diversity

MDPI

Article

Characterization of 15 Earthworm Mitogenomes from Northeast China and Its Phylogenetic Implication (Oligochaeta: Lumbricidae, Moniligastridae)

Huifeng Zhao [1,2], Shuanghu Fan [1], Nonillon M. Aspe [3], Lichao Feng [4] and Yufeng Zhang [1,5,*]

1 Hebei Key Laboratory of Animal Diversity, College of Life Science, Langfang Normal University, Langfang 065000, China
2 Northeast Asia Biodiversity Research Center, Northeast Forestry University, Harbin 150040, China
3 College of Marine and Allied Sciences, Mindanao State University at Naawan, Naawan 9023, Misamis Oriental, Philippines
4 Department of Plant Sciences, Jilin Agricultural Science and Technology College, Jilin 132101, China
5 College of Environment, Northeast Normal University, Changchun 130117, China
* Correspondence: qqzyf123@126.com

Abstract: Earthworms are an important ecological group, especially in agricultural regions in Northeast China. However, fewer studies focus on this group of organisms compared with other faunal groups. Here, we sequenced 15 new mitogenomes of *Aporrectodea tuberculata* Eisen, 1874, *A. trapezoides* Duges, 1828, *Eisenia nordenskioldi* Eisen, 1878 and *Drawida ghilarovi* Gates, 1969 in Northeast China using a high-throughput sequencing platform. These incomplete linear and double-stranded mitogenomes vary from 14,998 bp to 16,123 bp in size and include 37 genes and a putative control region. Intraspecific genetic divergence was quantified in the lumbricid species, and a control region in *D. ghilarovi* was reported for the first time by comparison to the mitogenomes of the congeners. Phylogenetic analysis based on coding genes and ribosomal DNA datasets using BI and ML inferences showed the non-monophyly of *Aporrectodea* and polyphyly of *E. nordenskioldi*. Future works should examine the taxonomy, phylogeny and population genetics not only of Lumbricidae but also the other earthworm families on the global scale using mitogenomic and nuclear data.

Keywords: earthworms; phylogeny; Lumbricidae; mitogenomes; non-coding region

Citation: Zhao, H.; Fan, S.; Aspe, N.M.; Feng, L.; Zhang, Y. Characterization of 15 Earthworm Mitogenomes from Northeast China and Its Phylogenetic Implication (Oligochaeta: Lumbricidae, Moniligastridae). *Diversity* **2022**, *14*, 714. https://doi.org/10.3390/d14090714

Academic Editors: Luc Legal and Daniel Fernández Marchán

Received: 5 July 2022
Accepted: 24 August 2022
Published: 28 August 2022

1. Introduction

Earthworms play an important role in many ecosystem processes, such as soil aeration, turnover and drainage, as well as the composition and decomposition of organic matter [1]. However, for such an important ecological group, the taxonomy and phylogeny of earthworms, particularly Lumbricidae and *Drawida* Michaelsen 1900 of Moniligastridae, which are widespread across the Holarctic, are inadequate [2–4]. The reasons for the taxonomic insufficiencies may be due to the structural simplicity, which lacks complex appendices or highly specialized copulatory apparatuses [5]. The lack of characters has led to many morphologically similar species being lumped into a single species with various morphotypes or as a species complex that includes various taxa of uncertain taxonomic category. This is especially true for the species of *Aporrectodea* Orley, 1885 and *Eisenia* Malm, 1877 of Lumbricidae, and *Drawida* [2–12].

Eisenia nordenskioldi, a species widespread in Northern Asia and adjacent regions, is known for its high morphological, karyotypic and genetic variation [8,13]. Its distribution extends from tundra to forest–steppe and broad-leaved forests. The diagnostic features of this species include the following: the body has no stripes; the clitellum is faint, yellow, and saddle-shaped in xxvii–xxxii; the spermathecal pores are paired in 9/10 and 10/11 ventrally; the spermathecae are round; and the setal arrangement is lumbricine. *Aporrectodea trapezoides* Dugés 1828 and *A. tuberculata* Eisen, 1874 belong to the *Aporrectodea caliginosa*

species complex, the most abundant earthworm group from the Paleartic grassland and forest regions. One diagnostic feature of *A. tuberculata* is its lack of body pigmentation, while *A. trapezoides* is brown. The position of the clitellum for both of the species occurs within the same range of segments, but the form and position of the tubercula pubertatis differ—they appear as two protuberances in *A. tuberculata*, while they appear as two lateral bands in *A. trapezoides* [5]. Meanwhile, the vestiture of *Drawida ghilarovi* found in this region is characterized by color variations: light-bluish, brown and grey [13] and pitch-black [14,15]. The life-forms are considered as black-wetland worm and other colored forest worms.

The reported complete mitogenomes of earthworms range from 14,648 to 15,188 base pairs (bp), consisting of 37 genes, including 13 protein-coding genes (PCGs), 2 ribosomal RNA genes (rRNAs), 22 transfer RNA genes (tRNAs) and a putative control region (CR) [16–25]. Mitochondrial sequences are considered to be ideal genetic markers that are extensively employed for the study of systematics, phylogeography and species delimitation in animals [26–32]. In previous studies of the phylogeny in earthworms [5,33], the main clades were not robust, with low support values using single or several genetic makers. The advantages of using the mitogenome as a phylogenetic marker over other genes to infer an evolutionary relationship have been discussed in various works. For instance, mitogenomes have shown to provide resolution for an enormously broad range of phylogenetic depths, from shallow divergence times between populations of a single species to deep divergence within an entire phylum [34–36]. Mitogenomes have often proved to be useful in resolving formerly troublesome phylogenies, clarifying the relationships within phylogenetically difficult groups where rapid radiations made other markers ineffective [37,38]. Previously in earthworm mitogenomic studies, more attention was paid to the mitogenomic studies of pheretimoids (Megascolecidae) in the Oriental Realm [18,20,21], while only a few studies were completed on moniligastrids and lumbricids.

Here, 15 new mitogenomes of *Aporrectodea tuberculata* Eisen, 1874, *A. trapezoides* Duges, 1828, *Eisenia nordenskioldi* Eisen, 1878 and *Drawida ghilarovi* Gates, 1969 were sequenced, assembled and analyzed in order to shed light on the mitochondrial structures of these species to assess the phylogenetic relationships of the two genera of Lumbricidae and a genus of Moniligastridae.

2. Materials and Methods

2.1. Sample Preparation and DNA Extraction

Four earthworm species of Lumbricidae and Moniligastridae from Northeast China (Figure 1), namely: *A. tuberculata* (Heilongjiang: Anda: Renmin Park: 46.400° N, 125.330° E); *A. trapezoides* (Jilin: Jilin: 43.727° N, 126.628° E) and *E. nordenskioldi* (Jilin: Changchun: Nanhu Park: N43.859, E125.308; Heilongjiang: Bei'an: Renmin Park 48.2536° N, 126.5040° E; Heilongjiang: Wudalianchi: 48.5794° N, 125.9435° E) of Lumbricidae, and *D. ghilarovi* (Jilin: Changchun: Jingyuetan National Scenic Area: 43.490° N, 125.790° E) of Moniligastridae were collected by hand in July–August, 2021. See Table S1 for detailed information concerning the samples. All of the samples were preserved in 95% ethanol, which was replaced with new ethanol every week during the fieldwork, and stored at room temperature (15–25 °C) in the lab. One to six specimens of each species were taken for mitogenomic analysis. The genomic DNA was extracted using TIANamp Micro DNA Kit (TIANGEN, Beijing, China) according to the manufacturer's protocol. The DNA concentrations were measured with a BIO DL MicroDrop spectrophotometer (Beijing, China). Table 1 shows the earthworm mitogenomes used in this study.

Figure 1. The distribution of earthworm samples used in this study. The brown circles indicate the distribution of *Aporrectodea* species, the green circles indicate the distribution of *Eisenia nordenskioldi* and the red circle indicates the distribution of *Drawida ghilarovi*.

Table 1. Mitogenomes used for the phylogeny of earthworms in this study.

