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Phylogeny of the *Eisenia nordenskioldi* complex based on mitochondrial genomes

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ABSTRACT

Eisenia nordenskioldi is an earthworm widespread in Northern Asia and adjacent regions. It is known for its high morphological, karyotypic, and genetic variation, and contains two subspecies, the pigmented *E. n. nordenskioldi* and the unpigmented *E. n. pallida*. We obtained almost complete sequences of mitochondrial genomes (without the control region and parts of the flanking tRNAs) for six genetic lineages of *E. n. nordenskioldi*, two for *E. n. pallida*, as well as for three congeneric outgroups. These genomes had gene content and arrangement typical for Annelida. Nucleotide and amino acid diversity among *E. nordenskioldi* lineages was almost as high as between them and the outgroup species. *E. nordenskioldi* was split into two clades, one containing *E. n. nordenskioldi* lineages 6 and 9, and the other comprised the rest of the lineages. We could not resolve relationships of these two clades with *E. tracta*; most datasets used recovered this species as the sister group to *E. n. nordenskioldi* lineages 6 and 9, but not with high statistical support. According to mtDNA data, neither *E. n. nordenskioldi* nor *E. n. pallida* are monophyletic, and the same could be true for *E. nordenskioldi* as a whole, which suggest a revision of the systematics of *E. nordenskioldi* complex.

1. Introduction

Eisenia nordenskioldi (Eisen, 1879) is a polymorphic earthworm species widespread in Northern Asia [1,2]. Its distribution extends from tundra to forest-steppe and broad-leaved forests. In the major part of its distribution, *E. nordenskioldi* is commonly divided into two subspecies, the pigmented *E. n. nordenskioldi* (Eisen, 1879) and the unpigmented *E. n. pallida* Malevič, 1956 [2], although as many as nine are sometimes reported if one includes *species inquirendum* and *species incertae sedis* [3].

Mitochondrial genomes are widely used for phylogenetic studies [4,5]. They are relatively easy to sequence, and are thus a simple way to obtain a dataset of orthologous genes [6]. Mitochondrial genomes were widely studied in the earthworm family Megascolecidae, with 22 published mtDNAs [7–11]. Not much is known on mitochondrial genomes from other earthworm families. For example, Lumbricidae is the most important earthworm family in the temperate zone, containing many

widespread and invasive cosmopolitan species [12]. Only two mitochondrial genomes were sequenced in this family from *Lumbricus terrestris* Linnaeus, 1758 [13] and *Aporrectodea rosea* (Savigny, 1826) [14].

Earthworms are known for high nucleotide diversity characteristic for mtDNA sequences: many species studied to date were found to contain several genetic lineages diverged by 5–17% that are found in specimens with similar morphology, sometimes even within a single population [15–17]. While this high genetic diversity is useful for phylogeographic and population genetic analysis, mitochondrial phylogenies are often unresolved or unaccountable on higher levels, e.g. often splitting genera that are morphologically solid [16,18,19]. It is unclear if this is the result of limited information provided by the *cox1* barcode or of peculiar evolution of earthworm mtDNA. Information on complete mitochondrial genome sequences would thus help to clarify this issue.

Table 1
Earthworm taxa and genetic lineages studied in this work.

Taxon	Location	location on Fig. 1
E. n. nordenskioldi (Eisen, 1879)		
lineage 1	Russia, Buryatia Rep., Ukyr, N51.159 E104.133	5
lineage 2	Russia, Novosibirsk oblast, Novosibirsk, N54.835 E83.117	2
lineage 3	Russia, Khabarovsk Krai, Lesopil'noye, N46.707 E134.295	6
lineage 5	Russia, Omsk oblast, Omsk	1
lineage 6	Russia, Omsk oblast, Omsk	1
lineage 9	Russia, Buryatia Rep., Ukyr, N51.159 E104.133	5
E. n. pallida Malevič, 1956		
lineage 2	Russia, Khabarovsk Krai, Lesopil'noye, N46.707 E134.295	6
lineage 6	Russia, Altai Krai, Izlap, N52.913 E86.629	3
E. tracta Perel, 1985		
	Kazakhstan, East Kazakhstan oblast, near Semipalatinsk	4
E. balatonica (Pop, 1943)		
	Kazakhstan, East Kazakhstan oblast, near Semipalatinsk	4
E. spelaea (Rosa, 1901)		
	Hungary, Köszeg, Pogányok, N47.371 E16.501	7

