



Article Patterns of Genetic Variation in the *Eisenia nordenskioldi* Complex (Oligochaeta: Lumbricidae) along an Elevation Gradient in Northern China

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Abstract: *Eisenia nordenskioldi* is the dominant earthworm species in many tundra and boreal habitats. Nothing is known about the genetic diversity of this species along the elevation gradient in China. This study sampled 28 individuals in the *E. nordenskioldi* complex from Wuling Mountain, northern China, to examine their external morphology and genetic diversity. Mt. Wuling is the southern limit of the distribution of the *E. nordenskioldi* complex. The specimens from Mt. Wuling were classified into three groups along an elevation gradient. Mismatch distribution analysis suggested that the Pleistocene glaciations possibly did not significantly affect the distribution of earthworm species in this region. We also found that elevation affected the genetic diversity, but not the external morphology of *E. nordenskioldi*. Given the altitudinal genetic diversity within the *E. nordenskioldi* complex, the phylogeography of this species provides important information for the zoogeographic reconstruction of the mountains in northern China. With the relatively limited sample size, the result is not conclusive, and further studies need to be conducted in the future to verify the results.

Keywords: COI barcoding; Eisenia nordenskioldi; elevation gradient; Wuling Mountain

1. Introduction

The vertical zonation of mountain climates results in the formation of diverse, unique habitats for animals, plants, and microorganisms, giving rise to vertical differences in the biome along altitude gradients [1]. Xu et al. [2] showed that altitude did not affect the overall abundance of epigeic soil animals on Mt. Dongling but did affect the distribution of various functional feeding groups of animals at different altitudes. Few studies have examined earthworm diversity along an altitude gradient [3]. One suggested that the difference in earthworm species richness along an elevation gradient in the mountains of northeastern Puerto Rico was due to a combination of biotic and soil physical and chemical factors [4]. No study has examined the intra or inter-specific genetic diversity of *Eisenia* along an elevation gradient in northern China.

In northern China, Mt. Wuling is the main peak in the Yanshan Mountains, which have a typical warm-temperate, semi-humid, continental monsoon climate. It is a nature



Citation: Zhang, Y.; Zhang, Y.; Wu, H.; Li, C.; Aspe, N.M.; Wu, D. Patterns of Genetic Variation in the *Eisenia nordenskioldi* Complex (Oligochaeta: Lumbricidae) along an Elevation Gradient in Northern China. *Diversity* **2022**, *14*, 35. https://doi.org/10.3390/d14010035

Academic Editors: Michael Wink and Daniel Fernández Marchán

Received: 25 November 2021 Accepted: 26 December 2021 Published: 7 January 2022

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Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). reserve with a total area of 143 km² and forest coverage of 76.2%. The vegetation and soil types vary along the elevation gradient [5].

Eisenia nordenskioldi, an earthworm species that is widespread in Northern Asia and adjacent regions, is known for its high morphological, karyotypic, and genetic variation [6,7]. The diagnostic features of this species include having purple dorsally and faint yellow ventrally; the body has no stripes; the intersegmental furrows in adult individuals are faint yellow; the clitellum is faint yellow, saddle-shaped in xxvii–xxxii; the spermathecal pores are paired in 9/10 and 10/11 ventrally; setae lumbricine, ab > bc, aa > bc; the spermathecae are tiny and ball-shaped; the gizzard is located in xvii–xviii. The *Eisenia nordenskioldi* complex contains two subspecies, the pigmented *E. n. nordenskioldi* and the unpigmented *E. n. pallida* [6,7]. Several genetic diversity and phylogeographic studies on *E. nordenskioldi* have been conducted previously [7–14]. In this study, we analyzed the genetic diversity of the *E. nordenskioldi* complex from Mt. Wuling along its elevation gradient and discussed its phylogeography.