Family	Taxa	Accession Number	Body Color	Habitat	References
Lumbricidae	*Aporrectodeacaliginosa*	CM035405	-	-	Unpublished
	Aporrectodea tuberculata	OL840316–7, OM687883–6	Light grey	Forest	This study
	Aporrectodea trapezoides	OM687882	Dark brown	Farmland	This study
	Aporrectodea rosea	MK573632	-	-	[24]
	Bimastus parvus	MZ857199	-	-	[39]
	Dendrobaena octaedra	MZ857197	-	-	[39]
	Eisenia andrei	MZ857198	-	-	[39]
	Eisenia balatonica	MK642872	-	-	[8]
	Eisenia nana	MK618511	-	-	[8]
	Eisenia nordenskioldi	OL840314–5, OM687887–90	Light reddish	Forest	This study
	Eisenia nordenskioldi	MK642869	-	-	[8]
	Eisenia nordenskioldi	MZ857200	-	-	[39]
	Eisenia nordenskioldi nordenskioldi	MK618509	-	-	[8]
	Eisenia nordenskioldi nordenskioldi	MK618510	-	-	[8]
	Eisenia nordenskioldi nordenskioldi	MK618513	-	-	[8]

Table 1. *Cont.*

Family	Taxa	Accession Number	Body Color	Habitat	References
	Eisenia nordenskioldi nordenskioldi	MK642867	-	-	[8]
	Eisenia nordenskioldi nordenskioldi	MK642868	-	-	[8]
	Eisenia nordenskioldi pallida	MK618512	-	-	[8]
	Eisenia spelaea	MK642870	-	-	[8]
	Eisenia tracta	MK642871	-	-	[8]
	Lumbricus rubellus	MN102127	Pink	Forest	[23]
	Lumbricus terrestris	LTU24570	-	-	[16]
	Octolasion tyrtaeum	MZ857201	-	-	[39]
	Amynthas aspergillus	KJ830749	-	-	[19]
	Amynthas carnosus	KT429008	-	-	[21]
	Amynthas corticis	KM199290	-	-	[18]
	Amynthas cucullatus	KT429012	-	-	[21]
	Amynthas gracilis	KP688582	-	-	[18]
	Amynthas hupeiensis	KT429009	-	-	[21]
	Amynthas jiriensis	KT783537	-	-	[40]
	Amynthas longisiphonus	KM199289	-	-	[18]
	Amynthas moniliatus	KT429020	-	-	[21]
	Amynthas morrisi	KT429011	-	-	[21]
	Amynthas pectiniferus	KT429018	-	-	[21]
Megascolecidae	*Amynthas robustus*	KT429019	-	-	[21]
	Amynthas redactus	KT429010	-	-	[21]
	Amynthas instabilis	KT429007	-	-	[21]
	Amynthas rongshuiensis	KT429014	-	-	[21]
	Amynthas spatiosus	KT429013	-	-	[21]
	Amynthas triastriatus	KT429016	-	-	[21]
	Metaphire schmardae	KT429015	-	-	[21]
	Metaphire californica	KP688581	-	-	[18]
	Metaphire guillelmi	KT429017	-	-	[21]
	Metaphire posthuma	MW222472	-	-	[41]
	Metaphire vulgaris	KJ137279	-	-	[20]
	Perionyx excavatus	EF494507	-	-	[42]
	Tonoscolex birmanicus	KF425518	-	-	[17]
	Drawida ghilarovi	OL840312–3	Gray	Forest	This study
Moniligastridae	*Drawida gisti*	MN539609	-	-	[25]
	Drawida japonica	KM199288	-	-	[21]
Naididae	*Tubifex tubifex*	MW690579	-	-	[43]
Outgroup	*Nais communis*	MW770354	-	-	[44]

2.2. Next Generation Sequencing

The genomic DNA was fragmented using the Covaris S220 Focused Ultrasonicator (Covaris, Woburn, MA, USA), and the 300–400 bp fragments were captured by magnetic beads. After repairing the blunt ends, adenylating 30 ends and ligating adapters, the fragmented DNA was enriched, and the PCR products were captured again by magnetic beads. The PCR products were sequenced by BGISEQ500 platform, and a sequence library was constructed. The raw data were cleaned by removing the following: (1) the adapters; (2) the reads that contained five and more Ns; (3) the reads that were shorter than 75 bp or the average Phred values lower than Q15; (4) the reads of the Phred values lower than Q20 with 4 bp slide windows; and (5) the reads that contained polyN (A, T, C, G) that were longer than 50 bp.

2.3. Data Assembly and Mitogenome Annotation

The clean reads were assembled by MitoZ v2.4 (Meng et al., Shenzhen, China) [45], NOVOplasty 4.3.1 (Dierckxsens et al., Brussels, Belgium) [46] and GetOrganelle 1.6.0 (Jin et al., Kunming, China) [47]. Annotation was performed using MitoZ and the MITOS2 webserver [48]. The mitochondrial genome was set as *cox1* to begin with, following the other mitogenomes of earthworm with "Mitogenome_reorder.py" provided by MitoZ v2.4, and visualized by Circos 0.69 (Krzywinski et al., Vancouver, Canada) [49].

2.4. Statistics of the Earthworm Mitogenomes

The nucleotide composition of the whole mitogenome, which includes 13 PCGs, 22 tRNAs, 2 rRNAs and a putative control region (CR), were counted. The relative synonymous codon usage (RSCU) of the PCGs of the four earthworms were calculated in MEGA5 [50]. The nucleotide compositional skew was estimated according to the formula: AT skew = [A − T]/[A + T], GC skew = [G − C]/[G + C]) [51]. Genetic divergence within species, including genetic variants and indels, was detected by DNASP v5 (Librado, Barcelona, Spain) [52]. The tandem repeats were detected using the webserver "TANDEM REPEATS FINDER" (https://tandem.bu.edu/trf/trf.basic.submit.html, accessed on 3 January 2022).

2.5. Phylogenetic Analysis

To investigate the phylogeny of earthworms with the mitogenome data, we reconstructed the phylogenetic relationships among the earthworm species using two datasets (PCG: 13 PCGs, including all three codons, and PCGrRNA: 13 PCGs and 12S, 16S rRNAs) with Bayesian inference (BI) and maximum likelihood (ML) methods under a partitioned strategy. A total of 46 sequenced data are available online and 15 newly sequenced data are used here to perform the phylogenetic analysis. The details of the taxa are shown in Table 1. The mitogenomic phylogenetic trees of the earthworms were reconstructed representing 13 genera of earthworms belonging to three families: Megascolecidae, Lumbricidae and Moniligastridae. *Tubifex tubifex* Müller, 1774 and *Nais communis* Piguet, 1906 of Naididae (freshwater oligochaetes) were used as the outgroups in this study.

The best substitution models based on the Akaike information criterion (AIC) were selected by jModeltest 2.1.7 (Darriba et al., Vigo, Spain) [53] for the two datasets, and the selected models were listed in Table S2. BI analysis was performed with the chosen models above using MrBayes 3.2.6 (Ronquist et al., Florida, USA) [54]. To ensure that the average standard deviation of split frequencies was less than 0.01, two million generations were run with sampling every 1000 generations. All of the parameters achieved an effective sample size (ESS) larger than 200 that was checked by Tracer v1.7.2 (Rambaut et al., Edinburgh, UK) [55] after combining two runs and adjusting the burn-in. The consensus trees were calculated, including burning in the former 25% calculation. The ML trees were constructed by RAxML 8 (Stamatakis, Heidelberg, Germany) [56] using the default rapid hill-climbing algorithm, and 1000 replicates of bootstrap with GTRCAT model were generated. All of the phylogenetic trees were visualized using Figtree v1.4.4 (Rambaut, Edinburgh, UK) [57].

3. Results

3.1. Mitogenome Organization and Nucleotide Composition of the Four Earthworm Species

All 15 mitogenomes assembled by the three bioinformatics tools are incomplete. The annotations of *A. tuberculata*, *A. trapezoides*, *E. nordenskioldi* and *D. ghilarovi* are listed in Tables S3 and S4, and the maps of mitogenomes of the four species are visualized in Figure 2. The ratio of AT, GC, AT skew and GC skew of whole mitogenomes, PCGs, tRNAs, rRNAs and CR are listed in Table S5. The accession numbers of the 15 new mitogenomes are OL840312–16 and OM687882–90 (see Table 1 for details). The total length of the six mitogenomes of *A. tuberculata* varies from 15,058 bp to 15,129 bp; that of *A. trapezoides* is 14,998 bp; that of the six mitogenomes of *E. nordenskioldi* vary between 15,290 bp and 16,123 bp; and that of the two mitogenomes of *D. ghilarovi* vary between 15,080 bp and 15,061 bp.

