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Genetic Variation and Phylogeography of *Lumbriculus variegatus* (Annelida: Clitellata: Lumbriculidae) Based on Mitochondrial Genes

Tingting Zhou [1,2], Jiefeng Yu [1,2], Yongjing Zhao [1], Dekui He [3], Hongzhu Wang [1] and Yongde Cui [1,*]

[1] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China
[2] University of Chinese Academy of Sciences, Beijing 100049, China
[3] The Key Laboratory of Aquatic Biodiversity and Conservation, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China
* Correspondence: ydcui@ihb.ac.cn

Abstract: Lumbriculus variegatus is a typical cold-water worm and is mainly distributed in the Tibetan Plateau and Northeast in China. The current study aimed to explore the genetic diversity and phylogeography of *L. variegatus* sampled from different geographical regions based on concatenated (COI + 16S rRNA, 879 bp) genes. Among 63 *L. variegatus* specimens, 29 haplotypes were identified with high haplotype diversity (h = 0.923) and nucleotide diversity (π = 0.062). The Bayesian phylogenetic analysis and Median-joining haplotype network revealed two lineages, or species, of *L. variegatus*. Taxa belonging to lineage I was mainly distributed in the Tibetan Plateau of China, North America, and Sweden, while lineage II composed taxa from Northeast China, southern China, and Sweden. The analysis of molecular variance indicated that the genetic difference was mainly due to differences between lineages. Neutrality tests showed that the overall *L. variegatus* have a stable population since the time of origin. Divergence time analysis suggested that *L. variegatus* originated from the Triassic period of Mesozoic in 235 MYA (95%HPD: 199–252 MYA), and the divergence between different lineages of *L. variegatus* began from the next 170 million years.

Keywords: divergence time; genetic diversity; lineage differentiation; the most recent common ancestor

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1. Introduction

The family Lumbriculidae belonging to Clitellata is one of the most diverse taxa within aquatic oligochaeta. The majority of species in the family Lumbriculidae are endemic in the Holarctic region, except for two species, Lumbriculus variegatus (Müller, 1774) [1] and Stylodrilus heringianus Claparède, 1862 [2], which are distributed nearly across all continents. Lumbriculus variegatus is primarily resident of cool water environments [3] and occupies a variety of habitats, ranging from rivers to lakes, marshes to ponds, and plains to plateaus (40–4900 m above sea level). Besides, these small worms are typical passive dispersers, whose survival is more affected by local environmental conditions. Considering the huge environmental variations across geographic regions, it remains unclear whether these are cryptic species among different regional populations.

Due to asexual reproduction by fission and easy culture, *L. variegatus* is used as a model organism for experiments on the accumulation of pollutants and other toxic substances [4]. At present, most of the studies on *L. variegatus* have focused on the fields of ecophysiology, toxicology, phylogeny [5,6], and the phylogeographic history of its diversity, with little discussion. There are few studies on the biogeography of aquatic oligochaetes, including *L. variegatus*, and the research is mainly concentrated on terrestrial earthworms, such as the families Hormogastridae and Megascolecidae [7–9]. As for the study on the origin and evolution of Clitellata, Erséus et al. [10] constructed the phylogenetic relationships of

Clitellata using transcriptome data and proposed that the most recent common ancestor of Clitellata lived in the freshwater of the Late Paleozoic Devonian period (419–359 million years ago, MYA). Yet, this conclusion was obtained mainly based on family-level phylogenetic relationships. To date, few empirical studies have explored the phylogeny of different geographic populations from the species-level phylogenetic relationships. Given the global distribution characteristics and potential phylogeny divergence, disentangling the genetic diversity patterns can shed light on the origin of *L. variegatus*.

With the development of biogeography, studies explored geographical patterns and genetic diversity of species from the perspective of phylogeny (i.e., phylogeography) [11–13]. Phylogeography is an integrative field of science linking micro- and macro-evolutionary processes, allowing us to infer the ecological drivers shaping the geographical distribution patterns of genealogical lineages within a species. Specifically, molecular markers, in particular mitochondrial genes, are used widely to infer the phylogenetic relationships and the divergence time, and thus, could provide new insights into gene divergence of specific animal populations across different regions [14]. Compared with the nuclear gene, mitochondrial DNA, with its simple structure and moderate evolution rate, is an ideal molecular marker for studying population genetic structure, phylogeographic pattern, and evolutionary history [15]. The cytochrome *c* oxidase subunit I (COI) gene is one of the most reliable markers for studying taxonomy and phyletic evolution [7] and is also the best candidate gene for DNA barcoding [16]. The 16S ribosomal RNA (16S rRNA) is also a common gene for the classification and identification of aquatic oligochaetes.