2. Materials and Methods

2.1. Sample Collection and DNA Extraction

E. nordenskioldi is a common species widely distributed in tundra and boreal habitats in northeast Asia [6]. Nature reserves were selected with high, medium, and low latitudes in North China in order to discuss the genetic differentiation of this species at different altitudes. A total of 28 specimens of the E. nordenskioldi complex were collected by hand sorting in litter from four elevations in natural forest habitats on Mt. Wuling: 1000–1200, 1200–1400, 1400–1700, and 1700+ m (Table 1, Figure 1). Total DNA was extracted from the tail muscle of individuals fixed in 95% ethanol using a DNA extraction kit (Sangon, Shanghai, China) according to the manufacturer's instructions. Total genomic DNA was extracted using the Invitrogen Genomic DNA extraction kit according to the manufacturer's protocol. A COI gene fragment was amplified by PCR using primers LCO1490 (5' GGT CAA CAA ATC ATA AAG ATA TT 3') and HCO2198 (5' TAA ACT TCA GGG TGA CCA AAA AA 3'), as described by [15]. The PCR products were sequenced directly using the Big Dye Terminator v3.1 Cycle Sequencing kit using these primers. Pairwise (p) distances were calculated using MEGA X [16]. Other samples of E. nordenskioldi were collected from nature reserves in northeast China (DQH, BS, SH, and LTDZ). The specimens from Russia were not collected, COI sequences from Russian earthworms were retrieved from GenBank (accession no. KU708313-708411 [9]), and those sequences of earthworms from Mongolia were taken from Blakemore [17]. The specimens collected from forests in northern China are stored in 95% ethanol in the Hebei Key Laboratory of Animal Diversity, Langfang Normal University, China. The net weight of the earthworm individuals was measured after ethanol fixing for two weeks.

Table 1. Collected locations of Eisenia nordenskioldi complex in North China
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Location	Code	No. Specimens	N. Latitude	E. Longitude	Elevation m	Habitat
Mt. Wuling Nature Reserve in Xinglong, Hebei	L-1	11	40.5634	117.4873	1116	Litter in mixed-forest
Mt. Wuling Nature Reserve in Xinglong, Hebei	L-2	8	40.5609	117.4777	1310	Litter in mixed-forest
Mt. Wuling Nature Reserve in Xinglong, Hebei	M3	7	40.5694	117.4743	1478	Litter in coniferous
Mt. Wuling Nature Reserve in Xinglong, Hebei	H4	2	40.5878	117.4795	1740	Litter in coniferous
Danginghe Nature Reserve in Heilongjiang	DQH	7	46°37′	129°22′	482	Litter in mixed-forest
Baoshan Nature Reserve in Heilongjiang	BS	5	48°57′	128°52′	405	Litter in mixed-forest
Shuanghe Nature Reserve in Heilongjiang	SH	9	52°59′	125°22'	354	Litter in mixed-forest
Laotudingzi Nature Reserve in Heilongjiang	LTDZ	2	41°19′	124°54′	906	Litter in mixed-forest



Figure 1. Geographic distribution of *E. nordenskioldi* from Mt. Wuling and northeast China. (**A**) is the location mark of northeast China. As shown in the legend, different groups are represented by different colors, the number of samples at the collection point is represented by the size of the dot, and the different color areas of the dot represent the proportion of different groups in the sampling point. The location of Wuling Mountain is indicated by a red five-pointed star. (**B**) is an enlarged view of the sampling point of Wuling Mountain, and the altitude has been marked. (**C**) represents the geographic distribution of *E. nordenskioldi* from Mt. Wuling and northeast China, the position of the dot and the sampling position (see Table 1 for coordinates).