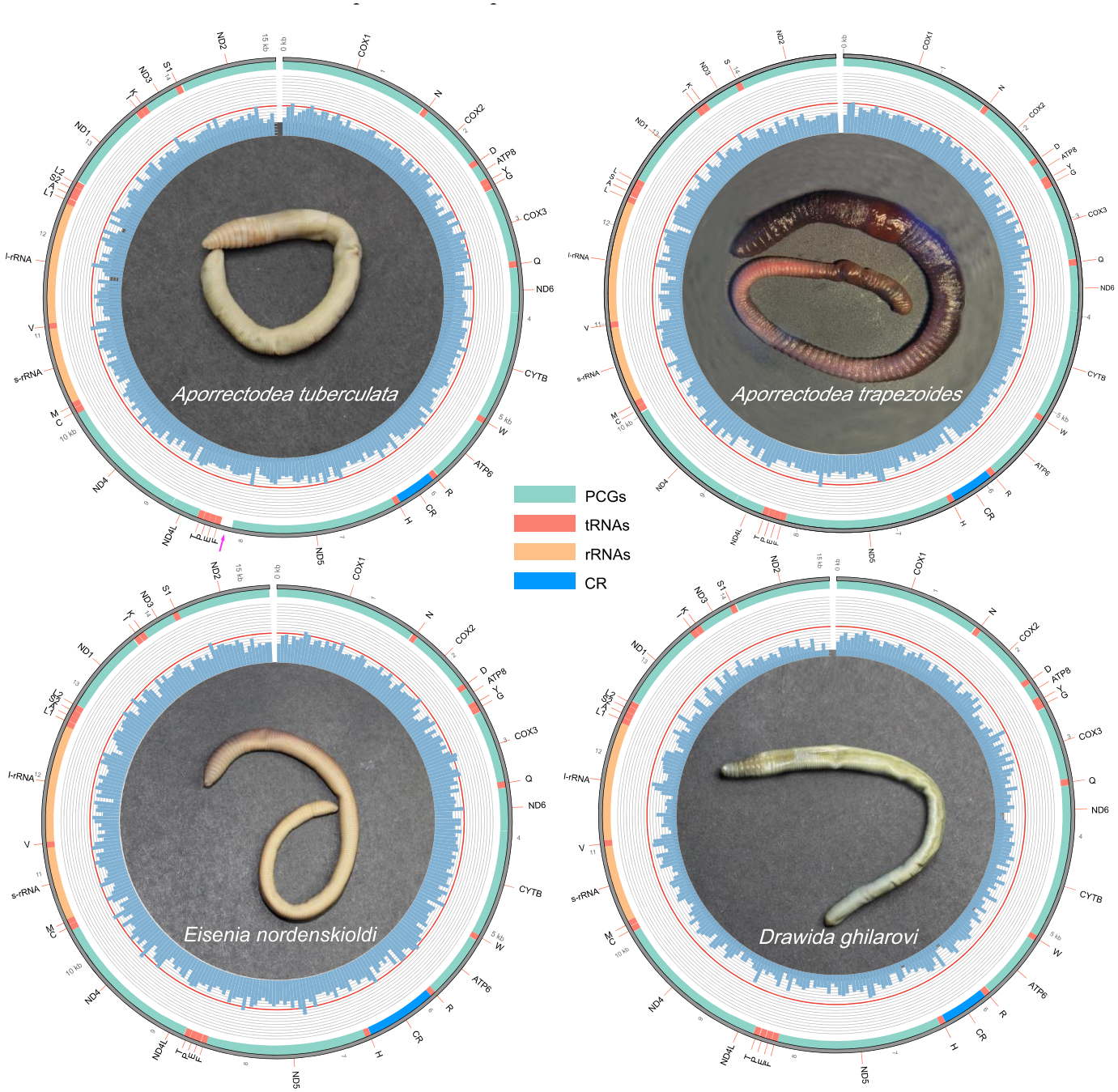

Figure 2. Map of mitogenomes of *A. tuberculata*, *A. trapezoides*, *E. nordenskioldi* and *D. ghilarovi*. The inner circles indicate the GC content in every 50-site window, and the outer circle shows the arrangement of the genes; *cox1* was set as the start of mitogenome. All genes are coded on the majority strand.

The intraspecies genetic divergence is quite high in the six mitogenomes from the same population of *A. tuberculata*, with 447 variants in 13 PCGs, 6 tRNAs, 2 rRNAs, CR and *introns*, and eight indel events were also detected in *trnW*, *trnR*, *trnT*, *rrnL* and CR (see Table S5). The genetic polymorphism of the two mitogenomes of *E. nordenskioldi* from Changchun (Nanhu Park) contains only five variants and three indels in *cox1*, *cox2*, *nd4*, *nd5*, *rrnL* and CR (see Table S5). Seven variants in *atp8*, *cox1*, *cytb*, *rrnL* and CR, and three indels in *trnR* and CR were also detected in the four mitogenomes within *E. nordenskioldi* lineages from Bei'an-Wudalianchi (see Table S5). Meanwhile, there was only one indel in the CR in the two mitogenomes of *D. ghilarovi* from Changchun (Jingyuetan National Scenic Area) (see Table S5).

The nucleotide compositions of the 15 new mitogenomes were similar within families while different between families (see Table S6 for details, Supplementary Materials). In Lumbricidae, the AT content of the whole mitogenomes of the lumbricids *A. tuberculata*, *A. trapezoides* and *E. nordenskioldi* ranged from 61.0% to 64.5%; that of the moniligastrid *D. ghilarovi* was 72.5%; the GC content of the three lumbricid species ranged from 35.5% to 39.0%; that of the *D. ghilarovi* was much lower (27.5%). The AT skews of the lumbricids ranged from −4.7% to 2.0%, while that of the moniligastrid was lower (−7.9%); the GC skews of the lumbricids ranged from −19.2% to −21.2%, and that of *D. ghilarovi* was much higher (−8.7%).

3.2. Protein-Coding Genes and Codon Usage in the Four Earthworm Species

In the 15 new mitogenomes, there was a large difference in the PCG length between families, a little difference between species and no difference within species. The lengths of the PCGs of the lumbricids *A. tuberculata*, *A. trapezoides* and *E. nordenskioldi* were 11,116 bp, 11,110 bp and 11,114 bp, respectively; that of the moniligastrid *D. ghilarovi* was 11,134 bp. The PCGs of the four earthworm species exhibited similar initiation and termination codons (Tables S3 and S4). All of the initiation codons of the four earthworms were ATG. Most of the termination codons were TAR, and *atp8*, *cox1*, *cox3*, *nd2* and *nd4l* in all four species, *nd1* in *Aporrectodea*, *nd6* in *E. nordenskioldi* and *nd4* in *D. ghilarovi* were incomplete stop codon T or TA (Tables S3 and S4). These truncated stop codons are commonly used in mitogenomes in Metazoa, and are modified to a complete stop codon by the post-transcriptional polyadenylation [58]. The RSCU values of the four earthworm species are shown in Figure 3. The codons CGA-Arg and UCU/A-Ser2 in the four earthworm species and the UUA-Leu2 in *D. ghilarovi* were the most common.

3.3. RNA Genes of the Four Earthworm Species

The secondary structures of 22 tRNAs of the 3 lumbricids, *A. tuberculata* (OL840316), *A. trapezoides* (OM687887) and *E. nordenskioldi* (OL840314) are illustrated in Figure 4. All of the tRNAs can fold as a typical cloverleaf structure, except the *trnA*, *trnG* and *trnV* whose lack the TψC loop and *trnS1* which lacks the DHU stem. Figure 5 shows the secondary structures of the tRNAs of the moniligastrid *D. ghilarovi*: *trnN*, *trnD*, *trnG*, *trnK*, *trnT*, *trnY* and *trnV* lack the TψC loop, and *trnS1* lacks the DHU loop. In addition, the identified wobble GT base pairs and unmatched base pairs AA, CA, CT and TT in different tRNA stems are shown in Figures 4 and 5, and they might be restored during the post-transcriptional editing processes [59].

The entire lengths of the rRNAs of the four species are similar, ranging from 2034 bp to 2044 bp. The rRNAs of the four species showed a positive AT skew and negative GC skew in the three lumbricids, which was positive in moniligastrid *D. ghilarovi*. The rRNAs A+T% was 62.4–65.6% in lumbricids and 72.9% in *D. ghilarovi* (see Table S3 for details).

