris* in warmer conditions confirmed our hypothesis that more favourable climate conditions increase the activity and performance of earthworms. Similar patterns were observed for epigeic earthworms, where higher temperatures increased performance and litter consumption (Uvarov et al., 2011). Interestingly, the difference between colder and warmer temperature was most distinct in earthworms from the East indicating that low initial biomass of these individuals was especially disadvantageous under more severe and prolonged winter conditions.

Soil water content was lowest under warm and moist conditions with West earthworms. Presumably, this was due to the unfavourable low soil moisture during summer, and initially larger burrows of the West earthworms with their higher biomass. Earthworm burrows, in particular the vertical ones formed by anecic species, were shown to function as preferential flow pathways for soil surface water (Edwards and Bohlen, 1996; Shipitalo, 2004), thereby reducing soil water content and indirectly negatively affecting plant seedling development (Eisenhauer et al., 2012).

The three-way interaction effects of the experimental treatments on soil microbial parameters indicate the complexity of direct treatment effects and earthworm-mediated effects on microorganisms. We found significant changes in litter consumption and soil water content in response to earthworm activity, which are known to be essential for soil microbial activity (Eisenhauer et al.,

2011). In fact, earthworm activity can have opposing effects on soil microbial properties with litter fragmentation and burial into the soil favouring microorganisms (Brown 1995), while earthworm-induced reductions in soil water content being detrimental (Eisenhauer 2010). Therefore, high variability within treatments may have been due to such opposing effects of litter decomposition and water drainage through burrows (Scheu and Parkinson, 1994; Shipitalo et al., 2004; Hale et al. 2005; Eisenhauer et al., 2012). Taken together, the results indicate that earthworm effects on soil microbial properties can be highly context-dependent and affected by the interplay of abiotic and biotic processes.

4.4.4 Caveats – genetic identity and diversity

Although the origin of *L. terrestris* significantly affected their performance (biomass gain and offspring number), activity (litter consumption), and ecological effects (soil water content and microbial functions), none of them were related to genetic diversity or identity. Therefore, significant effects of earthworm origin have to be attributed to other characteristics like physiology or behaviour that are linked to genetic diversity and/or identity (Tsutsui et al., 2003; Gamfeldt et al., 2005; Mattila & Seeley, 2007; Hughes et al., 2008).

The lack of effects of genetic diversity/identity likely were due to the "random" genetic composition of earthworms in the mesocosms. The population sampled from the West comprised three genetic lineages, which increased genetic variance among mesocosms. In contrast, populations sampled from Centre and East contained only one genetic lineage, resulting in lower nucleotide diversity and mean pairwise distances in these treatments compared to West. The pattern was similar for genetic diversity, though less pronounced. Nucleotide diversity allows a direct comparison of occurring genetic variance within each mesocosm (among the individuals within a mesocosm), reducing the genetic differences in the West and increasing them in the Centre and East.

4.4.5 Conclusions

The main determinant for *L. terrestris* performance in the present experiment was seasonality of temperature and precipitation. Although earthworm performance depended on the initial biomasses of the experimental populations, *L. terrestris* performed well in each of the climate treatments, but best under climate conditions common in central and western Europe but not in North America. Thereby, the study exemplifies the ability of this common invader species to adapt to environmental conditions. Significant effects of earthworms on soil water content suggest that earthworm invasion into northern North America may interact with other environmental changes in transforming

ecosystems (Bohlen et al. 2004, Frelich et al. 2012), such as exacerbating climate change effects (Eisenhauer et al. 2012). Importantly, the magnitude of exotic earthworm effects on soil microbial functions is likely to depend on climate conditions and their changes with time. Such interactive effects may trigger important ecosystem functions by changing microbial activity and nutrient cycling in invaded ecosystems.

Although genetic diversity and identity differed between West and Centre/East populations, this variability did not significantly influence earthworm performance or microbial functions. In future studies, the role of genetic identity and diversity should be tested by not only basing the experimental design on different source populations, but by determining and controlling the genetic structure of earthworm communities of experimental treatments.