2.2. Sequence Analysis

COI DNA diversity statistics, including nucleotide and haplotype diversities, Tajima's D neutrality test statistics, nucleotide mismatch distribution, gene flow, and genetic differentiation analysis results, were calculated using DnaSP 5.0 (Barcelona, Spain) [18]. Bayesian analysis was performed using MrBayes v3.2.6 (http://nbisweden.github.io/MrBayes/ index.html). Two simultaneous independent analyses were run from different random starting trees using four chains of metropolis-coupled Monte Carlo simulations for 50,000,000 generations, sampling a tree every 1000 generations. MrModetest suggested a model based on hierarchical likelihood ratio tests for COI sequences. The combined COI dataset was analyzed in MrBayes using a gamma shape parameter of 0.007692. For the COI tree, the sequences of *Eisenoides carolinensis* (Michaelsen, 1903) (FJ214226), *Eisenia zebra* (Michaelsen, 1903) (FJ214229), and *Eisenia andrei* (Bouché, 1972) (AY874508) were used as an outgroup. Haplotype networks were reconstructed using TCS v1.21 [19]. Haplotype and nucleotide diversities were calculated using MEGA (https://www.megasoftware.net). MrModeltest indicated the best model as GTR+I+G [20].

3. Results

3.1. Distribution of the E. nordenskioldi Complex along an Elevation Gradient

Group 1 (G1) was widely distributed not only in Mt. Wuling but also in northeast China. Group 2 (G2) was found only at low and mid-elevations on Mt Wuling. Groups 3 and 4 existed only in northeast China. Group 5 (G5) was distributed only at low elevation on Mt. Wuling. Some low-elevation locations from Mt. Wuling had their own set of haplotypes; these are described as cold intolerant with a narrow distribution (Figures 1 and 2). The topological relationships of the Mt. Wuling clusters were classified into three groups (G1, G2, and G5) along an elevation gradient: G1 could be described as cold tolerant as it is widely distributed in many zones (Mt. Wuling and northeast China) with very low temperature; G2 was adapted to either cold or warm habitats on Mt. Wuling, and gene flow was more frequent within this group; G5 existed only in a narrow lowaltitude zone (Figures 1 and 2). Gene flow estimated from the sequence data had values of DeltaSt = 0.05159, GammaSt = 0.08542, and Nm = 0.68. A haplotype network (Figure 3) was constructed using the 635-bp COI fragment of 128 individuals from different locations not only in Mt. Wuling but also in other adjacent regions (northeast China, Mongolia, and far east Russia). This indicated that the E. nordenskioldi complex in northern China comprises at least five exclusive groups, in accordance with the BI tree.



Figure 2. Bayesian Inference phylogenetic tree constructed using COI nucleotide sequences. *E.n.p* (*Eisenia nordenskioldi pallida*) L1, L2, L3, L4, and L5 are lineages of Russian Groups (Shekhovtsov et al., 2016); Different branch color indicates a different post-inspection probability; Different label color indicates a different locations unit (OTU); Different background color indicates different altitudes of Mt. Wuling.



Figure 3. Haplotype networks for *E. nordenskioldi*. Geographic information of DQH, BS, SH, LTDZ, L1, L2, M3, and H4 are shown in Table 1; Circle sizes are proportional to the number of individuals having this haplotype; COI sequences of Russian and Mongolian Groups were retrieved from Genbank.

3.2. External Morphological Data of E. nordenskioldi Complex from Mt. Wuling

Morphological data (body length, width, and net weight) were obtained for 28 individuals from the *E. nordenskioldi* complex. The respective body length, width, and net weight of the individuals were as follows: 32.99 ± 12.79 mm, 3.85 ± 0.73 mm, and 0.21 ± 0.13 g for Cluster L-1 (low altitude); 41.19 ± 10.53 mm, 4.21 ± 0.68 mm, and 0.36 ± 0.16 g for Cluster L-2 (low altitude); 43.43 ± 17.24 mm, 3.94 ± 1.39 mm, and 0.28 ± 0.22 g for Cluster M3 (intermediate altitude); and 46.5 ± 2.26 mm, 4.65 ± 0.64 mm, and 0.46 ± 0.09 g for Cluster H4 (high altitude) (Figure 4).



Figure 4. Morphological data of E. nordenskioldi at an elevation gradient from Mt. Wuling.