3.4. Putative Control Region and Non-Coding Region

All of the putative CRs were located between *trnR* and *trnH* in the four earthworms. The lengths of CRs were different within and among species; the CR length varied from 374 to 444 bp among the individuals in *A. tuberculata*; 440 bp in *A. trapezoides*; 757 and 761bp in the *E. nordenskioldi* lineage from Changchun; 1569–1578 bp in the *E. nordenskioldi* lineage from Bei'an-Wudalianchi; and 468 and 487 bp in *D. ghilarovi* (see Table S3 for the details). There were three tandem repeats that were detected (AT, AATACA, ATACAAATATAT) in the CRs of *A. tuberculata* (OM687883 and/or OM687883 and/or OM687886); two copies of a 175 bp tandem repeat were detected in the Bei'an-Wudalianchi lineage of *E. nordenskioldi;* no tandem repeat was detected in the Changchun lineage of *E. nordenskioldi*; and 35–40 copies of AT were detected in *D. ghilarovi*.

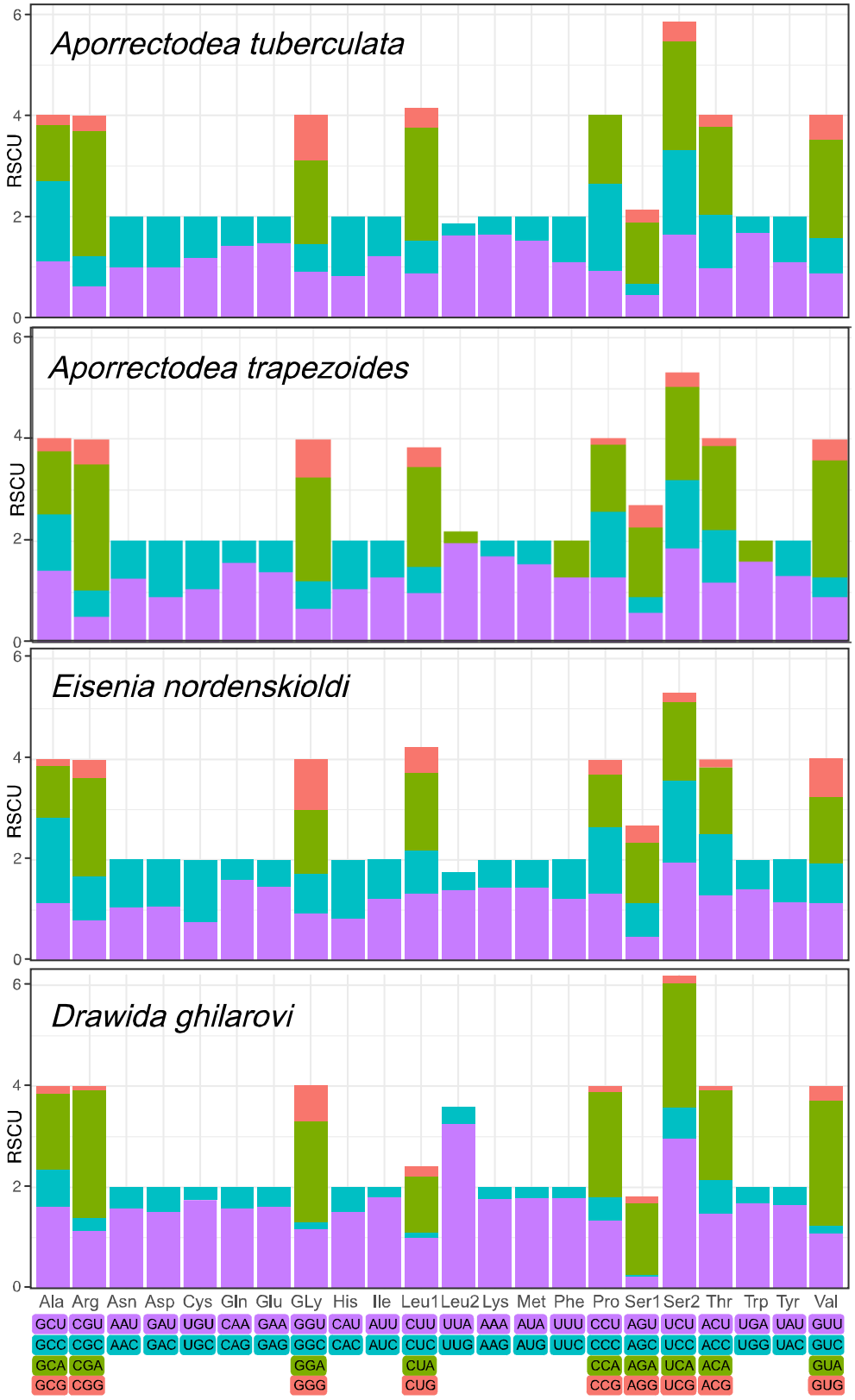

Figure 3. Relative synonymous codon usage (RSCU) in the PCGs of the four newly sequenced earthworm species. Codon families are indicated below the X axis.

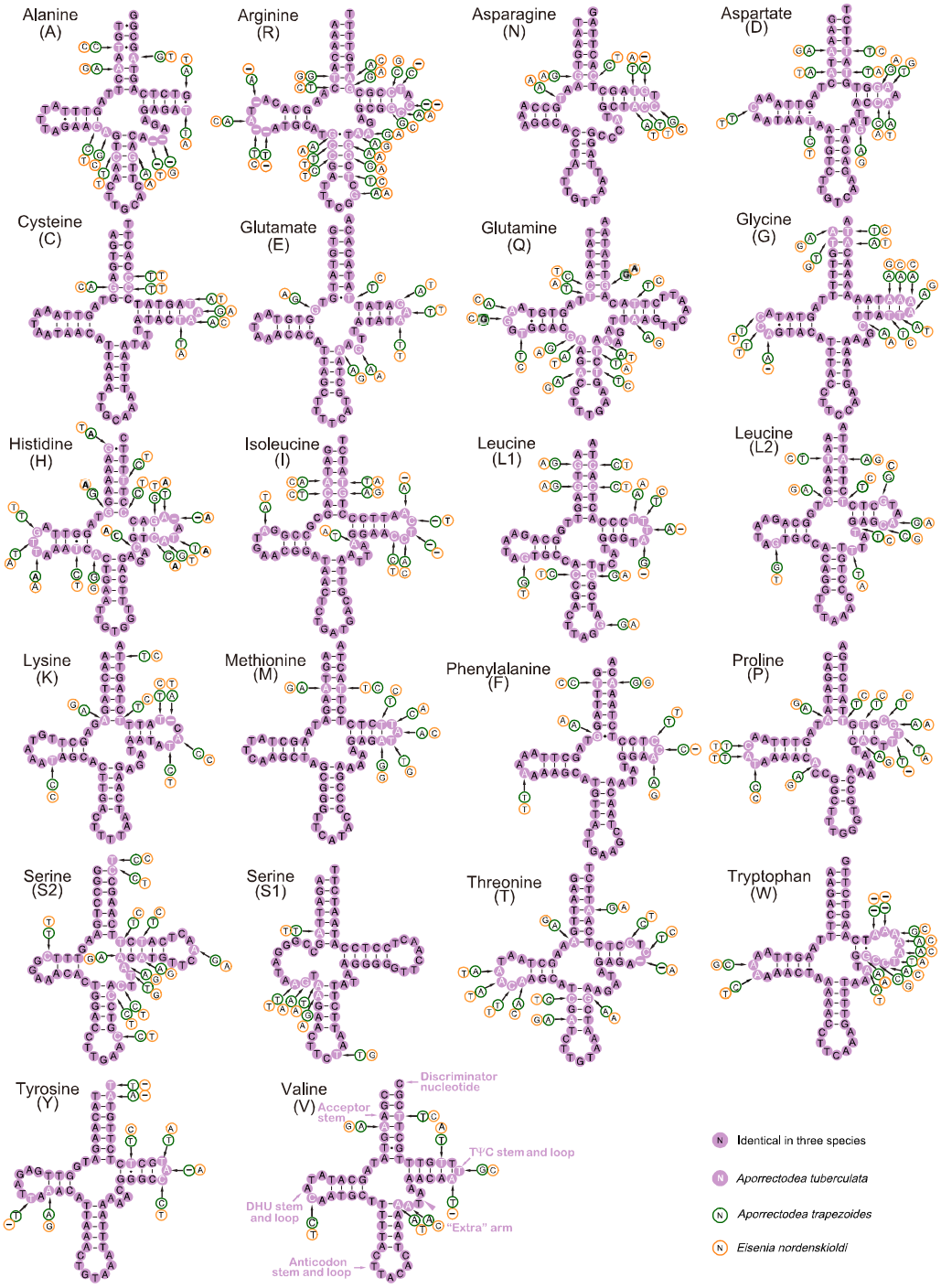

Figure 4. Predicted secondary structure for the tRNAs of the three lumbricid species. The tRNAs are labeled with the abbreviations of their corresponding amino acids. Dashes indicate the Watson-Crick base pairs; dots indicate the wobble GT pairs and gaps indicate non-match bases.