In this study, we selected *L. variegatus* to uncover the phylogeographic patterns, divergence time, and underlying drivers from the view of the phylogeography perspective. To do this, a total of 63 specimens were collected from different regions of the Tibetan Plateau, Northeast China, southern China, Sweden, and the United States. Then, we explored the phylogeographic patterns of *L. variegatus* based on the mitochondrial genes (COI and 16S rRNA) using molecular analysis.

2. Materials and Methods

2.1. Specimens Collection, DNA Extraction and PCR Amplification

The specimens were collected by a dip net from 2018 to 2021. The individuals of *L. variegatus* were obtained from rivers, lakes, and wetlands of Tibetan Plateau, Northeast China, and southern China (Figure 1). Besides for the sequences for sampled specimens, we also downloaded the sequences (including seven outgroups) from GenBank (see Table S1). We used scalpel and tweezers in the fume hood to cut the body into two parts. The anterior end (approximately two thirds) of the specimens was deposited in 10% formalin solution as vouchers, and the posterior end was stored in absolute ethanol for DNA extraction.

The Qiagen blood tissue kit was used to extract total genomic DNA according to the standard manual (DNeasy Blood and Tissue Handbook). Mitochondrial COI and 16S rRNA were amplified with primer set LCO1490-GGTCAACAAATCATAAAGATATTGG/HCO2198-TAAACTTCAGGGTGACCAAAAAATCA [17,18] and 16SARL-CGCCTGTTTATCAAAAACAT/16SBRH-CCGGTCTGAACTCAGATCACGT [19]. All reactions were 25 μL with 8.5 μL of ddH_2O, 12.5 μL of Q5 Polymerase, 1 μL of 10 μmol/L of primer pair mix, and 2 μL of template DNA. PCR conditions for COI started with pre-denaturation at 98 °C for 30 s, followed by 35 cycles of 10 sec at 98 °C, 45 sec at 46 °C, and 90 sec at 72 °C, and then, a final extension at 72 °C for 2 min. The thermocycling procedure for 16S rRNA was similar, but the annealing temperature was at 60 °C. In addition, 5 μL PCR products were checked by 1% agarose gel electrophoresis, and the remaining fragments were used for sequencing.

2.2. Population Genetic Analysis

Based on the concatenated sequence of COI and 16S rRNA, the software of DnaSP6.0 was used to analyze the sequence composition and diversity index of *L. variegatus* in different geographical regions. The target parameters included variable sites, singleton variable sites, parsimony informative sites, haplotype number, haplotype diversity, and

nucleotide diversity [20]. The genetic distances between and within groups based on the Kimura two-parameter model were calculated using the MEGA6.0 [21]. The genetic differentiation index (F-statistics, F_{st}) was estimated using analysis of molecular variance (AMOVA) as implemented in Arlequin3.5 [22], which can detect gene flow in different populations. We also explored whether these target populations experienced expansion historically based on Tajima's D [23] and Fu's Fs [24] values.