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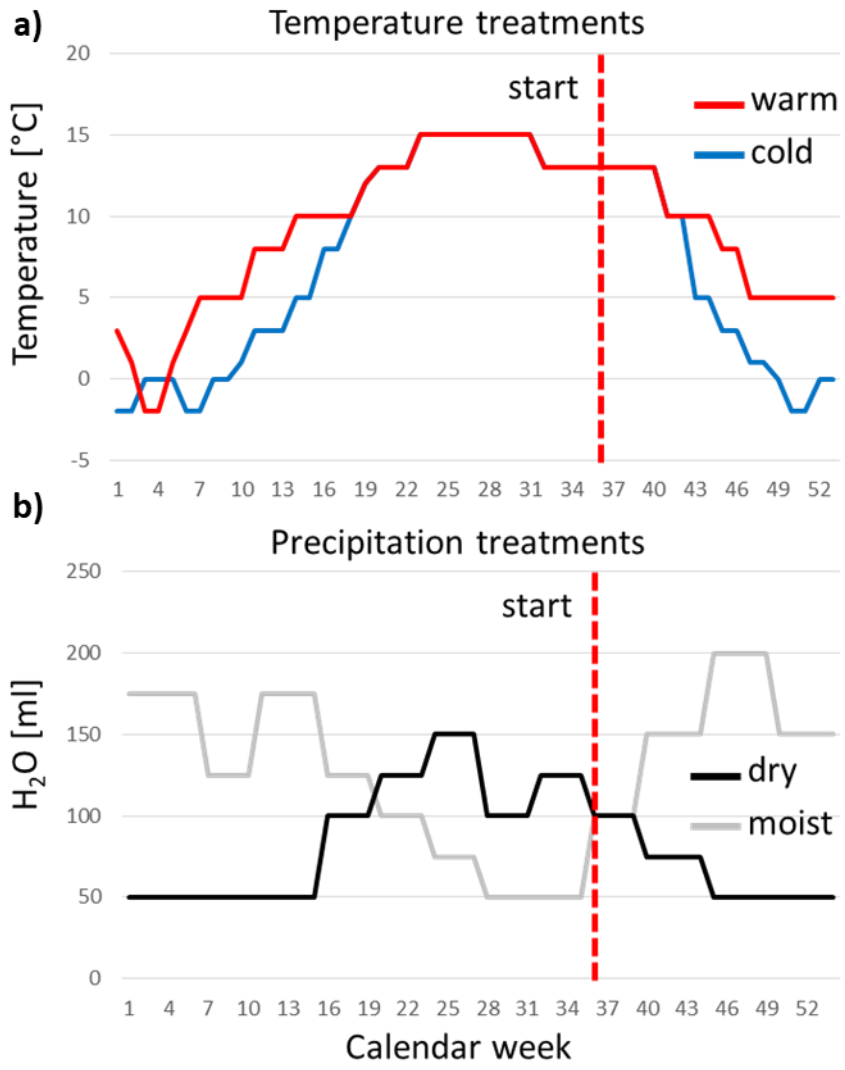

Figure S.1: Temperature settings for climate chambers simulating the natural temperature profile for a) the warm treatment (red) present at the West location; and the cold treatment (blue) present at the Centre and East locations. b) Precipitation settings simulating the natural profile for moist (West; grey) and dry (East/Centre; black) locations.

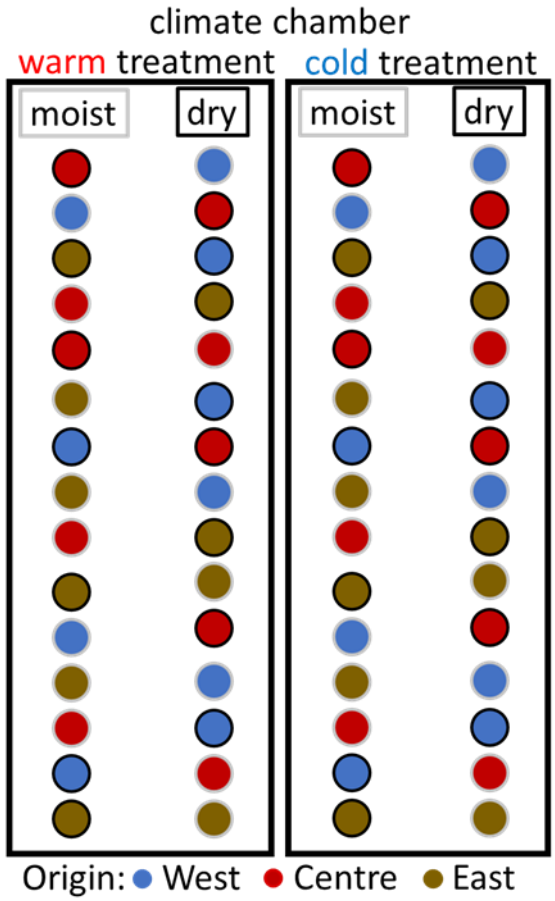

Figure S.2: Scheme of the full factorial experimental design. Two climate chambers were run parallel, with the warm and cold temperature profile. In each chamber, half of the mesocosms were treated with the dry watering scheme, the other half with the moist profile. Replicates were randomised.