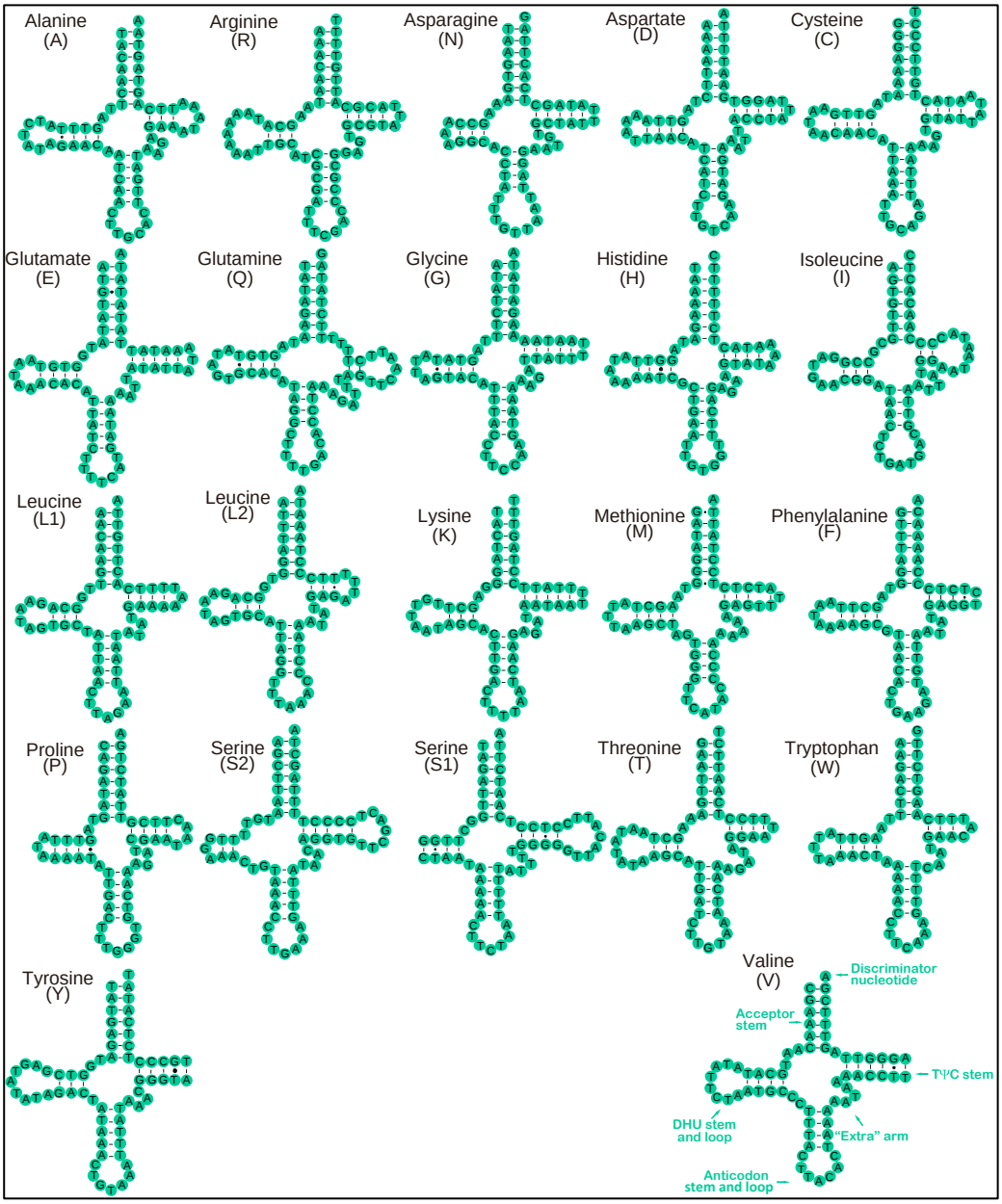

Figure 5. Predicted secondary structure for the tRNAs of *D. ghilarovi*. The tRNAs are labeled with the abbreviations of their corresponding amino acids. Dashes indicate the Watson–Crick base pairs, dots indicate the wobble GT pairs and gaps indicate non-match bases.

3.5. Phylogenetic Analysis

The *Aporrectodea tuberculata* species in this work is similar to the lineages of North Europe (Denmark, Finland and Poland) (Figure S1) [5], while *A. trapezoides* belongs to the lineage of a parthenogenetic group that occurs in a large region in Europe (Serbia, France and Spain) [5] based on the *cox2* gene data (Figure S1). On the other hand, *Drawida ghilarovi* in this study is separate from that of the *cox1* data from Russia (Figure S2) [2,7].

The phylogenetic trees of BI and ML reveal that the three families are reciprocal monophyletic with high support (both posterior probabilities (PP) = 1 and bootstrap (BS) = 100, as shown in Figure 6). For Megascolecidae, the species of *Amynthas* and *Metaphire* are scattered throughout a multi-clade, which mostly agrees with Zhang et al.'s study [21]. For the details on the trees of Megascolecidae, see Figures S3–S6.

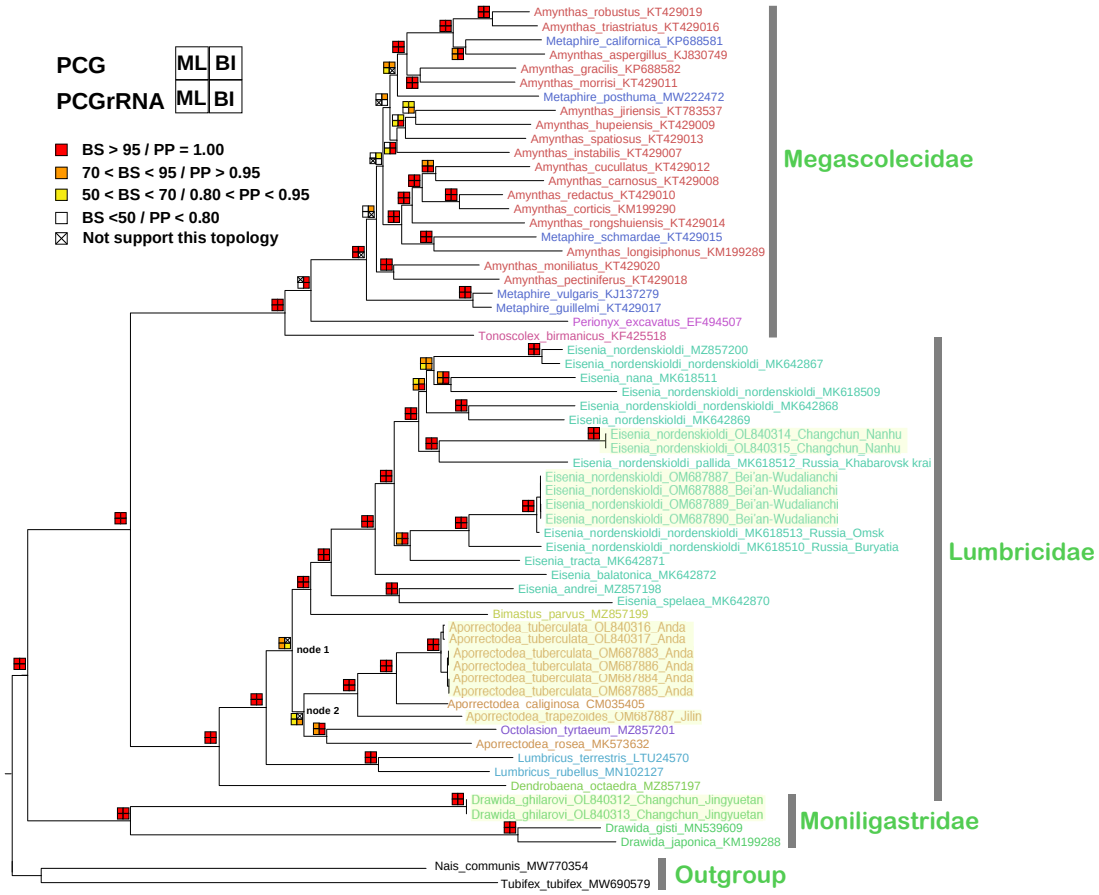

Figure 6. Phylogenetic trees of earthworm using mitogenome data. The four squares near the node indicate the support value: top left, ML of PCG; bottom left, ML of PCGrRNA; top right, BI of PCG; bottom right, BI of PCGrRNA. The newly sequenced mitogenomes are highlighted, and genera delimited by the current taxonomic base are distinguished from each other by a particular color.