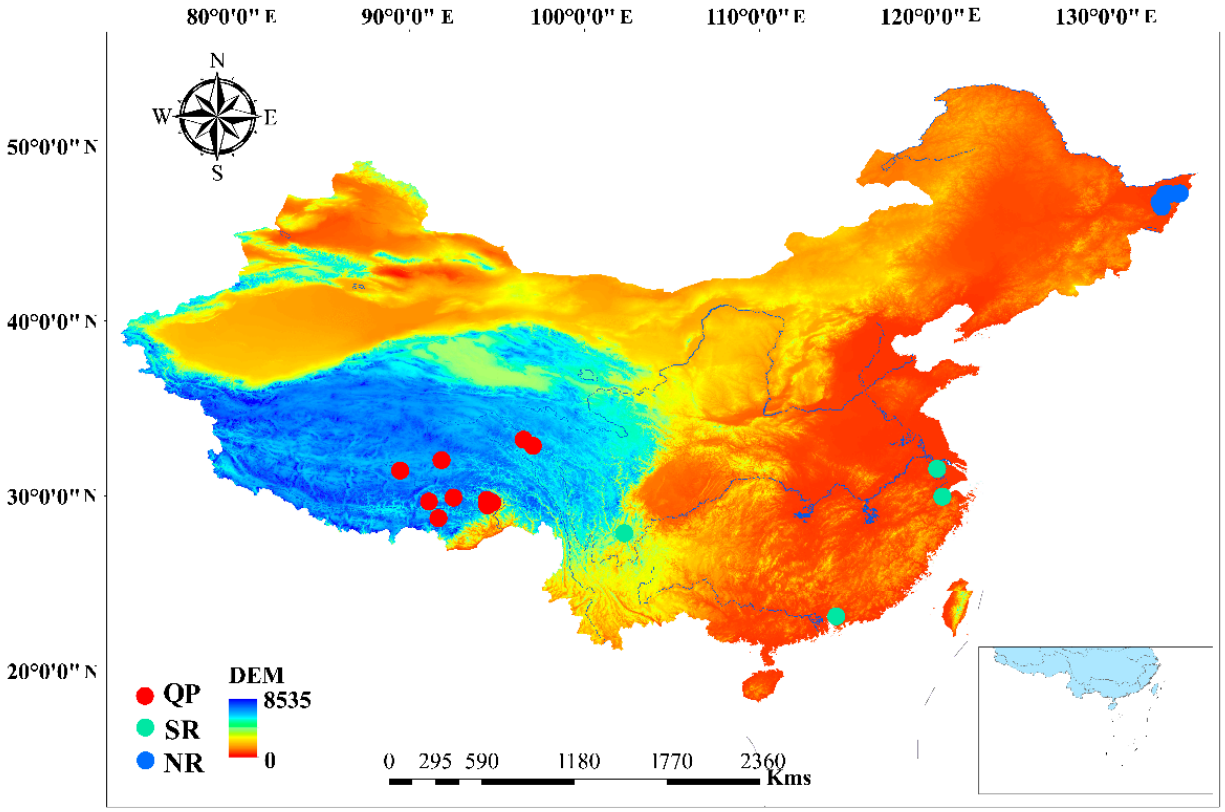

Figure 1. Map of China showing the sampling localities of the species *Lumbriculus variegatus* used in this study. QP, SR, and NR portions in the circle represent the Tibetan region, Southern China, and Northeast China.

2.3. Phylogenetic Analysis

The sequences were aligned by ClustalW in MEGA6.0 [21]. Phylogenetic relationships were assessed using Bayesian inference in the software Phylosuite1.2.1 [25]. The best-fit substitution model (GTR + F + I + G4) was selected based on BIC criteria [26]. Two runs were performed with four MCMC chains for 2×10^6 generations, sampling one tree every 100 generations. The setting of the burn-in fraction was set to 0.25. The first 4000 trees (standard deviation of split frequencies is below 0.01 as the convergence diagnostics) were discarded as burn-in phase, and the nodal support was assessed by calculating the mean posterior probabilities (PP) values of each node of the resulting consensus tree after burn-in. The generated phylogenetic trees were submitted to the iTOL website (https://itol.embl.de/itol.cgi, accessed on 1 December 2022) for further online editing.

2.4. Haplotype Analysis

The median-joining network was constructed using PopART1.7 software [27]. The software of DnaSP6.0 and Arlequin3.5 act as aid tools to generate haplotype data file and haplotype frequencies in populations file, respectively [20,22]. Both of the active data were saved in NEXUS file format to match PopART1.7. The data format contained two module data and traits. Here, ntax, nchart, and ntraits represent the number of haplotypes,

sequence length, and group count. Besides, the lineage geographical pattern of the tree was discussed.

2.5. Biogeographic Analysis

Based on the constructed Bayesian results, the molecular clock correction was performed using the divergence time of Naididae and Lumbriculidae (354 MYA), and Lumbricidae and Megascolecidae (157 MYA) [10]. Firstly, jModelTest2.1.7 software was used to select the optimal nucleotide substitution model and to estimate the frequency of each base pair. Then, BEAST2.6.6 [28] was used to estimate the divergence time of different geographic populations of *L. variegatus*. In this step, the Relaxed Clock Log Normal model was selected, and the average value and 95% highest posterior density interval (HPD) were considered as the divergence time. The base substitution model is the GTR, the number of Gamma Categories was 4, the Yule Model was selected as the dot plot model, and the length of the Markov chain (MCMC) was 5×10^8. Subsequently, Tracer1.7.2 software [29] was used to evaluate the operation results. If the effective sample size (ESS) of each parameter was greater than 200, it meant that the results were ideal. Otherwise, the parameters were adjusted again, and the operation continued. At last, we converted the file format in Tree Annotator2.6.6 and annotated the Tree file in FigTree1.4.