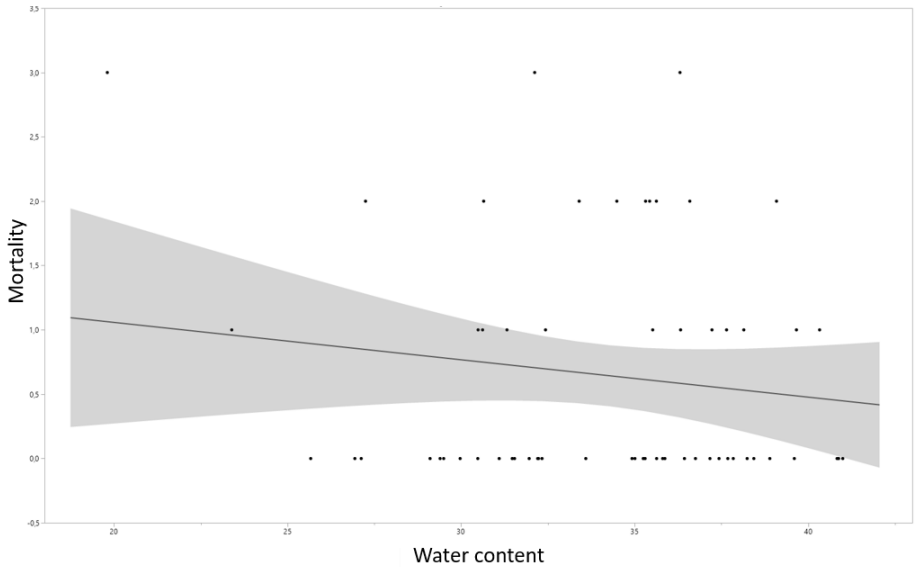

Figure S.3: Correlation of mortality and water content; p=0.067.

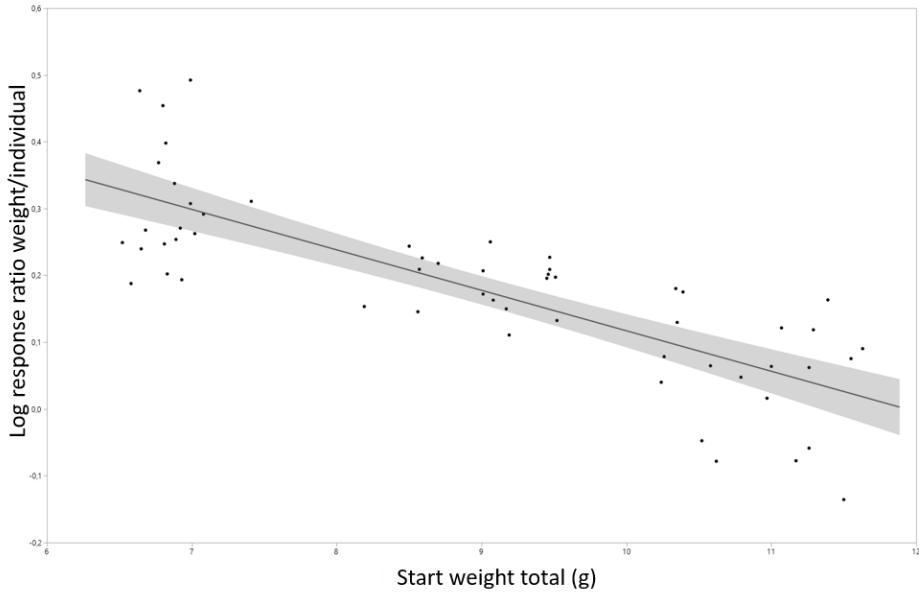

Figure S.4: Correlation of weight gain and start weight; p=0.001.

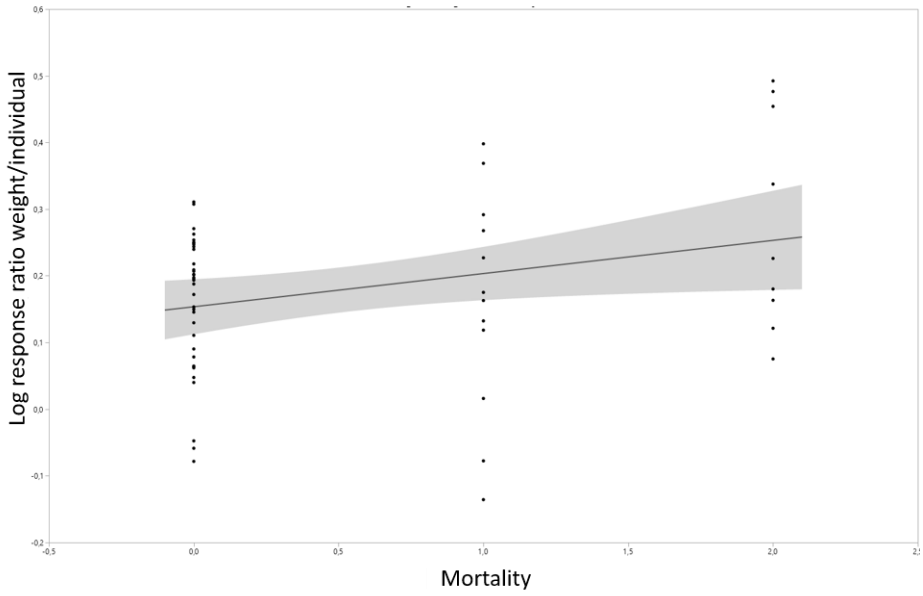

Figure S.5: Correlation of weight gain and mortality; p=0.001.