In this study we obtained mitochondrial genomes for eight genetic lineages of *E. nordenskioldi*: six for *E. n. nordenskioldi* and two for *E. n. pallida*. We also sequenced mitochondrial genome of three congeneric outgroup species, *E. spelaea* (Rosa, 1901), *E. balatonica* (Pop, 1943), and *E. tracta* (Perel, 1985). We used the obtained data to construct several datasets in order to study the phylogeny of *E. nordenskioldi* and specifically to determine whether this species and two of its subspecies are monophyletic.

2. Materials and methods

2.1. Earthworm specimens

Live earthworm specimens were collected in 2017–2018; collection locations are provided in Table 1 and Fig. 1. A total of 10–15 specimens of each taxon/lineage were collected; several specimens were fixed and identified morphologically; afterwards, a single specimen with typical morphology was taken for RNA extraction. Species and lineage identification was performed via DNA barcoding by sequencing a fragment of the *cox1* gene as described earlier [20].

2.2. Sequencing and assembly of mitochondrial genomes

Total RNA was extracted by the Trizol method [21]; DNA, by a silica column kit (BioSilica, Novosibirsk, Russia). Poly-A RNA was purified using TruSeq Stranded mRNA Library Prep Kit (Illumina, USA). Sequencing was performed using NextSeq 400 Mid Output Kit (Illumina, USA) generating single-end reads.

Sequence reads were de novo assembled using Trinity v2.4.0 [22] with the default parameters. Mitochondrial sequences were identified by performing blastn, blastp, and blastx [23] searches against various earthworm mitochondrial genomes (*L. terrestris*, *A. rosea*, and those obtained in this study). Mitochondrial contigs were assembled using *L. terrestris* mitochondrial genome (Genbank Accession Number NC_001673) as the template. The assembled genomes included one to six gaps, 20 to 1150 bp long. Primers were designed based on the flanking regions, and PCR was performed using Herculase (Agilent Technologies, USA). PCR reactions had the following profile: 95 °C, 2 min; 35 cycles of 95 °C, 20 s; 58 °C, 20 s; 72 °C, 1 min; and the final step of 5 min at 72 °C. The resulting products were used to check the validity of genome assembly and to close the gaps by Sanger sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Sequencing was performed in two directions with the primers used for PCR, as well as additional nested primers in case of long PCR products.

The control region of earthworm mtDNA is notoriously hard to amplify, and it is of little use for phylogenetic studies [10]. Thus in this study we limited ourselves to the partial genomes containing all 13 expected protein-coding, two rRNA genes, and 20 of the 22 tRNA genes, without the control regions and portions of the flanking tRNA genes, tRNA-Arg and tRNA-His.

The obtained mtDNA sequences were annotated manually in BioEdit v. 7.0.5.3 [24] using the published mitochondrial genomes as the template; they were deposited in GenBank as MK618509-MK618513 and MK642867-MK642872.

2.3. Phylogenetic analysis

Although mtDNA is inherited as a single molecule, phylogenetic inferences based on different parts of the genome may differ. In order to verify the obtained phylogenies, we constructed several datasets extracted from the mitochondrial genomes:

a) complete genome alignment (mtDNA, 13989 bp);
b) coding sequences of the 13 protein-coding genes (cds, 11084 bp);
c) amino acid sequences of the 13 protein-coding genes (protein, 3665 aa);
d) amino acid sequences of eleven protein-coding genes, without the fast-evolving *atp8* and *nd2* (3075 aa);
e) ribosomal 12 S and 16 S genes (rRNA, 1795 bp);
f) concatenated cds + rRNA dataset (12879 bp).