3. Results

3.1. Sequence Divergence Analysis

The aligned sequences ranged from 685 to 715 bp for COI while 500 to 531 bp for 16S. The mean base composition of sequences has a low G content (15.5%) and similar T and A contents (30.7% and 33.6%, respectively). The third codon position showed an obvious AT bias (64.3%), consistent with the invertebrate mitochondrial gene bases. The alignment sequences of 63 individuals contained 225 (25.60%) variable sites, of which 177 sites (20.14%) were parsimony-informative. All polymorphic sites exhibited two states, and the estimated transition/transversion (ti/tv) ratio was 2.47.

3.2. Distance and Population Structure Analysis

Concatenated sequences represented 29 unique haplotypes (*h*). The haplotype diversity (H*d*) and nucleotide diversity (π) were 0.923 and 0.062, respectively. Tajima's D test showed significant negative values only for lineage I ($p \leq 0.01$), indicating that *L. variegatus* has undergone a population expansion (Table 1). The genetic differentiation coefficient (F_{st}= 0.745) showed that *L. variegatus* in different lineages have differentiated to some degree. The average genetic distance (K2P) ranged from 0.057 (I) to 0.141 (II) within a lineage and up to 0.147 between lineages (Table 2). Almost all genetic variation was distributed between lineages, and 82.65% of the variation was partitioned between groups and 17.35% among populations within groups (Table 3).

Table 1. Genetic diversity and neutrality test of Lumbriculus variegatus based on the concatenated sequences of gene COI and 16S.

Lineage	Specimen Number	Haplotype Number	Haplotype Diversity (H*d*)	Nucleotide Diversity (π)	Tajima's D Test (D)	Tajima's D Test (*p*)	Fu's Fs Test (D)	Fu's Fs Test (*p*)
I	46	15	0.859	0.007	−1.964 **	0.010	−1.061	0.364
II	17	14	0.971	0.062	0.431	0.693	1.560	0.777
Total	63	29	0.923	0.062	−0.767	0.352	0.249	0.571

Note: ** $p \leq 0.01$.

Table 2. Genetic distances within lineages (on diagonal), genetic distances among lineages (below diagonal) and lineages pairwise F_{st} (above diagonal) based on combined COI and 16S gene sequences.

Lineage	I	II
I	0.057	0.745
II	0.147	0.141

Table 3. Analysis of molecular variance among population and within population in *Lumbriculus variegatus*.

Source of Variation	df	Sum of Squares	Variance Components	Percentage of Variation (%)
Among populations	1	1021.652	40.809	82.65
Within populations	61	522.427	8.564	17.35
Total	62	1544.079	49.373	

3.3. Phylogenetic Relationships

The Bayesian phylogenetic relationships of *L. variegatus* were constructed by selecting two Lumbricidae, two Megascolecidae, one Moniligastridae, and two Naididae as outgroups. In the tree, Drawida japonica was sister to the clade consisting of Amynthas triastriatus, Perionyx excavatus, Aporrectodea rosea, and Lumbricus rubellus with strong nodal support (PP = 1.00). Twenty-nine haplotypes of *L. variegatus* clustered together with high Bayesian posterior probabilities (PP = 1.00). The BI tree based on the concatenated sequences revealed a clear separation between the groups of *L. variegatus* (Figure 2). There were two major clades. Lineage I included most of the specimens found in the Tibetan Plateau and Sweden together with America. The remaining members formed lineage II, containing taxa collected from Northeast China, southern China, and Sweden.