3.3. Genetic Diversity and Differentiation of the Mt. Wuling E. nordenskioldi Complex

COI sequences were determined for 28 E. nordenskioldi specimens from Mt Wuling. The complete alignment included 635 sites (i.e., no length polymorphism was detected). There were 432 conserved sites and 203 variable sites, of which 182 were parsimony informative, and 21 were singleton sites. The overall average p distance was 0.174. The p distance was highest between L1 and H4 (0.208) and lowest between M3 and H4 (0.072). The p distance was highest within L1 (0.174). The genetic p distance was high both within and among the four elevation groups on Mt. Wuling. Figures 5 and 6 compare basic genetic diversity parameters among the four elevation groups, including the numbers of segregation sites, haplotypes and polymorphic sites, nucleotide and haplotype diversities, the total number of mutations, and average number of nucleotide differences. Mismatch distribution analysis suggested that the earthworm species had not experienced expansion (Figure 7). Table 2 shows the genetic diversity of E. nordenskioldi from different regions. Our E. nordenskioldi sequences fell into five groups in the Bayesian phylogeny, strongly supported by Bayesian posterior probabilities (Figures 1 and 2). Figures 2 and 3 present the data for G1 and G5 of the *E. nordenskioldi* complex showing both high haplotype and nucleotide diversities because of the elevation gradient. The probability obtained from a permutation test with 1000 replicates (PM test) was significant (p < 0.05) (Table 3), indicating genetic differentiation among G1, G2, and G5 (Figures 1 and 2).



Figure 5. Genetic diversity of *E. nordenskioldi* at elevation gradients from Mt. Wuling. L1 1000–1200 m; L2 1200–1400 m; M3 1400–1700 m; H4 1700 m; S = Number of segregating sites; K: Average number of nucleotide differences.



Figure 6. Genetic diversity of *E. nordenskioldi* at elevation gradients from Mt. Wuling. L1 1000–1200 m; L2 1200–1400 m; M3 1400–1700 m; H4 1700 m; *pi* = Nucleotide diversity; *h* = Haplotype diversity.



Figure 7. Mismatch curve of nucleotide pairwise based on COI sequences. Exp, expected value; Obs, observed value.

	Russia	Mongolia	N.E China	Mt. Wuling
S	221	508	545	545
h	56	3	22	28
Κ	84.953	413.667	352.158	342.783
Μ	381	826	1598	1373
Np	221	577	545	545
Hd	0.979	1	0.996	1
Pi	0.156	0.759	0.646	0.629

Table 2. Comparison among Eisenia nordenskioldi complexes from different regions.

S = Number of segregating sites; h = Number of haplotypes; Pi = Nucleotide diversity; Hd = Haplotype diversity. Np: Number of polymorphic sites, M: Total number of mutations; K: Average number of nucleotide differences.

Table 3.	Results	from	Tajima's	Neutrali	ity Test
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S	ps	Θ	π	D
545	1	0.174417	0.512729	6.288815

Abbreviations: S = Number of segregating sites, ps = S/n, $\Theta = ps/a1$, $\pi =$ nucleotide diversity, and D is the Tajima test statistic). NOTE: The analysis involved 174 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 545 positions in the final dataset. Evolutionary analyses were conducted in MEGA X. Not significant p > 0.10.

4. Discussion

4.1. Morphological and Genetic Variations within the E. nordenskioldi Complex According to Elevation

Eisenia nordenskioldi is known to have very high genetic diversity and contains several cryptic genetic lineages [7–14]. The *E. nordenskioldi* complex is separated into two species: *E. nordenskioldi* from northern and western Russia and *Eisenia nordenskioldi* from southern and southeastern Russia [13]. Both species need to be described and will be future work. The sequences of pigmented *E. n. mongol* and *E. n. onon* [17] were included within *E. n. nordenskioldi* from Russia, while unpigmented *E. n. pallida* from Korea [21] formed a separate branch within *Eisenia nordenskioldi* from southern and southeastern Russia. Our groups were morphologically similar to *Eisenia* from southern and southeastern Russia, characterized by body-color (purple dorsally and faint yellow ventrally), spermathecae shape (ball-shaped), and clitellum position (saddle-shaped in xxvii–xxxii).