For Lumbricidae, the phylogenetic relationship between *Aporrectodea*, *Octolasion* and *Lumbricus* and the species within *Eisenia* is unclear. Clusters ((*Eisenia*, *Bimastus*) and (*Aporrectodea*, *Octolasion*)) formed with a low support value (node 1 in Figure 6). *Aporrectodea* is paraphyletic in both of the ML trees and the BI tree from the PCGrRNA dataset, as the topology shows (((*A. tuberculata*, *A. caliginosa*), *A. trapezoides*) (*A. rosea*, *Octolasion*)) (node 2 in Figure 6). *Aporrectodea* is also polyphyletic, as the topology shows ((*Eisenia*, *Bimustus*), (*A. rosea*, *Octolasion*)), ((*A. tuberculata*, *A. caliginosa*), *A. trapezoides*) in the BI tree of the PCG dataset with a low support value (PP = 0.66), as seen in the bottom right of Figure 6.

4. Discussion

4.1. Organization of a Partial Mitochondrial Genome

The 15 newly sequenced mitogenomes showed no variation in the number of genes, the strand location and the arrangement of genes (Table S3 and Figure 2) as compared with the mitogenomes of earthworm species in other works [13–22]. The 15 mitogenomic sequences that were assembled by the three tools failed to completely circularize, although lengths fell under the ranges of those circular mitogenomes [16–25]. As shown in Figure 2, the gap in the sequences was between *nd2* and *cox1*. In the future, circular mitogenomes may be assembled using the Pacbio or Nanopore sequence platform that can produce reads longer than 10 kb [60], covering the polyNs or short tandem repeats of mitogenomes in earthworms.

The mitogenomes of *A. tuberculata* are distinguished from *A. trapezoides* and *A. rosea* [24] by containing a non-coding region (noted by the pink arrow on Figure 2). The mitogenome

sequences of *D. ghilarovi* is differentiated from *D. gisti* [25] and *D. japonica* [21] by the presence of a putative CR (Figure 2). The putative control region is often absent in partial sequences due to certain technical difficulties in deciphering it by the traditional PCR method [21,25]. More mitogenomes of *Drawida* should be sequenced in the future by next generation sequencing to confirm the existence of CR in these earthworms.

4.2. Genetic Diversity of Lumbricids in Northeast China

Quantities of DNA polymorphs were detected in the mitogenomes of lumbricids that were collected from the gardens of downtown areas in Northeast China. This indicates that these gardens may be refuges for lumbricids. There were fewer earthworms collected in the farmland than in the gardens. This may be due the overuse of herbicides or pesticides that may have resulted in a reduction in earthworms in the farmland [61]. To test for the most common type of refuge for lumbricids in Northeast China, more earthworm samples need to be studied in the future.

4.3. Phylogenetic Inference

The paraphyly of *E. nordenskioldi* and the non-monophyly of *Aporrectodea* are supported by both BI and ML analyses. The non-monophyly may be explained by the following points: (1) the mitochondrial loci are a maternal inheritance, their rate of divergence is faster than that of the nuclear loci, and that may lead to the non-monophyletic topology of *Aporrectodea* and *Octolasion*; (2) taxonomical characters of *E. nordenskioldi*, *Aporrectodea*, *Octolasion* and *Lumbricus* are not synapomorphic, which results in the chaos of the taxonomy in Lumbricidae; (3) the number of species analyzed using mitogenomic data was inadequate for acquiring a clearer picture of the systematic relationships of *Aporrectodea*, *Octolasion* and *Lumbricus*. The non-monophyly of *E. nordenskioldi* agrees with the data from Shekhovtsov et al. [8], while the non-monophyly of *Aporrectodea* agrees with the data from Pop et al. [62], Pérez-Losada et al. [5] and Shekhovtsov et al. [4,63]. This suggests that certain morphological characters that are used to define these taxa could be homoplasious. A revision in the taxonomic identification and classification is needed, taking into consideration other morphological and ecological characteristics.

Our mitogenomic phylogenetic inference of Lumbricidae agrees with the study by Domínguez et al. [64], which contained the nuclear loci (*18S* and *28S* rDNAs), the morphological characters and a large number of species that reach 76. The mitogenomic phylogeny of Lumbricidae here makes sense and aligns with the former attempts that have consistently indicated the need for extensive revision of the taxonomy of Lumbricidae.

5. Conclusions

Fifteen new mitogenomes from four Palearctic earthworm species are provided here. We reported the presence of CR in *D. ghilarovi* for the first time. Quantities of variants were detected among lumbricid species, particularly in the population of *A. tuberculata*. Phylogenetic analysis of BI and ML indicates the non-monophyletic taxa in Lumbricidae. Future works should examine the taxonomy, phylogeny and population genetics not only of Lumbricidae but also of the other earthworm families on a global scale using mitogenomic and nuclear data.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14090714/s1, Figure S1: NJ tree of COX2 of available online and this study of *Aporrectodea*; Figure S2: NJ tree of COX1 of all available online and this study of *Drawida ghilarovi*; Figure S3: ML tree of PCG dataset; Figure S4: ML tree of PCGrRNA dataset; Figure S5: BI tree of PCG dataset; Figure S6: BI tree of PCGrRNA dataset; Table S1: The information of specimens in the study; Table S2: The best substitute models were selected by jModeltest 2.1.7 for the genes in PCGrRNA and PCG datasets; Table S3: Organization of the mitogenomes of the lumbricid species *A. tuberculata* (OL840316), *A. trapezoides* (OM687887) and *E. nordenskioldi* (OL840314); Table S4: Organization of the mitogenomes of *D. ghilarovi* (OL840312; Table S5: The nucleotide sites of the variants and indels of

earthworms in this study; Table S6: Statistics of mitogenomes of *A. tuberculata* (420Ra-f), *A. trapezoides* (DL377), *E. nordenskioldi* (374NHa-b, 451Ra-b, 456Ra-b) and *D. ghilarovi* (374JYa-b).

Author Contributions: Sampling, S.F., L.F. and Y.Z.; data curation and formal analysis, H.Z.; funding acquisition and investigation, Y.Z.; writing—original draft, H.Z. and Y.Z.; 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 Project of Hebei Education Department (QN2021126), the project of Northeast Asia Biodiversity Research Center (2572022DS09) to H.Z., the National Science and Technology Fundamental Resources Investigation Program of China (2018FY100301) to Y.Z. and National Natural Scientific Fund of China (42071059).