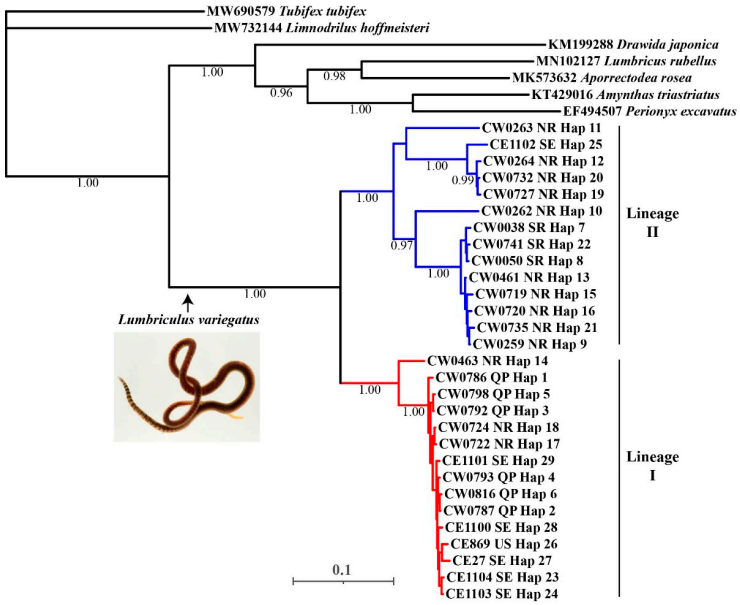

Figure 2. Bayesian inferences (BI) tree of Lumbriculus variegatus lineages based on the concatenated sequences of gene COI and 16S. Seven worms of Naididae, Lumbricidae, Moniligastridae, and Megascolecidae were designated as the outgroup. The numbers below the branch point to the Bayesian posterior probability. BI posterior probabilities > 0.95 is indicated; QP, SR, NR, SE and US portions in the circle represent the Tibetan region, Southern China, Northeast China, Sweden and North America. Hap denotes Haplotype.

3.4. Haplotype Analysis

The median-joining network (Figure 3) showed clearly that 29 unique haplotypes, among a total of 63 specimens from five regions, separated into two lineages. Lineage I, as the most basal divergent lineage, was radiate in appearance. One haplotype of Hap 2, shared by specimens from the Tibetan Plateau of China, Sweden, and the United States. This lineage was located in the center of the network structure, and was the haplotype with the highest frequency of sharing and occurrence. There are fewer common haplotypes in lineage II.