It was indicated that altitudinal stratification has an effect on the genetic diversity of megascolecid worms from mountains in Taiwan, China [22]. The studies of Shekhotsov et al. [7–14] have not discussed the genetic diversity of *E. nordenskioldi* complex (Lumbricidae) in relation to its altitudinal distribution. In this work, we tackled the pattern of genetic variation in *E. nordenskioldi* along elevational gradients in northern China. Our results showed that elevation affected the genetic diversity, but not external morphological diversity, within *E. nordenskioldi*. The *E. nordenskioldi* complex from Mt. Wuling is not monophyletic and consists of three morphologically similar altitude groups (G1, G2, and G5) that have high genetic divergence (Figures 1 and 2).

4.2. Distribution of the E. nordenskioldi Complex from Mt. Wuling

Dong et al. [23] found two exclusive lineages for *Amynthas triastriatus* of the Megascolecidae in southeast China: Lineage A is distributed mainly at high altitudes and Lineage B mainly at low altitudes. Similarly, regarding the distribution of the *E. nordenskioldi* complex from Mt. Wuling (Figure 1), we hypothesized that G1 tolerates low temperatures in the tundra and high boreal habitats, while G5, which may be close to *E. n. pallida*, is intolerant to low temperatures and has a narrow regional distribution. We found that Mt. Wuling harbors high earthworm diversity. In the haplotype network (Figure 3), Mt. Wuling haplotypes were relatively close to Mongolian ones but far from Russian or northeast Chinese ones. This suggests a dispersal route from Siberia to Mongolia to Inner Mongolia to Mt. Wuling.

4.3. Phylogeography of the E. nordenskioldi Complex from Mt. Wuling

The Pleistocene glaciation resulted in dramatic shifts in animal habitats and a dramatic reduction in intra-specific diversity [24]. The cold Pleistocene glacial periods caused alpine insects to disperse into lowland regions [25]. Insects, such as bees and beetles, have significantly stronger dispersal ability compared with soil invertebrates, such as earthworms. Genetic differences were observed among different elevations on Mt. Wuling, which had their own haplotype sets (Figure 3). This indicated that the Pleistocene glaciations did not significantly affect the distribution of earthworm species. The analysis of allelic gene frequency and nucleotide mismatch implied that the events shaping the phylogeography of Mt. Wuling *E. nordenskioldi* occurred long before the last glacial maximum. Given this altitudinal genetic diversity within *E. nordenskioldi*, the phylogeography of this species provides important information for the zoogeographic reconstruction of the mountains in northern China.

5. Conclusions

Mt. Wuling is the southern limit of the distribution of the *E. nordenskioldi* complex. Our results showed that elevation has an effect on the genetic diversity of the *E. nordenskioldi* complex but not on the morphological diversity. The *E. nordenskioldi* complex in northern China has at least five exclusive genetic groups, and those of Mt. Wuling were classified into three groups (G1, G2, and G5) along an elevation gradient. Mismatch distribution analysis indicated that Pleistocene glaciations did not significantly affect the distribution of earthworm species in this region. Future work should examine the distribution and dispersal of the *E. nordenskioldi* complex in the Palearctic.

Author Contributions: Data curation, Y.Z. (Yiming Zhang); Formal analysis, C.L.; Funding acquisition, D.W.; Investigation, H.W.; Writing—original draft, Y.Z. (Yufeng Zhang); Writing—review and editing, N.M.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Science and Technology Program of Hebei Province China (C2019408050), National Natural Scientific Fund of China (42071059) and the National Science and Technology Fundamental Resources Investigation Program of China (2018FY100300).

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of Hebei Key Laboratory of Animal Diversity, Langfang Normal University.

Data Availability Statement: "MDPI Research Data Policies" at https://www.mdpi.com/ethics, accessed on 25 November 2021.

Acknowledgments: We are grateful to Jiahua Hao, Tingting Xiao, and Qin Xu for helpful comments on the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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