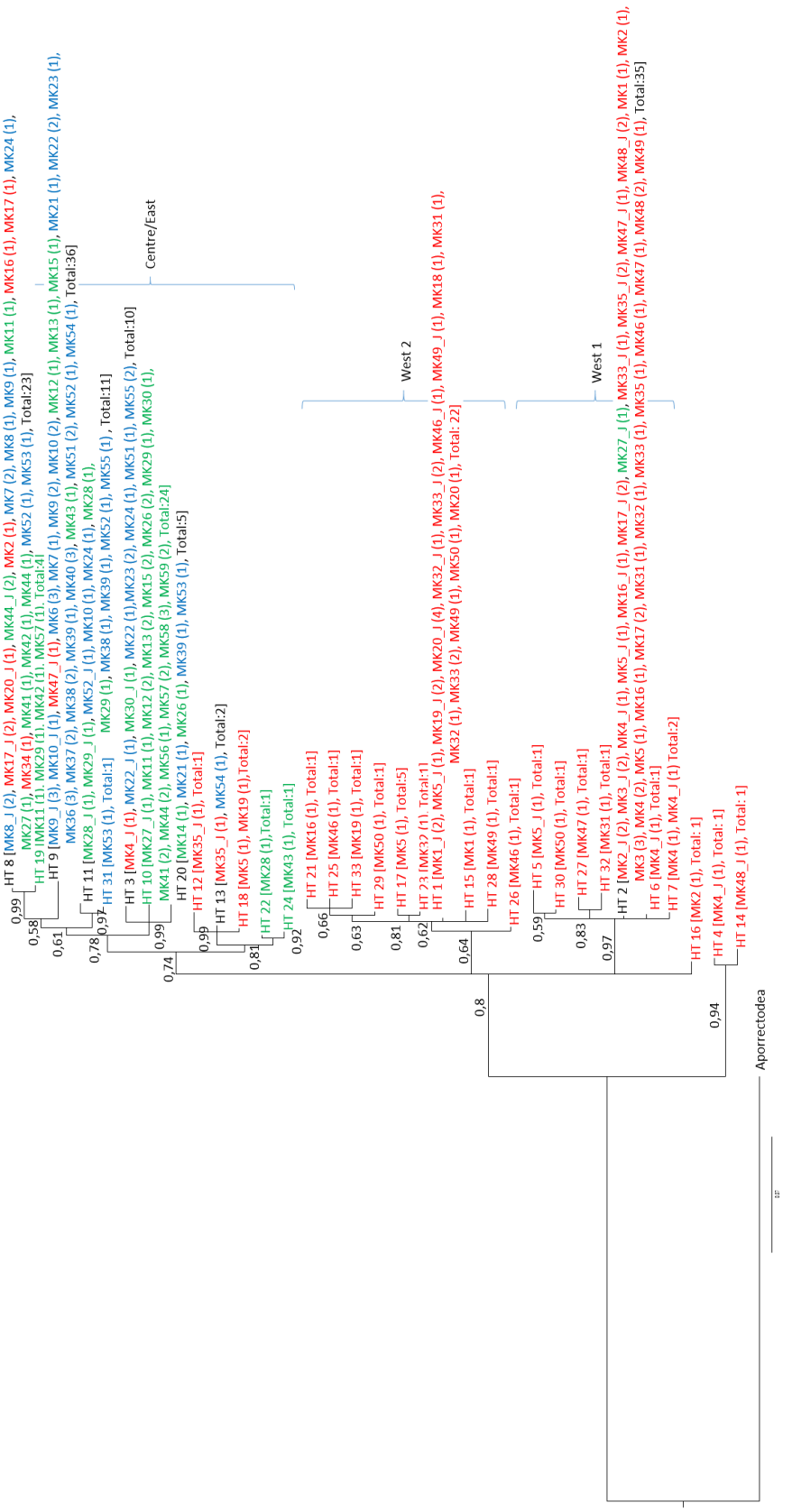

Figure S.6: Bayesian phylogenetic COI tree for the combined adult and juvenile dataset. red=West, green=Centre, blue=East.

