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# MORPHOLOGICAL AND GENETIC ANALYSIS OF *DENDRODRILUS RUBIDUS* (*BIMASTOS RUBIDUS*) (*OLIGOCHAETA*, *LUMBRICIDAE*) IN RUSSIA AND BELARUS

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**Abstract.** *Background.* The systematic position of the earthworm *Dendrodrilus rubidus* (*Bimastos rubidus*) has been changed many times due to differences in its anatomy and external morphology, which were revealed in individuals that at first glance belonged to the same species. Deviations from existing diagnoses also made taxonomic determination difficult. The research goal is to conduct comparative morphometric and molecular genetic analyses of *D. rubidus* (*B. rubidus*) individuals from different regions of Russia and Belarus to identify accurate diagnostic features. *Materials and methods.* For the study, we collected four samples of adult individuals of *D. rubidus* (*B. rubidus*), depending on geographical location. The worms were collected in the territories of Russia (European part, Western Siberia, North-Western Caucasus) and Belarus. Morphometric analysis was carried out according to the main qualitative and quantitative anatomical and morphological features. The variability of the mitochondrial cytochrome oxidase I (*cox 1*) gene used in molecular taxonomy was evaluated using molecular-genetic analysis. Further the data obtained from the two analyses were compared. *Results and conclusions.* Previously, it was believed that only two subspecies of *D. rubidus* (*tenuis* and *subrubicundus*) were present in the studied territories, which are currently considered to be species forms. During the study, we found three forms of this species: *tenuis*, *rubidus* and *subrubicundus*. The revealed anatomical and morphological differences make it possible to determine the species forms of adult individuals of *D. rubidus*, taking into account possible deviations from generally accepted diagnoses. There were no significant molecular-genetic differences between the different species forms.

**Keywords:** *Dendrodrilus rubidus* (*Bimastos rubidus*), morphometric analysis, cytochrome oxidase I (*cox 1*), subspecies, species form

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# МОРФОЛОГИЧЕСКИЙ И ГЕНЕТИЧЕСКИЙ АНАЛИЗ *DENDRODRILUS RUBIDUS* (*BIMASTOS RUBIDUS*) (*OLIGOCHAETA*, *LUMBRICIDAE*) НА ТЕРРИТОРИЯХ РОССИИ И БЕЛАРУСИ

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**Аннотация.** Актуальность и цели. Систематическое положение дождевого червя *Dendrodrilus rubidus* (*Bimastos rubidus*) многократно подвергалось изменениям из-за различий в анатомии и внешней морфологии, которые выявлялись у особей, на первый взгляд относившихся к одному виду. Отклонения от имеющихся диагнозов также затрудняли таксономическое определение. Цель данной работы – провести сравнительный морфометрический и молекулярно-генетический анализы особей *D. rubidus* (*B. rubidus*) из разных регионов России и Беларуси для выявления точных диагностических признаков. **Материалы и методы.** Для исследования нами составлены четыре выборки взрослых особей *D. rubidus* (*B. rubidus*) на основании географической принадлежности. Черви были собраны на территориях России (Европейская часть, Западная Сибирь, Северо-Западный Кавказ) и Беларуси. Морфометрический анализ проводили по основным качественным и количественным анатомо-морфологическим признакам. С помощью молекулярно-генетического анализа оценивали изменчивость митохондриального гена цитохромоксидазы I (*cox 1*), использующегося в молекулярной таксономии. Далее проводилось сопоставление полученных данных по двум анализам. **Результаты и выводы.** Ранее считалось, что на исследуемых территориях присутствуют только два подвида *D. rubidus* (*tenuis* и *subrubicundus*), которые в настоящее время принято считать видовыми формами. В ходе исследования нами были обнаружены три формы данного вида: *tenuis*, *rubidus* и *subrubicundus*. Выявленные анатомо-морфологические различия позволяют определять видовые формы взрослых особей *D. rubidus* с учетом возможных отклонений от общепринятых диагнозов. Достоверных молекулярно-генетических различий между разными формами вида обнаружено не было.