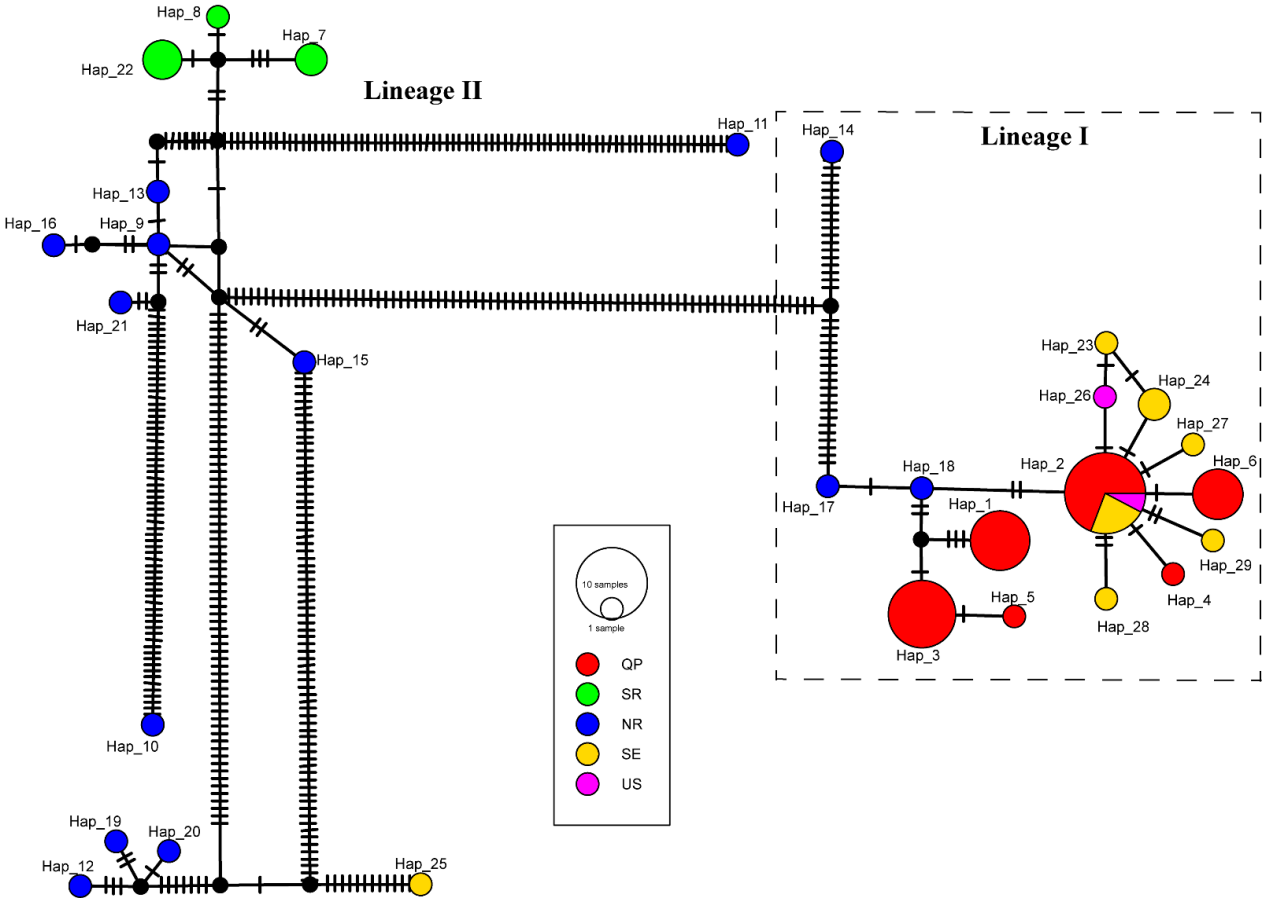

Figure 3. The haplotype network of Lumbriculus variegatus lineages based on the concatenated sequences of gene COI and 16S. "Hap" denotes Haplotype; subsequent numbers mean haplotype number; QP, SR, NR, SE, and US portions in the circle represent the Tibetan region, Southern China, Northeast China, Sweden, and North America.

3.5. Biogeographic Analysis

The evolutionary process of *L. variegatus* was inferred using molecular clock analysis based on seventy mitochondrial gene sequences. We carried out molecular clock correction based on the divergence time of Lumbricidae and Megascolecidae (157 MYA) as well as Naididae and Lumbriculidae (354 MYA). The results showed that the linear topology of molecular clock was consistent with Bayesian tree structure, corresponding to two major lineages (Figure 4). The most recent common ancestor of *L. variegatus* existed during the Mesozoic Triassic, about 235 MYA ago (95%HPD: 199–252 MYA). Since then, lineages I and II began to diverge.

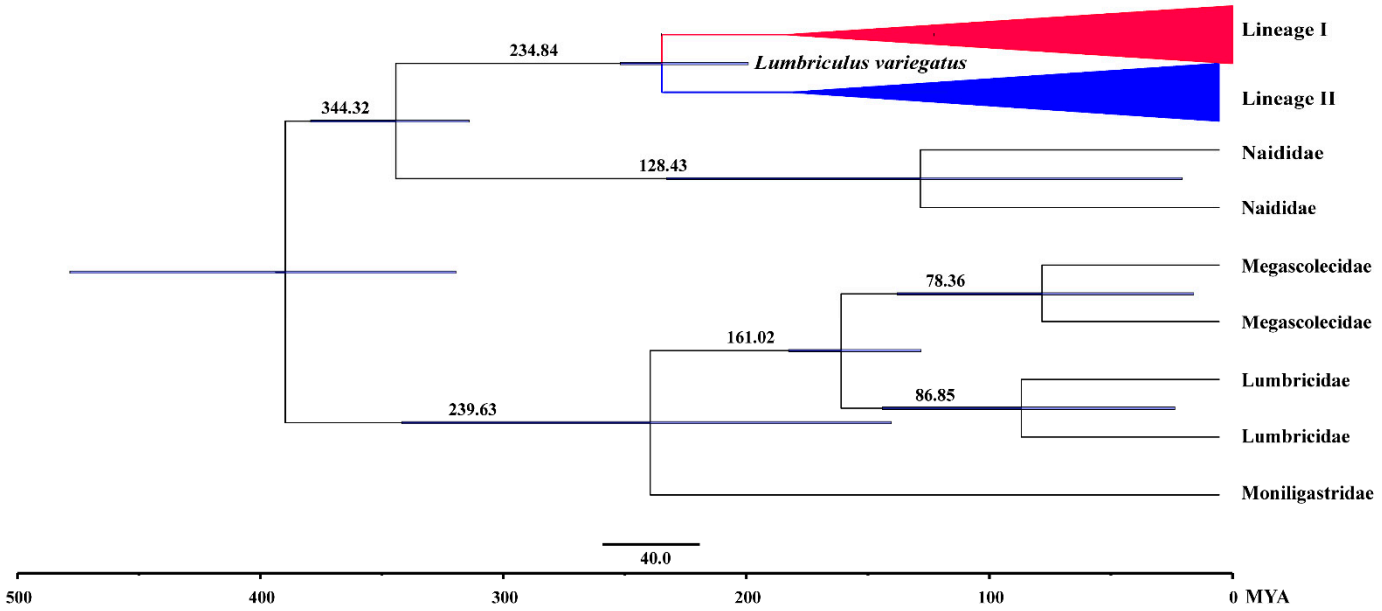

Figure 4. Divergence times among Lumbriculus variegatus derived from the Bayesian relaxed-molecular. Node bars indicate 95% high probability density of the divergence time estimates. Two major lineages (I and II) of *L. variegatus* are indicated.