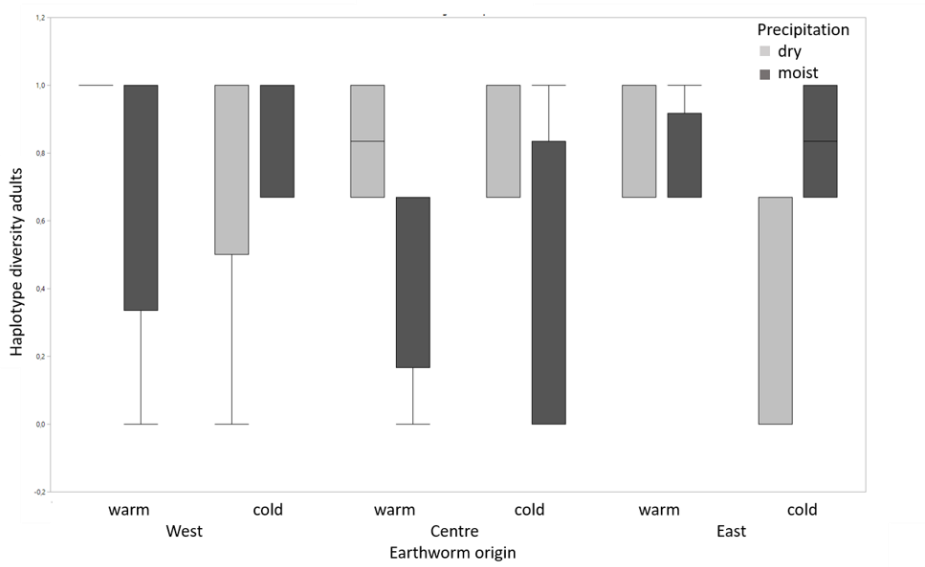

Figure S.7: Haplotype diversity of adults vs. three-way interaction of temperature, precipitation and earthworm origin.

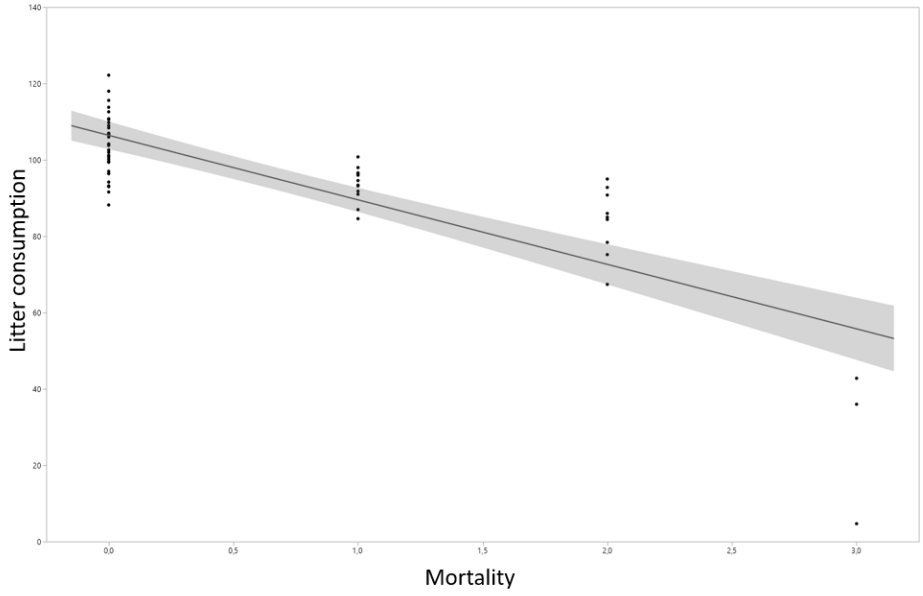

Figure S.8: Correlation of litter consumption and mortality; p=0.001.

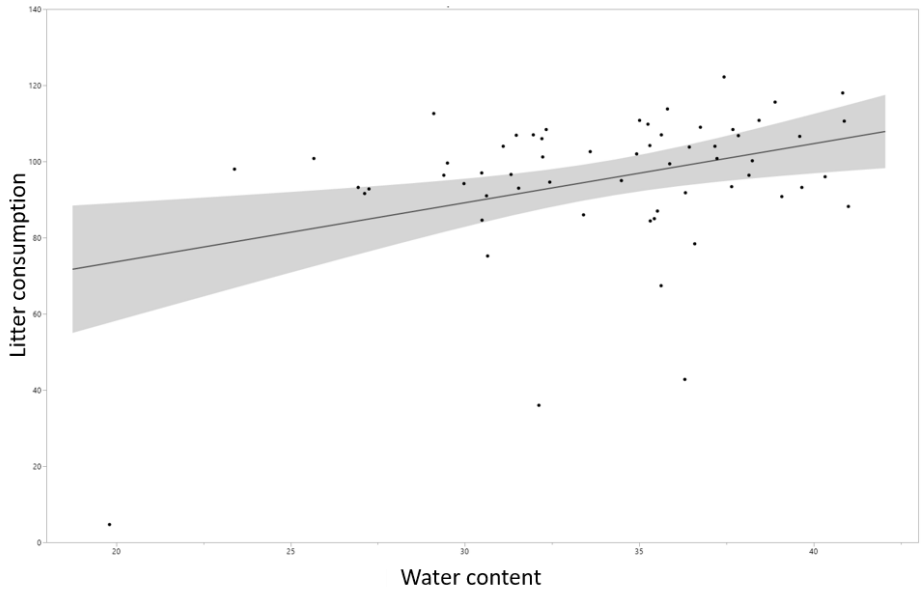

Figure S.9: Correlation of litter consumption and water content; p=0.001.