**Ключевые слова:** *Dendrodrilus rubidus* (*Bimastos rubidus*), морфометрический анализ, цитохромоксидаза I (*cox 1*), подвид, форма вида

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## Introduction

Earthworms *Dendrodrilus rubidus* (*Bimastos rubidus*) are typical cosmopolitans. According to existing living form classifications, they are referred to as litter dwellers [1], or *epigeic* worms [2]. They inhabit predominantly the forest litter in both deciduous and coniferous forests; they are one of the main inhabitants of the forest deadwood (sometimes being the only representatives of earthworms there, they are even known to be called a trivial name of "tree worm"), and also live in the upper layers of the humus horizon of soil and humus heaps [3–6]. A number of experimental studies showed that *D. rubidus* can survive in habitats contaminated with heavy metals [7, 8] and their egg cocoons are extremely resistant to cold weather [9, 10] which, to some extent, explains the ecological plasticity of the species.

The systematic position of *D. rubidus* has been changed many times. One of the reasons for this was the difference in anatomy and partly external morphology revealed in worms that, at first glance, belonged to the same species. For example, tubercula pubertatis could be absent or barely visible, whereas spermathecae were either represented by two pairs, or absent at all [11–13]. In the first generic systems of the Lumbricidae family, the species was named *Enterion rubidum* (Savigny, 1826), then *Bimastus constrictus* (Rosa, 1845);

once the above differences had been identified, the single species was divided into two different species, *Bimastus tenuis* (Eisen, 1874) and *Dendrobaena subrubicunda* (Eisen, 1874). Later, as the systematic position of the Lumbricidae family was being revised, P. Omodeo identified a new subgenus (later a monotypic genus) of *Dendrodrilus* in 1956 that included the type species *D. rubidus* represented by two forms, *D. r. tenuis* and *D. r. subrubicundus* [14] classified as subspecies by some sources [13]. By the end of the last century, researchers started dividing the species into as many as the following four forms [11, 13, 15, 16]:

*D. r. tenuis*: no spermathecae or tubercula pubertatis; the clitellum occupies segments 26–31 (according to some studies, it occupies segments 25–26–27 to 31–32) [11, 13];

*D. r. rubidus*: spermathecae (segments 9–10) intact, but usually empty and rudimentary, sometimes they may be absent [16]; tubercula pubertatis in segments 29–30 are shaped as fuzzy narrow bands separated by a groove between segments 29/30; the clitellum occupies segments 25–26 to 31 as well as 1/n of segment 32;

*D. r. subrubicundus*: two pairs of well-developed spermathecae filled with seminal fluid in segments 9–10, tubercula pubertatis clearly visible as wide rectangular bands in segments 28–30; the clitellum occupies segments 25–26 to 31–32;

*D. r. norvegicus*: two pairs of well-developed and visible spermathecae in segments 9–10, tubercula pubertatis completely absent or represented by a narrow band in segments 29–30; the clitellum occupies segments 26 to 32.

It is also noteworthy that the forms coming from different regions have a pronounced difference in chromosome sets. For example, a study in the UK found 34 chromosomes in *D. r. rubidus*, 48 in *D. r. tenuis*, and 68 in *D. r. subrubicundus*, while identifying 34 chromosomes in *D. r. subrubicundus* from Italy, as well as 102 chromosomes in *D. r. rubidus* from Greenland and *D. r. tenuis* from the Eastern Alps [11]. S. V. Mezhzherin et al. found earthworm races with 34, 68, 85 and 102 chromosomes in Ukraine, but the study does not specify to which form the studied samples belong to [17]. The *D. r. norvegicus* form has not been studied for chromosomes; however, electrophoretic analysis of isozymes showed that it differs from *D. r. rubidus* in four enzymes. It also was proved experimentally that *D. r. norvegicus* may be remarkably adaptable to harsh cold weather conditions – about 60 % of cocoons produced by the worms developed very slowly, and juvenile species hatched after 110 days, which was not observed in any earthworm species. It has been suggested that *D. r. norvegicus* and other forms are at an early stage of species divergence [15]. As for the other three forms, recent studies in North America have shown that there are no differences in individuals of different forms at the molecular genetics level. Therefore, *D. r. tenuis*, *D. r. rubidus* and *D. r. subrubicun-*

*us* are not different species or subspecies but the same species. Moreover, phylogenetic analysis has shown that the monotypic genus of *Dendrodrilus* is included in *Bimastos*; therefore, the modern literature now classifies all the mentioned forms as *Bimastos rubidus* (Savigny, 1826) [16].