All obtained alignments had gaps and ambiguously aligned regions, and thus had to be processed by gblocks, a program that removes poorly aligned or insufficiently conserved regions [25]. The dataset lengths listed above refer to the processed alignments. GTR + I + G was chosen as the most appropriate substitution model for all nucleotide datasets by MrModeltest based on the Akaike information criterion [26]. Bayesian phylogeny was inferred using MrBayes v.3.2.6 [27]. For each dataset, 10, 000, 000 generations were performed; two independent analyses were run from different random starting trees; 25% of generations were discarded as burn-in.

Maximum Likelihood trees were built using RaxML v.8.0 [28]. Substitution models were the same as for the Bayesian analysis; 1000 bootstrap replicates were performed for each dataset.

3. Results

3.1. Mitochondrial genomes

The size of the obtained partial mitochondrial genomes ranged from 14473 bp in *E. n. pallida* lineage 6 to 14658 bp in *E. spelaea*; longer sequences contained partial assembled sequences of the control region.

All newly obtained genomes had gene content and arrangement typical for earthworms [10,13]. All genes were encoded on a single DNA strand. The only detected variation was a deletion about 55 bp long in the region between the *cytb* and tRNA-Trp genes compared to the *L. terrestris* genome (NC_001673). AT-content of *E. nordenskioldi* lineages ranged within relatively narrow limits (62.67–64.30%), and that of the other species was also close: 63.37% for *E. balatonica*, 65.69% for *E. tracta*, and 59.88% for *E. spelaea* (Table S1).

Different parts of the genome had almost no length variation (Table S1), but different substitutions rate (Fig. 2). *Cox1* was the most slowly evolving gene, while the short *atp8* gene had the highest substitution rate: p-distances of 16.7% and 28.5% on nucleotide level, respectively, and 1.2% and 33.9% for the amino acid sequence. Ribosomal RNA was also characterized by high substitution rate: p-distance was 21.2% for the *E. nordenskioldi* lineages, 22.2% for all *Eisenia* species, and 23.2% for the studied Lumbricidae sample, respectively.
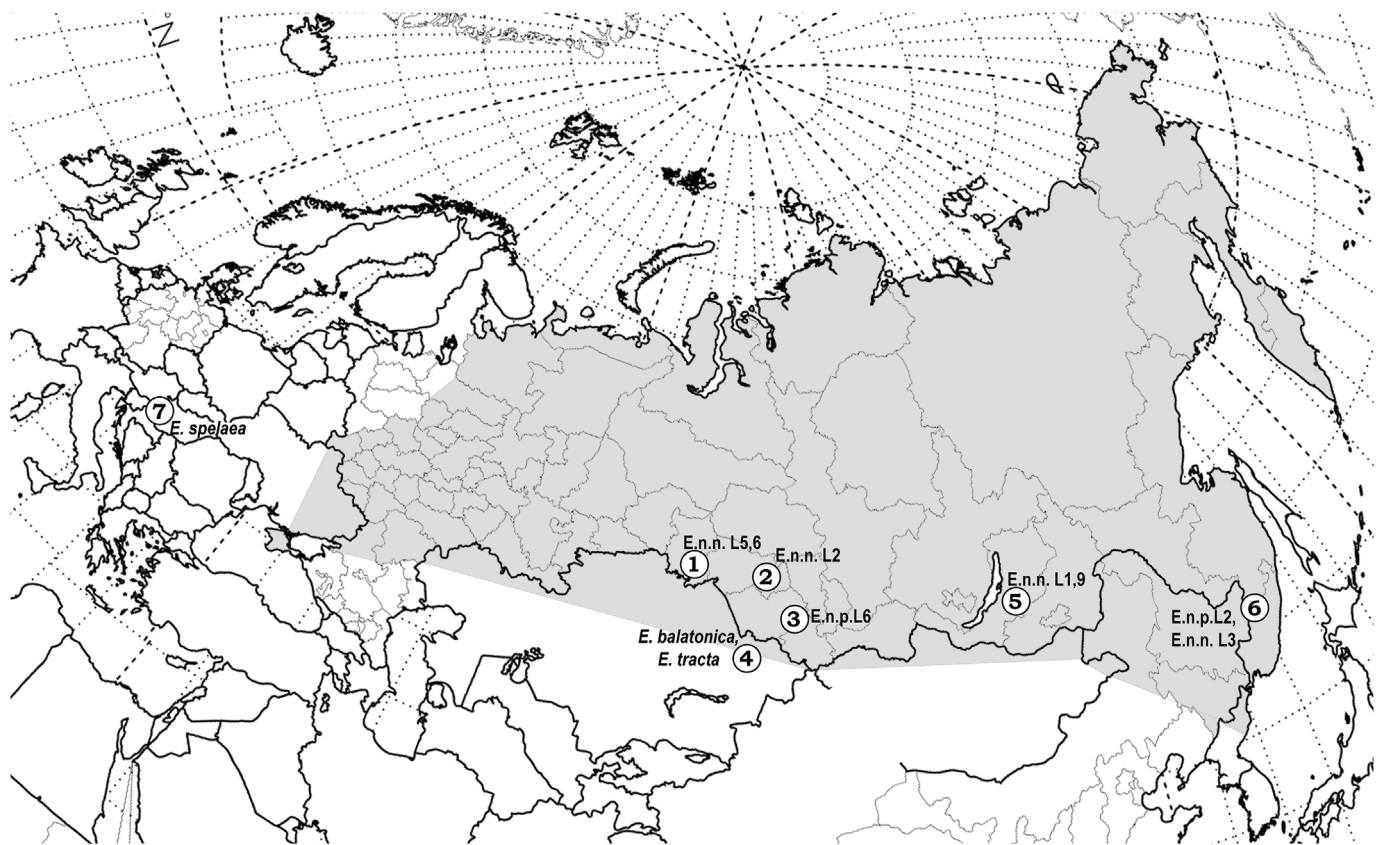
Fig. 1. Sampling points of *E. nordenskioldi* lineages and outgroups. Location numbers refer to Table 1.