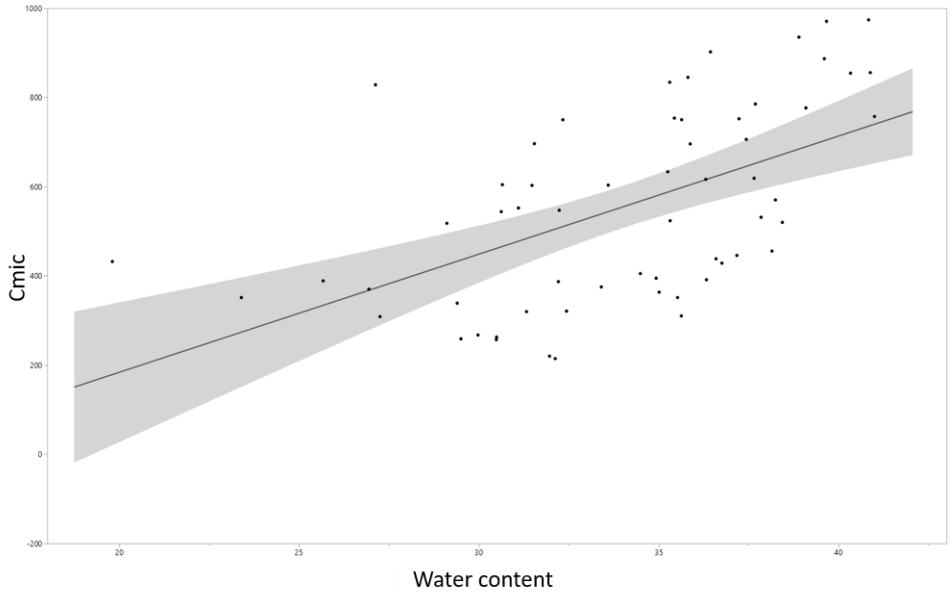

Figure S.10: Correlation of Cmic and water content; p=0.001.

CHAPTER 5

GENERAL DISCUSSION

European earthworms invaded large areas across the northern North American continent within the past 400 years. This quick invasion was facilitated by passive transport, considering the low active dispersal of soil-living animals. The role of anthropogenic dispersal has been investigated for a few earthworm species at local and regional scale. In this study, I investigated the dispersal of two European earthworm species at continental scale. Both species are now wide-spread across northern North America; they are closely related but differ in mobility (anecic vs. epigeic). To understand if North American populations of these two species are connected or if they established from independent introductions events, I analyzed the genetic diversity and structure of populations at a large scale, ranging from the east coast to the west coast of northern North America. Further, to identify potential drivers and obstacles for earthworm invasions I correlated genetic patterns with geographic dispersal barriers, regional climate, and included several factors related to human activities, such as dumping of fishing baits, road networks and human population densities. The role of regional climate as obstacle for earthworm invasion was further investigated in a mesocosm experiment using earthworm populations from different areas in North America.

5.1 Genetic structure and dispersal

Results of this study provide major advances in the understanding of genetic diversity and structure of earthworm invasion in North America, and into their dispersal mechanisms. Populations of both species did not differ genetically between the East and West of the North American continent. Rather, common lineages are widespread and dominant in all sampling regions disregarding dispersal barriers, suggesting that passive long-distance dispersal is important and harmonises the genetic structure across the continent. The genetic structure of the investigated invasive European earthworms in northern North America does not reflect the historical colonisation routes by European settlers. However, the three eastern sampling regions were genetically more coherent and differed to some degree from the two other regions. This pattern probably is a relic of the historic colonisation of European settlers that today likely is superimposed by recent long-distance dispersal, like bait dumping, global trade, industrialised agriculture, mass-mobility and leisure activities. Additional factors characteristic for certain regions, such as climate or land use, may also homogenise genetic structure of earthworms.

5.2 Dispersal mechanisms - bait

Genetic data of 104 individuals purchased from bait shops across northern North America, showed high genetic similarity among field and bait shop individuals. The presence of bait shop genotypes in field populations, even in distant sampling regions (Chapter 2 and 3), suggests passive long-distance dispersal by commercial bait distributors that sell earthworms across large areas and across state borders. Passive dispersal is the most important mechanism for earthworm invasions (Marinissen & van den Bosch, 1992; Terhivuo & Saura, 1997; Cameron et al., 2008; Eijsackers, 2011; Chatelain & Mathieu, 2017) and a number of studies demonstrated that bait dumping is an important introduction vector in areas close to lakes, including boat launches for fishing (Holdsworth et al., 2007; Keller et al., 2007; Cameron et al., 2008). According to my personal observation, the two largest bait distributors in North America sold bait to shops in all sampling regions. However, released earthworms sold as fishing baits does not explain the dominance of widespread lineages in all sampling regions, because some dominant field genotypes did not occur in bait populations.