According to various researchers, species is ubiquitous in Russia and Belarus and represented by two forms, *D. r. tenuis* and *D. r. subrubicundus* [3, 12, 18–20]. However, when determining these forms of earthworms, the above diagnostics cannot be applicable in some cases. The objective of this work is carrying out a comparative morphometric study of *Dendrodrilus rubidus* (*Bimastos rubidus*) individuals collected in various regions of Russia and Belarus to identify accurate diagnostic features in different forms. We also used molecular genetic analysis to search for potential differences among the forms.

### Materials and methods

Four samples of adult *Dendrodrilus rubidus* (*Bimastos rubidus*) individuals were used as material for this study. The worms were taken from quantitative records and fauna collections from soil and deadwood of 2018–2020 in the following regions: European part of Russia (Moscow Oblast, Bryansk Oblast and Sverdlovsk Oblast), Western Siberia (Novosibirsk Oblast), North-Western Caucasus (Karachay-Cherkess Republic) and the Republic of Belarus (Vitebsk Region, Gomel Region, Mogilev Region and Minsk Region) (Fig. 1, Table 1).

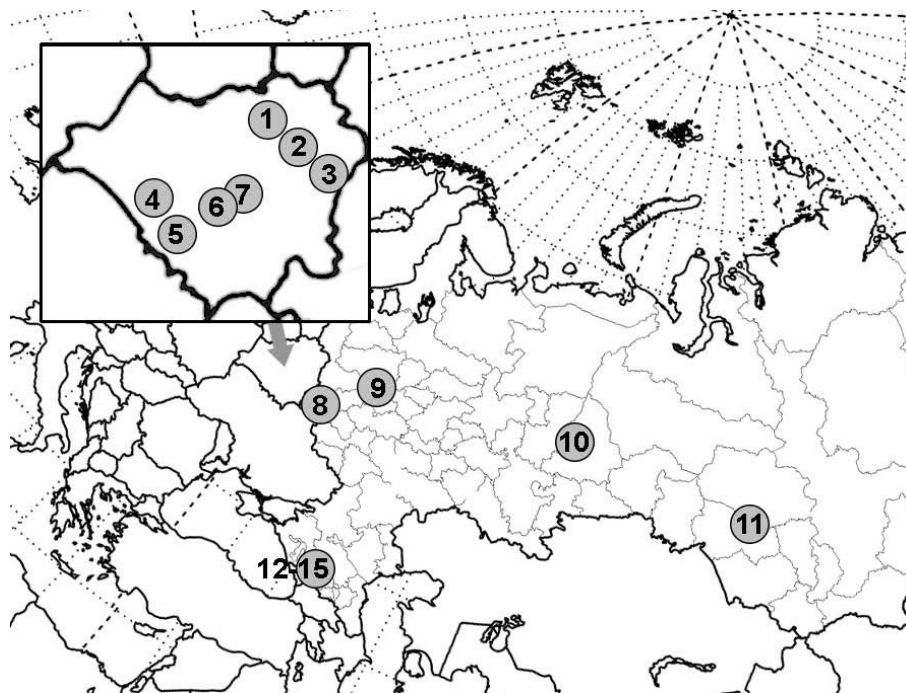


Fig. 1. Areas of collection for *D. rubidus* specimens (Numerical designations are given in Table 1)



Table 1

Areas of sampling for *D. rubidus* specimens. Population numbers correspond to the dots in Figure 1