3.2. Phylogeny of E. nordenskioldi

Phylogenetic analysis demonstrated that different dataset yielded somewhat different tree topologies, and that Bayesian analysis gave much higher confidence levels for most branches than Maximum Likelihood. The genus *Eisenia* was monophyletic in all cases except for the rRNA dataset, which grouped *Eisenia spelaea* together with *A. rosea*. *E. balatonica* was always the sister group of the clade containing *E. nordenskioldi* lineages and *E. tracta* (Fig. 3). The position of the latter differed for different datasets: cds, cds + rRNA, and complete mtDNA datasets recovered *E. tracta* as the sister group to *E. n. nordenskioldi* lineages 6 and 9; the protein dataset, as the sister group to all *E. nordenskioldi* lineages; and the rRNA dataset could not resolve its position relative to *E. nordenskioldi*. Since the results for the protein dataset contradicted those for nucleotide datasets, we performed additional analysis using protein data without the most diverse proteins, ATP8 and ND2. The resulting trees grouped together *E. tracta* and *E. n. nordenskioldi* lineages 6 and 9. As seen of Figs. 3 and 4, bootstrap supports for the branching of these groups was low. We should conclude that mtDNA data cannot unambiguously resolve the trichotomy of the two *E. nordenskioldi* groups and *E. tracta*.

E. nordenskioldi invariably split into two clades with high statistical support, one containing *E. n. nordenskioldi* lineages 6 and 9, and the other, the rest of lineages. The latter group included lineages belonging both to *E. n. nordenskioldi* and *E. n. pallida*, and demonstrated no consistency in tree topology (Figs. 3 and 4). *Eisenia n. nordenskioldi* lineage 1 plus *E. n. pallida* lineage 6 was the only group present on all trees with ultimate bootstrap support and Bayesian probabilities. *Eisenia n. nordenskioldi* lineages 2 and 5 were recovered on all trees except for the rRNA dataset, but with much lower confidence. The third clade comprising *E. n nordenskioldi* lineage 3 and *E. n. pallida* lineage 2 was detected for the protein and cds datasets.

4. Discussion

4.1. Variation among earthworm mitochondrial genomes

The available data suggests that mitochondrial genomes of many Annelida, including Clitellata, are relatively stable in terms of gene arrangement [29–32]. This might be explained by the fact that the genome is apparently transcribed as a single transcript, so any inversions are immediately deleterious. In this study we found no diversity in gene order and arrangement among the studied genomes.