4. Discussion

4.1. Genetic Variation

Lumbriculus variegatus is a widespread species, found on all continents except Antarctica and South America (presumably because no studies have been done yet). According to the distribution data of *L. variegatus* on the Global Biodiversity Information Facility (GBIF), the latitude of most of them range from 23.1° N to 73.7° N, and few record southernmost extends to 6.2° N north [3].

Population genetic diversity is one of the important dimensions of biodiversity, a guarantee of the evolutionary potential of species, and the basis of species conservation [30]. Haplotype diversity and nucleotide diversity are often used to evaluate the genetic diversity of a specific population [31]. Population genetic diversity tends to be high if a population is in glacial refugia [32]. Lineage II had the highest haplotype diversity and nucleotide diversity, suggesting that Northeast China was glacial refugia. Taken as a whole, lineage I (37–150 m) is higher in elevation than lineage II (2927–4869 m). Most specimens were collected at higher altitudes, but less at low altitudes. It only shows that the *L. variegatus* is distributed mainly at a higher altitude. There were also collections at a lower altitude, but the number was too small. Grant and Bowen [33] found that haplotype diversity $\geq$0.5 and nucleotide diversity $\geq$0.005 indicated that population evolution has a long history and population structure is stable. Therefore, the present distribution pattern of *L. variegatus* has been formed after a long evolution of the population. The results of AMOVA showed that the main source of variation was among the populations (82.65%), indicating that the two populations had diverged. The genetic distance and the genetic differentiation index among the populations were relatively large, which also confirmed that the genetic divergence was mainly caused by the isolation of mountains or river systems. Geographical barriers, such as long geographical distances, hinder gene exchange among populations, especially for the weak passive dispersers. Thus, with the increasing of geographical distance, the variation among populations gradually increases, forming different populations with genetic differences [34]. When the genetic differentiation coefficient is greater than 0.15, it indicates that there has been a high degree of genetic differentiation among populations [35]. In the study, the population differentiation was significant (F_{st} > 0.15). Both the Neutrality test and Tajima's D could be used to test whether a group has expanded historically [36].

Fu [24] proposed that Tajima's D test was more likely to detect ancient mutations and reveal the history of ancient population expansion, while Fu's Fs test was more sensitive to the detection of recent population expansion. This suggested that the present distribution pattern of *L. variegatus* was induced by divergence from a long time ago, and no population expansion has occurred recently.

4.2. Cryptic Species

With the development of molecular technology, several studies have revealed that there were potential cryptic species in clitellate taxa, such as Tubifex tubifex, Limnodrilus hoffmeisteri, Nais communis, and so on [37–39]. The same case may occur in the taxa of *L. variegatus*. Our results showed that two genetic lineages of *L. variegatus* were well separated, as indicated by the Bayesian inferences tree and haplotype network. These results were in line with Gustafsson's [5] earlier findings. Furthermore, Gustafsson et al. also observed the chromosome number of *L. variegatus*. Clade I specimens were highly polyploid, and clade II individuals were diploid. Therefore, we infer that *L. variegatus* consists of two distinct cryptic species. However, we didn't detect any obvious morphological differences between the two species. Blind blood vessels and chaetae bifid with upper tooth reduced are important characteristics for the identity of *L. variegatus*. Beyond that, the pattern and position of genital elements remain to be explored. We, thus, expect that more studies on the morphological variations of organisms should be conducted because traditional biodiversity surveys commonly rely on morphological identification.