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 Hao Jiahua (Northeast Normal University, Changchun, China) for his assistance in the collection of specimens during the field work.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Edwards, C.A. *Earthworm Ecology*; CRC Press: Boca Raton, FL, USA, 2004. [CrossRef]
2. Ganin, G.N.; Atopkin, D.M. Molecular differentiation of epigeic and anceic forms of *Drawida ghilarovi* Gates, 1969 (Moniligastridae, Clitellata) in the Russian Far East: Sequence data of two mitochondrial genes. *Eur. J. Soil. Biol.* **2018**, *86*, 1–7. [CrossRef]
3. Shekhovtsov, S.V.; Berman, D.I.; Bulakhova, N.A.; Vinokurov, N.N.; Peltek, S.E. Phylogeography of *Eisenia nordenskioldi nordenskioldi* (Lumbricidae, Oligochaeta) from the north of Asia. *Polar Biol.* **2018**, *41*, 237–247. [CrossRef]
4. Shekhovtsov, S.V.; Derzhinsky, Y.A.; Poluboyarova, T.V.; Golovanova, E.V.; Peltek, S.E. Phylogeography and genetic lineages of *Aporrectodea rosea* (Lumbricidae, Annelida). *Eur. J. Soil. Biol.* **2020**, *99*, 103191. [CrossRef]
5. Pérez-Losada, M.; Ricoy, M.; Marshall, J.C.; Domínguez, J. Phylogenetic assessment of the earthworm Aporrectodea caliginosa species complex (Oligochaeta: Lumbricidae) based on mitochondrial and nuclear DNA sequences. *Mol. Phylogenet. Evol.* **2009**, *52*, 293–302. [CrossRef] [PubMed]
6. Ganin, G.; Anisimov, A.; Roslik, G.; Atopkin, D. The Russian Far East Endemic *Drawida Ghilarovi* (Oligochaeta, Moniligastridae): Polimorphism, Ecology Specifics And Karyotype. *Russ. J. Zool.* **2014**, *93*, 1070–1079. [CrossRef]
7. Atopkin, D.M.; Ganin, G.N. Genetic differentiation of black and grey colored forms of the earthworm *Drawida ghilarovi* Gates, 1969 (Moniligastridae, Oligochaeta) on Russian Far East. *Eur. J. Soil. Biol.* **2015**, *67*, 12–16. [CrossRef]
8. Shekhovtsov, S.V.; Golovanova, E.V.; Ershov, N.I.; Poluboyarova, T.V.; Berman, D.I.; Bulakhova, N.A.; Szederjesi, T.; Peltek, S.E. Phylogeny of the *Eisenia nordenskioldi* complex based on mitochondrial genomes. *Eur. J. Soil. Biol.* **2020**, *96*, 103137. [CrossRef]
9. Shekhovtsov, S.V.; Golovanova, E.V.; Peltek, S.E. Cryptic diversity within the Nordenskiold's earthworm, *Eisenia nordenskioldi* subsp. *nordenskioldi* (Lumbricidae, Annelida). *Eur. J. Soil. Biol.* **2013**, *58*, 13–18. [CrossRef]
10. Shekhovtsov, S.V.; Berman, D.I.; Peltek, S.E. Phylogeography of the earthworm *Eisenia nordenskioldi nordenskioldi* (Lumbricidae, Oligochaeta) in northeastern Eurasia. *Dokl. Biol. Sci.* **2015**, *461*, 85–88. [CrossRef]
11. Shekhovtsov, S.V.; Berman, D.I.; Bazarova, N.E.; Bulakhova, N.A.; Porco, D.; Peltek, S.E. Cryptic genetic lineages in *Eisenia nordenskioldi pallida* (Oligochaeta, Lumbricidae). *Eur. J. Soil. Biol.* **2016**, *75*, 151–156. [CrossRef]
12. Shekhovtsov, S.; Berman, D.; Golovanova, E.; Peltek, S. Genetic diversity of the earthworm *Eisenia nordenskioldi* (Lumbricidae, Annelida). *Vavilov J. Genet. Breed.* **2017**, *21*, 589–595. [CrossRef]
13. Vsevolodova-Perel, T.S. *The Earthworms of the Fauna of Russia*; Nauka: Moscow, Russia, 1997.
14. Ganin, G. *Structural and Functional Organization of Mezopedobiont Communities of the Southern Russian Far East*; Russian Academy of Sciences Far Eastern Banch, Institute of Water and Ecological Problems: Vladivostok, Russia, 2011; p. 380.
15. Ganin, G.; Anisimov, A.; Roslik, G.; Atopkin, D. Earthworms *Drawida ghilarovi* Gates, 1969 (Oligochaeta, Moniligastridae): 1. Polimorphism, geographic range, ecology specifics. *Amurian Zool. J.* **2013**, *4*, 401–404. [CrossRef]
16. Boore, J.L.; Brown, W.M. Complete sequence of the mitochondrial DNA of the annelid worm *Lumbricus terrestris*. *Genetics* **1995**, *141*, 305–319. [CrossRef] [PubMed]
17. Wang, A.R.; Hong, Y.; Win, T.M.; Kim, I. Complete mitochondrial genome of the Burmese giant earthworm, *Tonoscolex birmanicus* (Clitellata: Megascolecidae). *Mitochondrial DNA* **2015**, *26*, 467–468. [CrossRef] [PubMed]

18. Zhang, L.; Jiang, J.; Dong, Y.; Qiu, J. Complete mitochondrial genome of four pheretimoid earthworms (Clitellata: Oligochaeta) and their phylogenetic reconstruction. *Gene* **2015**, *574*, 308–316. [CrossRef] [PubMed]
19. Zhang, L.; Jiang, J.; Dong, Y.; Qiu, J. Complete mitochondrial genome of an *Amynthas* earthworm, *Amynthas aspergillus* (Oligochaeta: Megascolecidae). *Mitochondrial DNA A DNA Mapp. Seq. Anal.* **2016**, *27*, 1876–1877. [CrossRef] [PubMed]
20. Zhang, L.; Jiang, J.; Dong, Y.; Qiu, J. Complete mitochondrial genome of a Pheretimoid earthworm *Metaphire vulgaris* (Oligochaeta: Megascolecidae). *Mitochondrial DNA A DNA Mapp. Seq. Anal.* **2014**, *27*, 297–298. [CrossRef]
21. Zhang, L.; Sechi, P.; Yuan, M.; Jiang, J.; Dong, Y.; Qiu, J. Fifteen new earthworm mitogenomes shed new light on phylogeny within the *Pheretima* complex. *Sci. Rep.* **2016**, *6*, 20096. [CrossRef]
22. Conrado, A.C.; Arruda, H.; Stanton, D.W.G.; James, S.W.; Peter, K.; Brown, G.; Silva, E.; Dupont, L.; Taheri, S.; Morgan, A.J.; et al. The complete mitochondrial DNA sequence of the pantropical earthworm *Pontoscolex corethrurus* (Rhinodrilidae, Clitellata): Mitogenome characterization and phylogenetic positioning. *ZooKeys* **2017**, *688*, 1–13. [CrossRef]
23. Zhang, Q.; Liu, H.; Zhang, Y.; Ruan, H. The complete mitochondrial genome of *Lumbricus rubellus* (Oligochaeta, Lumbricidae) and its phylogenetic analysis. *Mitochondrial DNA B Resour.* **2019**, *4*, 2677–2678. [CrossRef]
24. Shekhovtsov, S.V.; Peltek, S.E. The complete mitochondrial genome of *Aporrectodea rosea* (Annelida: Lumbricidae). *Mitochondrial DNA B Resour.* **2019**, *4*, 1752–1753. [CrossRef]
25. Liu, H.; Xu, N.; Zhang, Q.; Wang, G.; Xu, H.; Ruan, H. Characterization of the complete mitochondrial genome of *Drawida gisti* (Metagynophora, Moniligastridae) and comparison with other Metagynophora species. *Genomics* **2020**, *112*, 3056–3064. [CrossRef] [PubMed]
26. Qi, M.; Zhao, H.; Yu, F.; Zhang, A.; Li, H. The First Mitogenomes of the Subfamily Odontiinae (Lepidoptera, Crambidae) and Phylogenetic Analysis of Pyraloidea. *Insects* **2021**, *12*, 486. [CrossRef] [PubMed]
27. Ruiz-García, M.; Pinilla-Beltrán, D.; Murillo-García, O.E.; Pinto, C.M.; Brito, J.; Shostell, J.M. Comparative mitogenome phylogeography of two anteater genera (*Tamandua* and *Myrmecophaga*; *Myrmecophagidae*, *Xenarthra*): Evidence of discrepant evolutionary traits. *Zool. Res.* **2021**, *42*, 525–547. [CrossRef] [PubMed]
28. Du, Z.; Hasegawa, H.; Cooley, J.R.; Simon, C.; Yoshimura, J.; Cai, W.; Sota, T.; Li, H. Mitochondrial Genomics Reveals Shared Phylogeographic Patterns and Demographic History among Three Periodical *Cicada* Species Groups. *Mol. Biol. Evol.* **2019**, *36*, 1187–1200. [CrossRef]
29. Hou, Q.; Tu, F.; Liu, Y.; Liu, S. Characterization of the mitogenome of *Uropsilus gracilis* and species delimitation. *Mitochondrial DNA A DNA Mapp. Seq. Anal.* **2016**, *27*, 1836–1837. [CrossRef]
30. Sun, X. Divergence across the mitogenomes of *Branchinella kugenumaensis* (Anostraca: Thamnocephalidae) with implications for species delimitation. *Mitochondrial DNA B Resour.* **2021**, *6*, 631–633. [CrossRef]
31. Zhao, H.; Chen, Y.; Wang, Z.; Chen, H.; Qin, Y. Two Complete Mitogenomes of Chalcididae (Hymenoptera: Chalcidoidea): Genome Description and Phylogenetic Implications. *Insects* **2021**, *12*, 1049. [CrossRef]
32. Zhao, H.; Chen, H.; Li, Y. A report of the complete mitochondrial genome of *Bisetocreagris titanium* (Arachnida: Pseudoscorpiones: Neobisiidae) from Yunnan Province, China. *Mitochondrial DNA Part B Resour.* **2021**, *6*, 3212–3213. [CrossRef]
33. Zhang, Y.F.; Ganin, G.N.; Atopkin, D.M.; Wu, D.H. Earthworm Drawida (Moniligastridae) Molecular phylogeny and diversity in Far East Russia and Northeast China. *Eur. Zool. J.* **2020**, *87*, 180–191. [CrossRef]
34. Saccone, C.; De Giorgi, C.; Gissi, C.; Pesole, G.; Reyes, A. Evolutionary genomics in Metazoa: The mitochondrial DNA as a model system. *Gene* **1999**, *238*, 195–209. [CrossRef]
35. Ramesh, A.; Small, S.T.; Kloos, Z.A.; Kazura, J.W.; Nutman, T.B.; Serre, D.; Zimmerman, P.A. The complete mitochondrial genome sequence of the filarial nematode *Wuchereria bancrofti* from three geographic isolates provides evidence of complex demographic history. *Mol. Biochem. Parasitol.* **2012**, *183*, 32–41. [CrossRef] [PubMed]
36. Kern, E.M.A.; Kim, T.; Park, J.-K. The Mitochondrial Genome in Nematode Phylogenetics. *Front. Ecol. Evol.* **2020**, *8*, 250. [CrossRef]
37. Yu, L.; Li, Y.-W.; Ryder, O.A.; Zhang, Y.-P. Analysis of complete mitochondrial genome sequences increases phylogenetic resolution of bears (Ursidae), a mammalian family that experienced rapid speciation. *BMC Evol. Biol.* **2007**, *7*, 198. [CrossRef] [PubMed]
38. Miya, M.; Nishida, M. The mitogenomic contributions to molecular phylogenetics and evolution of fishes: A 15-year retrospect. *Ichthyol. Res.* **2015**, *62*, 29–71. [CrossRef]
39. Liu, H.; Zhang, Y.; Xu, W.; Fang, Y.; Ruan, H. Characterization of Five New Earthworm Mitogenomes (Oligochaeta: Lumbricidae): Mitochondrial Phylogeny of Lumbricidae. *Diversity* **2021**, *13*, 580. [CrossRef]
40. Hong, Y.; Kim, M.J.; Wang, A.R.; Kim, I. Complete mitochondrial genome of the earthworm, *Amynthas jiriensis* (Clitellata: Megascolecidae). *Mitochondrial DNA A DNA Mapp. Seq. Anal.* **2017**, *28*, 163–164. [CrossRef]
41. Yu, X.; Yang, H.; Liu, J.; Qi, Y.; Sun, L.; Tian, X. A strategy for a high enrichment of insect mitochondrial DNA for mitogenomic analysis. *Gene* **2022**, *808*, 145986. [CrossRef]
42. Kim, D.W.; Lee, K.S.; Jee, S.H.; Seo, S.B.; Park, S.C.; Choo, J.K. Complete sequence analysis of the mitochondrial genome in the earthworm, *Perionyx excavatus*. *Integr. Biosci.* **2005**, *9*, A705.
43. Lee, J.; Jung, J. First record of the complete mitochondrial genome of Tubifex tubifex (Müller) 1774 (Annelida; Clitellata; Oligochaeta) and phylogenetic analysis. *Mitochondrial DNA B Resour.* **2022**, *7*, 1208–1210. [CrossRef]
44. Lee, J.; Jung, J. The complete mitochondrial genome of Nais communis Piguet, 1906 (Annelida; Clitellata; Naididae). *Mitochondrial DNA B Resour.* **2022**, *7*, 199–201. [CrossRef] [PubMed]