Nucleotide composition of mitochondrial genomes was also relatively stable: AT-content of *E. nordenskioldi* lineages was 62.67–64.30%, and of Lumbricidae (including the published *L. terrestris* and *A. rosea* genomes), 59.88–65.69% (Table S1). According to Zhang et al. [10], other earthworm families have higher AT-content: Megascolecidae, 62.6–67.6%, and Moniligastridae represented by the single sequenced *Drawida japonica* genome had AT-content as high as 69.7%; however, these differences might be partly due to the control region sequence.

However, earthworm mitochondrial genomes are notorious for high nucleotide diversity, with the majority studied species consisting of several genetic lineages, with up to 15–20% intraspecific nucleotide diversity based on the barcoding fragment of the *cox1* gene [14,16]. Our results demonstrate that *cox1* is the most slowly evolving mitochondrial gene, while the *atp8* and *nd2* genes have much higher nucleotide and amino acid diversity (Fig. 2). These results accord with those reported by Zhang et al. [10] for the *Amynthas* genus, who found pairwise p-distances of ca. 30%, 29%, and 17% for the nucleotide sequences of *atp8*, *nd2*, and *cox1* genes, respectively.

Pairwise distances between individual genes (Fig. 2) indicate that the diversity among *E. nordenskioldi* lineages is only slightly lower than between them and other species and genera. This is true for all studied lineages except for *E. n. nordenskioldi* lineages 6 and 9, could be seen on Fig. 2 as the three bottom outlier dots. The same was observed for
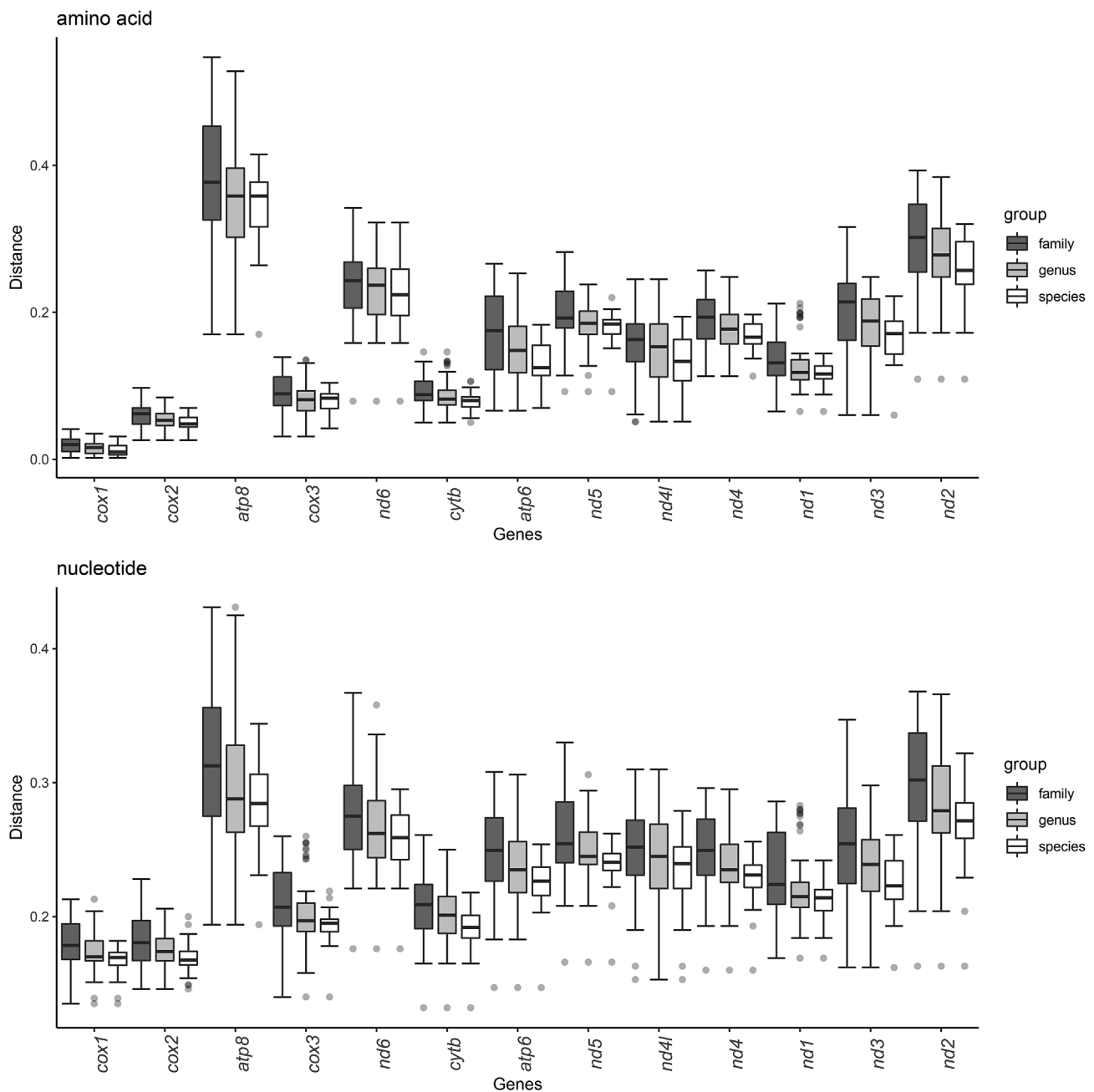
Fig. 2. Genetic p-distances for amino acid and nucleotide sequences for individual genes. White box plots, *E. nordenskioldi* lineages; light grey, *E. nordenskioldi* plus *E. tracta*, *E. balatonica*, and *E. spelaea*; dark grey, the whole *Eisenia* set plus *L. terrestris* and *A. rosea*. The band inside the box represents the median; upper and lower hinges correspond to the 25th and 75th percentiles; upper and lower whiskers, to 1.5 IQR from the upper and lower hinges; circles, to outliers.