5.3 Genetic structure as related to climate and agricultural use

The results of the climate chamber experiment (Chapter 4) and molecular variance of the continental samples (Chapter 2) indicated preferences of *L. terrestris* for warm and moist (summer) conditions, and that regional climate conditions affected the genetic structure of *L. terrestris* but not of *L. rubellus*. Notably, the epi-endogeic species *L. rubellus* and anecic species *L. terrestris* belong to different functional groups and also differ in ecological traits, i.e. in *L. rubellus* having a broader tolerance against frost and pH as compared to *L. terrestris* (Sims & Gerard, 1999; Addison, 2009). Further, genetic structure was more pronounced in the less mobile species *L. terrestris*, and genetic structure and diversity of lineages differed significantly in two sampling regions, Alberta and Minnesota. Both regions are characterised by continental climate that was least favourable in the experiment (Chapter 4), and in particular frost, drought and seasonality, affected the performance of *L. terrestris* in terms of growth, reproductive rate, mortality and the establishment of different haplotypes. It is possible that the harsh continental climate in Alberta and Minnesota facilitate the coexistence of several genetic lineages, while in more favourable climate regions competitive exclusion resulted in the dominance of only few lineages. Susceptibility of *L. terrestris* to cold and dry conditions in the experiment is confirmed by previous studies (Butt 1991; Edwards and Bohlen, 1996; Berry and Jordan, 2001; Tiunov et al., 2006; Uvarov et al., 2011). Several studies demonstrated that harsh environmental conditions promote genetic variation within populations of plant and invertebrate species (Doncaster et al., 2000; Tagg et al., 2005; Scheu & Drossel, 2007; Hughes et al. 2008).

For *L. terrestris,* genetic diversity and identity of genetic lineages were similar in these two sampling regions but different to all other regions. The presence of identical lineages in two regions that are ~1,900 km apart cannot be explained by climate alone, especially as distance among sampling regions was the most important factor after climate conditions that explained genetic structure of *L. terrestris*. In contrast to *L. rubellus* that primarily occupies forest and grassland habitats, *L. terrestris* tolerates regular disturbances and occurs with high densities in agricultural soils (Sims & Gerard, 1999; Tiunov et al., 2006; Addison, 2009). Notably, these two sampling regions, Alberta and Minnesota, belong to the North American corn-belt, a region of intense agricultural use. Transport of soil containing earthworms or their cocoons between fields, or to nearby forests with agricultural machinery is easy and transfer of soil-borne goods from fields, and cooperative use of machines by farmers or large companies bears the potential to transport earthworms over long distances. Recent long-distance dispersal that connects both regions is likely, as introductions of earthworms into Alberta are not older than about 30 years (Scheu & Parkinson, 1994; McLean & Parkinson, 1997; Cameron et al., 2007). This is also supported by results of Chapter 3 that demonstrated that populations of *L. terrestris* in Alberta are small and patchily distributed, indicating that earthworm populations established from multiple and independent introductions of few individuals. Populations were genetically homogeneous suggesting recent establishment as populations accumulate polymorphisms in microsatellite markers with time.

5.4 Genetic diversity, human agglomerations and road networks

Genetic diversity of the two invasive species was higher than expected and similar to the genetic diversity in their native range in Europe (Sechi, 2013; Giska et al. 2015; Martinsson & Erséus, 2017). In the studied North American regions, genetic diversity of earthworm populations positively correlated with human population density, in particular for *L. terrestris,* which is more closely associated with human activities, i.e. fishing and agriculture. This close association indicates that densely populated areas, i.e. cities or agglomerations of towns, additionally contribute to genetic diversity and dispersal of earthworm populations by unintentional passive transport. This pattern was also present at the regional scale in Alberta (Chapter 3) using microsatellite data, where genetic diversity outside the urban area of Calgary was significantly lower suggesting regional traffic and road networks as likely drivers of dispersal. Previous studies demonstrated that proximity to road networks to be the main driver of local and regional earthworm dispersal (Holdsworth et al., 2007; Cameron et al., 2007; Cameron & Bayne, 2009) and this study adds that genetic diversity also increases with proximity to human agglomerations. Genetic diversity is an important factor for successful establishment of invasive species, because it increases the likelihood that certain haplotypes, which are able to establish

in this particular area, are present. Additionally, centres of human activity act as sources of earthworm dispersal and diversity, highlighting their importance in the process of earthworm invasion.