Area of collection	<i>D. rubidus</i> form
1. Republic of Belarus, Vitebsk Region, Lepelsky district, near Domzheritsy village, N54.7515 E28.3041	<i>D. r. subrubicundus</i>
2. Republic of Belarus, Vitebsk Region, Chashniksky district, near Novolukomlskoe lake, N54.6495 E29.1085	<i>D. r. tenuis</i> , <i>D. r. subrubicundus</i>
3. Republic of Belarus, Vitebsk Region, Sennensky district, Pogrebyonka village, N54.83796 E30.3989	<i>D. r. subrubicundus</i>
4. Republic of Belarus, Brest Region, Stolinsky district, Tereblichy village, N51.8572 E27.4006	<i>D. r. tenuis</i>
5. Republic of Belarus, Gomel Region, Zhitkovichsky district, near Khlupin village, N52.0703 E28.1555	<i>D. r. tenuis</i> , <i>D. r. subrubicundus</i>
6. Republic of Belarus, Minsk Region, Starodorozhsky district, near Solon village, N53.0425 E28.1514	<i>D. r. tenuis</i>
7. Republic of Belarus, Mogilev Region, Osipovichsky district, floodplain of the Svisloch river, N53.4125 E28.4869	<i>D. r. subrubicundus</i>
8. Russia, Bryansk Oblast, Bryansky Les Reserve, N52.5488 E34.0497	<i>D. r. tenuis</i> , <i>D. r. rubidus</i>
9. Russia, Moscow, Valuevo settlement, N55.5777 E37.3163	<i>D. r. rubidus</i>
10. Russia, Sverdlovsk Oblast, Visimsky Nature Reserve, N57.3733 E59.7737	<i>D. r. subrubicundus</i>
11. Russia, Novosibirsk Oblast, Baryshevo settlement, N55.5777 E37.3163	<i>D. r. tenuis</i> , <i>D. r. subrubicundus</i> , <i>D. r. rubidus</i>
12. Russia, Karachay-Cherkess Republic, Zelenchuk river, N43.5284 E41.2182	<i>D. r. tenuis</i> , <i>D. r. rubidus</i>
13. Russia, Karachay-Cherkess Republic, Khusa-Kardonikskaya stanitsa, N43.7660 E41.5434	--/--
14. Russia, Karachay-Cherkess Republic, Kefal river, Leso-Kefal farm, N43.7558 E41.3981	--/--
15. Russia, Karachay-Cherkess Republic, Teberda National Biosphere Reserve, N43.5824 E41.3777	--/--

**Morphometric analysis.** For comparative analysis of anatomical and morphological features of the studied worms, we examined the following quantitative features: number of segments (NS), body length (L, mm), body width at the widest point, except the clitellum (W, mm), clitellum length (CL, mm), clitellum width (CW, mm), and weight of the fixed worm (Wt, g). The dimensions were measured using a stereomicroscope with rulers and graph paper and the margin of error of  $\pm 1$  mm for body length and  $\pm 0.1$  mm for other features; the weight was measured using analytical scales with a capacity of 50 g and the margin of error of  $\pm 0.001$  g. Before weighing, excess fixing solution was removed from the worms with filter paper; all worms were weighed with filled intestines. A number of qualitative features were studied as well like the position of the clitellum, the position of tubercula pubertatis, pigmentation, number of seminal vesicles, number of spermathecae, the position of the first dorsal pore, and visibility of tumescences (within/beyond segment 15). Then quantitative features of the samples were compared using the Mann–Whitney U test ( $p < 0.01$ ).

**Molecular-genetic analysis.** The DNA of earthworms was isolated by tissue lysis in guanidinium isothiocyanate followed by purification on BioSilica columns (Russia) [21]. A fragment of the

mitochondrial cytochrome c oxidase 1 (*cox1*) gene was amplified using universal primers HCO2198 (5'-TAAAC-TTCAG-GGTGA-CCAAA-AAATC-A-3') and LCO1490m (5'-TACTC-AACAA-ATCACAAAGA-TATTG-G-3') [22]. DNA sequencing was performed at the Interinstitutional Sequencing Center SB RAS using BigDye 3.1 (Applied Biosystems, USA). The sample also included 22 unique *D. rubidus* sequences from GenBank (KT705452, HQ983623, MH410150, KM612222, HM887558, MG976101, KX400643, FJ374776, KJ772496, FJ214209, JN869909–JN869912, JQ909058, JQ909068, JQ909070, JQ909071, JQ909074, JQ909085, JX531568, MK837022). Phylogenetic trees were built using the Maximum Likelihood algorithm in RAXML v.8.2.12 [23]; the GTRCAT model of nucleotide substitutions was used to build the trees. 1,000 bootstrap replicas were made. Phylogenetic trees were also developed using Bayesian analysis in MrBayes v.3.4 [24]. 20 million iterations were performed; of those, 25 % were discarded as burn-in.

## Results

**Morphometric analysis.** The study revealed different variations of both qualitative and quantitative features (Tables 2, 3).