ribosomal RNA genes. The average p-distance among *E. nordenskioldi* lineages was 21.2% for the 16 S rRNA gene. This is high compared to other studied earthworm species; e.g., 16 S rRNA of *Metaphire* species (Megascolecidae) were reported to differ by 5.5–14.2% [33].

Although pairwise genetic differences do not necessarily reflect the rate of evolution, the divergence times of *E. nordenskioldi* genetic lineages are probably high and happened not long after the divergence of species within the genus *Eisenia*.

4.2. Phylogeny and systematics of E. nordenskioldi complex

Previous studies indicated that the two subspecies, *E. n. nordenskioldi* and *E. n. pallida*, might not be monophyletic [34], and that genetic distances among *E. nordenskioldi* lineages are high [35]. These

inferences, however, were inconclusive because of the absence of appropriate outgroups and, in the former study, few genetic markers used. The data on mitochondrial and nuclear genes obtained by Shekhovtsov et al. [34] suggested that neither of the studied subspecies were monophyletic.

This study suggests that *E. nordenskioldi* may be polyphyletic, since there was no consistence in branching patterns among two groups of *E. nordenskioldi* lineages and *E. tracta*, and none of the groupings had high statistical support. This, and the high level of genetic distances among *E. nordenskioldi* lineages suggest that at least some of these lineages might deserve the species status. The third reason is that certain closely related species, i.e., *E. atlavinyteae*, *E. sibirica*, and *E. nana*, in practice turn out to belong to one of *E. nordenskioldi* lineages when the identification is verified by cox1 sequencing (our observations). These species
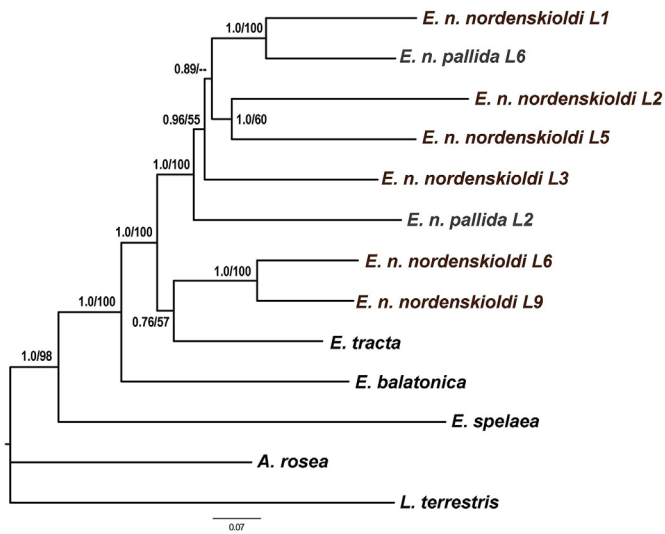
Fig. 3. Bayesian phylogenetic tree for the cds + rRNA dataset. Numbers near branches indicate Bayesian posterior probabilities/ML bootstrap values; brown lineage names are for pigmented lineages; grey, for unpigmented ones. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