5.5 Experimental climate effects on genetic lineages

None of the climate treatments studied in the laboratory simulating North American conditions was optimal for earthworm performance. Rather, earthworms performed best in climate treatments more similar to western and central Europe, suggesting phenotypic plasticity. In my experiment in Chapter 4, I demonstrated that earthworm populations from different regions, differed in regard to life-style strategies, i.e. biomass gain versus reproduction.

The use of molecular markers revealed patterns that are usually not taken into account when investigating invasive earthworms. In my experiment, the genetically most diverse individuals from the West had the highest reproductive rate suggesting that genetic variation can be an important factor for successful invasions, establishment and range expansion of earthworms. These results show that experimental studies that combine genetic diversity and identity of invasive species with environmental conditions provide additional information allowing a better understanding on the role of the genetic constitution for successful invasions.

Previous experimental studies indicated that genetic (haplotype) diversity is an important factor for successful invasion in plant and arthropod species (Hughes et al., 2008). Experiments by Crutsinger et al. (2007) indicated that genetic diversity and identity in plants can act as barriers for following invasions by other plant species. Further, environmental filtering can influence successful invasion through genetic identity and diversity, i.e. by selection of ecologically tolerant haplotypes (general haplotypes) that evade filtering, or by haplotypes exhibiting high levels of phenotypic plasticity. This indicates that accounting for genetic diversity and identity of *L. terrestris* in experimental settings will provide additional information on earthworm performance and the role of environmental filtering for establishment of earthworm populations.

5.6 Conclusions and outlook

The results of my study demonstrated how earthworms as belowground invaders with substantial differences in life history traits can be used to test general questions in invasion ecology, such as the role of genetic variation for successful invasion as well as the role of geographic and climatic dispersal barriers, and the role of humans in ecological invasions. The results of the study further provide evidence that the combination of molecular studies and large-scale sampling provides new insights

into mechanisms and vectors of earthworm invasion in North America, such as agriculture and human agglomerations.

For future research in below-ground invasions that apply multiple spatial scales population genetics and time series as sampling strategies, a number of new research topics emerge. Of particular importance for earthworm invasions is the study of factors, such as human agglomeration as drivers of genetic diversity, and as factor favouring dispersal into the surrounding countryside. Further, linkage between agricultural fields and adjoining forests may act as drivers of gene flow and play an important role in long-distance dispersal, with high potential to model genetic structure in invaded areas. Further, to consider climate as a factor influencing the genetic structure of an invasive species is new for exotic earthworms but needs further research, in particular in form of transplantation experiments under controlled conditions.

Geographic scales (local, regional, continental) are important to consider in the below-ground invasion of earthworms, because vectors and mechanisms that drive dispersal, establishment and genetic structure differ with scale. Human traffic on the regional and trans-regional scale differs in frequency transporting earthworms and thereby genetic diversity and connectivity among populations, both are relevant for connectivity among populations and affect invasive potential. Climate and frequent disturbances by agriculture were identified as main factors influencing invasion and establishment of earthworms in central northern North America but the significance of both factors remains unclear, because in this study their effects are auto-correlated and cannot be separated. However, molecular markers are essential to disentangle the contribution of human activities, such as agriculture or leisure, and human traffic, and abiotic factors, such as climate or dispersal barriers, which are necessary to understand the extremely successful earthworm invasions in North America.

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LIST OF PUBLICATIONS

Published in peer-reviewed journals

Klein, A., Cameron, E.K., 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. *Applied Soil Ecology*, 120, 265-272. https://doi.org/10.1016/j.apsoil.2017.08.009.

Finished manuscripts to be reviewed

Klein, A., Scheu, S., Eisenhauer, N., & Schaefer, I. Invasive lumbricid earthworms in North America – different life-styles, common dispersal? *Journal of Biogeography*.

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THESIS DECLARATION

Declaration of the author's own contribution to manuscripts with multiple authors

Chapter 2 comprises a manuscript that has been published in a peer-reviewed journal; Chapter 3 and 4 comprise manuscripts that are currently submitted to peer-reviewed journals.

I am the first author of all manuscripts. I have collected and analysed the data, written the manuscripts, developed the main ideas, created tables and figures, and contributed significantly to each study design.

Bastian Heimburger (M.Sc.), Maximilian Wolf (M.Ed.) and Laura Holla (B.Sc.) performed their Master- and Bachelor theses as part of this project that were supervised by me. They contributed to Chapters 2, 3 and 4 by collecting and analysing parts of the data, and produced additional results that were not included in my thesis but will be published in peer-reviewed journals in the near future.

All persons contributing to the manuscripts have been named so. All co-authors contributed to finalising the manuscripts.

Plagiarism declaration

I, Andreas Klein, declare that I have written this doctoral thesis independently. All persons contributing to the manuscripts have been named so. All sentences or passages quoted from other people's work have been specifically acknowledged by clear cross-referencing.

I have not submitted this thesis in any form for another degree at any university or institution.

_____

Andreas Klein

Göttingen, July 2018