45. Meng, G.; Li, Y.; Yang, C.; Liu, S. MitoZ: A toolkit for animal mitochondrial genome assembly, annotation and visualization. *Nucleic Acids Res.* **2019**, *47*, e63. [CrossRef] [PubMed]
46. Dierckxsens, N.; Mardulyn, P.; Smits, G. NOVOPlasty: De novo assembly of organelle genomes from whole genome data. *Nucleic Acids Res.* **2017**, *45*, e18. [CrossRef] [PubMed]
47. Jin, J.; Yu, W.; Yang, J.; Song, Y.; dePamphilis, C.W.; Yi, T.; Li, D. GetOrganelle: A fast and versatile toolkit for accurate de novo assembly of organelle genomes. *Genome Biol.* **2020**, *21*, 241. [CrossRef]
48. Donath, A.; Jühling, F.; Al-Arab, M.; Bernhart, S.H.; Reinhardt, F.; Stadler, P.F.; Middendorf, M.; Bernt, M. Improved annotation of protein-coding genes boundaries in metazoan mitochondrial genomes. *Nucleic Acids Res.* **2019**, *47*, 10543–10552. [CrossRef]
49. Krzywinski, M.; Schein, J.; Birol, I.; Connors, J.; Gascoyne, R.; Horsman, D.; Jones, S.J.; Marra, M.A. Circos: An information aesthetic for comparative genomics. *Genome Res.* **2009**, *19*, 1639–1645. [CrossRef]
50. Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **2011**, *28*, 2731–2739. [CrossRef]
51. Perna, N.T.; Kocher, T.D. Patterns of nucleotide composition at fourfold degenerate sites of animal mitochondrial genomes. *J. Mol. Evol.* **1995**, *41*, 353–358. [CrossRef]
52. Librado, P.; Rozas, J. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **2009**, *25*, 1451–1452. [CrossRef]
53. Darriba, D.; Taboada, G.L.; Doallo, R.; Posada, D. jModelTest 2: More models, new heuristics and parallel computing. *Nat. Methods* **2012**, *9*, 772. [CrossRef]
54. Ronquist, F.; Teslenko, M.; van der Mark, P.; Ayres, D.L.; Darling, A.; Hohna, S.; Larget, B.; Liu, L.; Suchard, M.A.; Huelsenbeck, J.P. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* **2012**, *61*, 53942. [CrossRef]
55. Rambaut, A.; Drummond, A.J.; Xie, D.; Baele, G.; Suchard, M.A. Posterior Summarization in Bayesian Phylogenetics Using Tracer 1.7. *Syst. Biol.* **2018**, *67*, 901–904. [CrossRef]
56. Stamatakis, A. RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **2014**, *30*, 1312–1313. [CrossRef] [PubMed]
57. Rambaut, A. Figtree 1.4.0. Available online: http://tree.bio.ed.ac.uk/software/figtree/ (accessed on 1 July 2022).
58. Ojala, D.; Montoya, J.; Attardi, G. tRNA punctuation model of RNA processing in human mitochondria. *Nature* **1981**, *290*, 470–474. [CrossRef] [PubMed]
59. Lavrov, D.V.; Brown, W.M.; Boore, J.L. A novel type of RNA editing occurs in the mitochondrial tRNAs of the centipede Lithobius forficatus. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 13738–13742. [CrossRef] [PubMed]
60. van Dijk, E.L.; Jaszczyszyn, Y.; Naquin, D.; Thermes, C. The Third Revolution in Sequencing Technology. *Trends Genet.* **2018**, *34*, 666–681. [CrossRef]
61. Hu, F.; Piao, Y.; Wang, H.; Pan, Y.; Fu, Y.; Song, W. The Investigation of the Usage of Herbicide in Heilongjiang Province. *J. Agric.* **2015**, *5*, 25–31.
62. Pop, A.A.; Csuzdi, C.; Wink, M.; Pop, V.V. *An Attempt to Reconstruct the Molecular Phylogeny of the Genus Allolobophora (sensu lato, Pop, 1941) Using 16S rDNA and COI Sequences (Oligochaeta, Lumbricidae)*; University Press: Cluj-Napoca, Romania, 2006.
63. Shekhovtsov, S.; Ermolov, S.; Poluboyarova, T.; Kim-Kashmenskaya, M.; Derzhinsky, Y.; Peltek, S. Morphological differences between genetic lineages of the peregrine earthworm: *Aporrectodea caliginosa* (Savigny, 1826). *Acta Zool. Acad. Sci. Hung.* **2021**, *67*, 235–246. [CrossRef]
64. Domínguez, J.; Aira, M.; Breinholt, J.W.; Stojanovic, M.; James, S.W.; Pérez-Losada, M. Underground evolution: New roots for the old tree of lumbricid earthworms. *Mol. Phylogenet. Evol.* **2015**, *83*, 7–19. [CrossRef]