have only slight differences in diagnosis from E. nordenskioldi, so they may well represent just morphological forms of the latter, but that needs to be proven with analysis of type specimens. The systematics of the *E. nordenskioldi* complex is thus obviously in need of revision.

It is hard to tell which genetic lineage of *E. nordenskioldi* is *bona fide E. nordenskioldi*. Eisen [36] described this species from populations from several locations from the Vaigach island (N 69°45') and along the Yenisei river (from N 71°55' to 60°50'). Only *E. n. nordenskioldi* lineage 9 was reported from Vaigach [37,38], so the clade containing *E. n. nordenskioldi* lineages 6 and 9 might be the real *E. nordenskioldi*. On the other hand, the other locations along the Yenisei are spread along 1200 km, so the specimens collected by Nordenskiöld and studied by Eisen [36] might well represent different *E. nordenskioldi* lineages.

It is even harder to determine what are the "real" *E. atlavinyteae*, *E. sibirica*, and *E. nana*, because they are found in the areas where multiple genetic lineages of *E. nordenskioldi* are known to live in sympatry, sometimes as many as four lineages in one soil sample [20]. Final systematic decisions would require sequencing analysis of type specimens, as was performed for *L. herculeus* by James et al. [39].

4.3. Pigmentation in E. nordenskioldi

Eisenia nordenskioldi was initially described based on pigmented specimens [36]. Malevič [40] described unpigmented (pale grey) with only a slight shade of reddish pigmentation on the anterior segments as *forma pallida*. Later on, Perel [41] isolated those forms into the subspecies *E. n. pallida* Malevič 1956, and thus divided the species into two
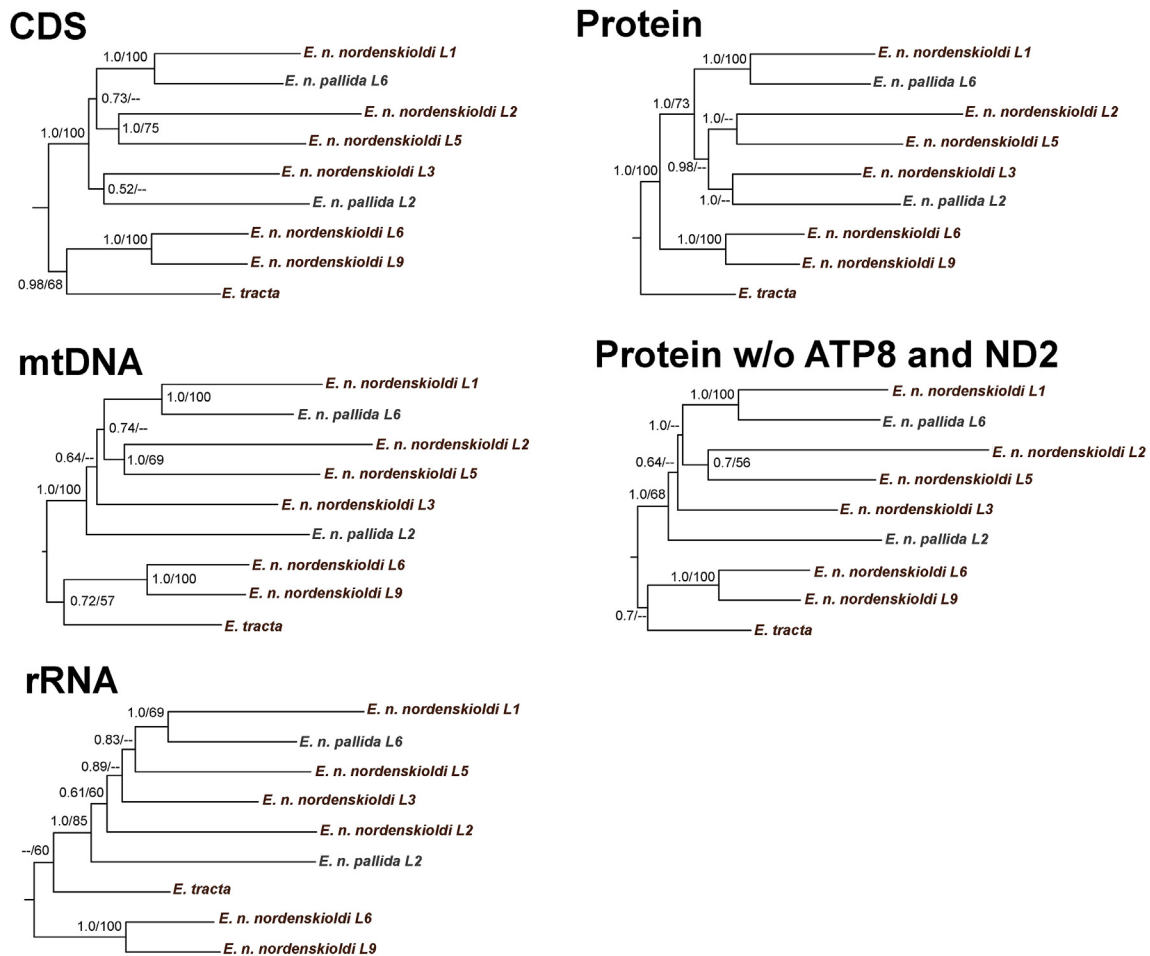