Thirteen species of the genus of Lumbriculus have been recognized by far. These species are morphologically identical, with chaetae sigmoid, bifid, and upper tooth reduced [40]. The identification of species within the genus depends on the distribution and pattern of the genitalia. Lumbriculus ambiguus (Holmquist, 1976) shares with *L. variegatus* the character of the atria paired in segment VIII, yet the former species has two pairs of testes and the latter only one pair [1,41]. Lumbriculus tetraporophorus (Popčhenko, 1976) is similar to Lumbriculus kolymensis (Morev, 1982) based on the atria in segments VIII and IX. They differ in the number of spermathecae with two and four pairs, respectively [42,43]. There are two groups of four species whose atria are located at segment X. In the group with only one pair of ovaries, the spermathecae of Lumbriculus alexandrovi (Popchenko, 1976) and Lumbriculus illex (Timm and Rodriguez, 1994) open in segments IX and XII, separately [42,44]. Two pairs of ovaries were found in Lumbriculus genitosetosus (Holmquist, 1976) and Lumbriculus inconstans (Smith, 1895), while the genital chaetae were modified in the former [41,45]. Both Lumbriculus japonicus (Yamaguchi, 1936) and Lumbriculus sachalinicus (Sokolskaya, 1967) have atria in segment XI. They are different because of the number of spermathecae, three pairs, and one pair, respectively [46,47]. The atria of Lumbriculus mukoensis (Yamaguchi, 1953) were paired in segment XII [48]. Three pairs of atria are located from segments X to XIII in Lumbriculus multiatriatus (Yamaguchi, 1937) [49]. Lumbriculus olgae (Sokolskaya, 1976) differs from *L. variegatus* by the connective position between the vas deferens and the atrium [50]. There is no gene sequence with all these species, except for *L. variegatus*. More molecular data are needed to support species classification and phylogenetic relationship construction in the future.

4.3. Population Historical Dynamics

Based on the reconstruction of the ancestral habitat of annelids, Erséus et al. [10] demonstrated the freshwater origin of Clitellata and revealed that aquatic oligochaetes emerged in the Late Paleozoic Devonian (419–359 MYA). Our molecular dating results are consistent with the previous findings. However, the origin period of the *L. variegatus* was (235 MYA) slightly earlier than Erséus et al.'s (179–226 MYA). A potential explanation was the difference in the target gene of interest. Erséus et al. inferred that the origin of Clitellata was based on the transcriptome data, while the current study was focused on two mitochondrial genes (COI + 16S), a phenomenon also shown in Novo et al. [51]. Novo et al. used multi-gene sequence analysis to conclude that the divergence between Hormogaster

and Eisenia was earlier than Erséus et al. [10]. We ascribe more reliability to Erséus's conclusion because of transcriptomes consisting of a lot of genetic information.

Our results revealed that the most recent common ancestor of the families Lumbriculidae and Naididae began to diverge in the Carboniferous period of the Paleozoic. The *L. variegatus* originated before the breakup of Pangaea in the Mesozoic Triassic period (c. 235 MYA). Then, the Pangaea broke apart, forming a clade from specimens of Tibet, Sweden, and the US. In the late Indosinian movement, the South China plate collided with the Sino-Korean plate, which had been merged into the Eurasian plate, forming the Qinling-Dabie Mountain collision belt. Lumbriculus variegatus spread to northeast China and ended up as a distinct species. Several potential reasons might account for the difference in divergence time between the current study (235 MYA) and previous findings. On one hand, *L. variegatus* is a complex of cryptic species, as discussed above. On the other hand, the genetic information carried by only two markers was too little to fully reflect reality [52]. More molecular information, such as mitochondrial genome and transcriptome, will be urgently needed for further analysis.

5. Conclusions

Our results suggested that there are two main lineages of *L. variegatus* that correspond to two species. Lineage I was comprised of the Tibetan Plateau of China, North America, and Sweden, while taxa from Northeast China, southern China, and Sweden formed lineage II. Besides, the divergence time showed that *L. variegatus* originated from the Triassic period. However, considering the relatively limited samples and molecular genes in the current study, we expect that more molecular data and diagnostic characteristics of *L. variegatus* should be developed to achieve a better understanding of biodiversity patterns.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15020158/s1, Table S1: List of specimens considered in the study, including of voucher numbers, collection information and GenBank numbers. New sequences are shown in bold. Missing data is marked with "-".

Author Contributions: T.Z.: conceptualization, data curation, formal analysis, methodology, investigation, and writing—original draft; J.Y.: methodology, and writing—review and editing; Y.Z.: project administration, and writing—review and editing; D.H.: software, and writing—review and editing; H.W.: methodology, and writing—review and editing; Y.C.: conceptualization, investigation, funding acquisition, and writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Not applicable.

Data Availability Statement: The molecular data presented in this study are available in the National Center for Biotechnology Information under the GenBank accession numbers OP535889-OP537054.

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

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