Fig. 4. Bayesian phylogenetic trees for different dataset showing only the relationships between *E. nordenskioldi* genetic lineages and *E. tracta*. Numbers near branches indicate Bayesian posterior probabilities/ML bootstrap values; brown lineage names are for pigmented lineages; grey, for unpigmented ones. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

subspecies based on the presence or absence of pigmentation. This seems to be an important morphological character for *E. nordenskioldi*, since these forms have different lifestyles, endogeic in unpigmented worms vs. epigeic or anecic in pigmented ones [42].

The division of the species into the pigmented and unpigmented subspecies implicitly suggests that both are reciprocally monophyletic. Simultaneously, the conventional viewpoint states that *E. n. pallida* is diploid, and *E. n. nordenskioldi* is polyploid, with reported ploidy level of 4n, 6n, and 8n [42,43], which implies that the lack of pigmentation is the ancestral state for this complex. However, it should be noted that chromosome studies were performed only on a few populations, especially for *E. n. pallida*.

Our results suggest that neither of the two subspecies is monophyletic, that *E. n. nordenskioldi* is paraphyletic relative to *E. n. pallida* for the studied dataset, and that the ancestor of the complex more likely was pigmented. However, as mentioned above, karyotypic diversity could actually be much higher, given the high genetic diversity that we know of now. We should also note that our sample contains only two of the six known *E. n. pallida* lineages.

Our results also suggest that the loss of pigmentation occurred independently at least twice. Based on the geographic distribution of genetic lineages [34,37,44], *E. n. pallida* lineage 2 is probably the *bona fide E. n. pallida*, which was described from the Far East, while the lineage from West Siberia might be redescribed as a new taxon.

5. Conclusions

In this study we added a significant amount of data on mitochondrial genomes for the earthworm family Lumbricidae. The obtained data suggests that neither of the two subspecies of *E. nordenskioldi* is monophyletic, with *E. n. nordenskioldi* being paraphyletic with respect to *E. n. pallida*. *Eisenia nordenskioldi* as a whole is also probably not monophyletic. These results suggest that a revision of the species is necessary, which might possibly include the isolation of *E. n. nordenskioldi* lineages 6 and 9 as *E. n. nordenskioldi*, and redescription of the branch comprising *E. n. pallida* lineages and the rest of *E. n. nordenskioldi* as a set of new taxa.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejsobi.2019.103137.

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