Table 2

 Variability of qualitative features of *Dendrodrilus rubidus*

Region	European part of the Russian Federation	Western Siberia	North-Western Caucasus (Russia)	Republic of Belarus
<b>Number of individuals</b>	11	12	15	16
<b>Pigmentation</b>	Purplish, head end is darker (all individuals)	Chestnut brown, head end is darker (all individuals)	Purplish, head end is darker (10 individuals) Purplish, almost evenly coloured (5 individuals)	Purplish, head end is darker (all individuals)
<b>First pore</b>	5/6 – all individuals	5/6 – all individuals	5/6 – all individuals	5/6 – 14 individuals 4/5 – 2 individuals
<b>Position of clitellum</b>	25–30 – 1 individual 25–31 – 4 individuals 25–31/32* – 1 individual 25/26–32** – 1 individual 26–31 – 3 individuals 27–32 – 1 individual	25/26–31 – 2 individuals 26–31 – 8 individuals 26–31/32 – 2 individuals	25–31 – 1 individual 25/26–31 – 2 individuals 26–31 – 11 individuals 26–32 – 1 individual	25–31 – 1 individual 26–31 – 15 individuals
<b>Tubercula pubertatis</b>	Not visible – 7 individuals Barely visible (28–30) – 2 individuals Barely visible (29–30) – 1 individual Visible (29–30) – 1 individual	Not visible – 5 individuals Barely visible (29–30) – 6 individuals Visible (29–30) – 1 individual	Not visible – 11 individuals Barely visible (28–30) – 1 individual Barely visible (29–30) – 2 individuals Visible (28–30) – 1 individual	Not visible – 8 individuals Barely visible (29–30) – 5 individuals Visible (29–30) – 3 individuals
<b>Number of spermathecae</b>	Absent – 10 individuals 2 pairs – 1 individual	Absent – 9 individuals 2 pairs – 3 individuals	Absent – all individuals	Absent – 13 individuals 2 pairs – 3 individuals
<b>Number of seminal vesicles</b>	2 pairs – 10 individuals 3 pairs – 1 individual	2 pairs – 9 individuals 3 pairs – 3 individuals	2 pairs – all individuals	2 pairs – 13 individuals 3 pairs – 3 individuals

\* – the clitellum partially occupies segment 32.

\*\* – the clitellum partially occupies segment 25.

Table 3

 Variability of quantitative features of *Dendrodrilus rubidus* ( $X \pm SE$ )

Region	European part of the Russian Federation	Western Siberia	North-Western Caucasus (Russia)	Republic of Belarus
<b>Number of individuals</b>	11	12	15	16
<b>Number of segments</b>	93 ± 5	93 ± 3	87 ± 4	94 ± 3
<b>Body length, mm</b>	27 ± 2	25 ± 1*	25 ± 1*	30 ± 1*
<b>Body width, mm</b>	2.4 ± 0.2	2.3 ± 0.1	2.6 ± 0.1	2.6 ± 0.1
<b>Clitellum length, mm</b>	2.4 ± 0.1	2.5 ± 0.1	2.4 ± 0.1	2.3 ± 0.1
<b>Clitellum width, mm</b>	2.4 ± 0.1	2.3 ± 0.1*	2.6 ± 0.1	2.8 ± 0.1*
<b>Weight, g</b>	0.08 ± 0.01	0.05 ± 0.01*	0.07 ± 0.01	0.08 ± 0.01*

\* – statistically significant differences between the samples,  $p < 0.01$ .

### Qualitative features

*Pigmentation.* The worms in samples from the European part of Russia, the North-Western Caucasus and the Republic of Belarus had the bright purplish pigmentation, whereas the pigmentation

of worms collected in the Western Siberia was chestnut brown (Fig. 2). In all worm samples, the head end was pigmented more intensely than the tail end, except for a few individuals from the Caucasus that had the purplish pigmentation almost evenly distributed along the body (Fig. 3).



Fig. 2. Pigmentation variations in *D. rubidus* (*B. rubidus*). At the top is an individual collected in the Western Siberia (chestnut brown pigmentation), whereas the one at the bottom is a worm collected in the North-Western Caucasus (purplish pigmentation)



Fig. 3. Pigmentation variations in *D. rubidus* (*B. rubidus*). Worms from the sample collected in the North-Western Caucasus: almost evenly distributed purplish pigmentation at the top, weakened pigmentation at the bottom

*Position of the first dorsal pore.* In all the studied individuals, the first dorsal pore is located in the intersegmental groove between segments 5 and 6, except for two individuals found in the Gomel Region (Belarus), whose first pore was located between segments 4 and 5.

*Tumescences around male genital openings.* Visibility of tumescences around male genital openings is similar for all worms of the studied regions: they do not protrude beyond segment 15. It turned out to be the only consistent qualitative feature.

*Spermathecae.* Analysis of crucial features to determine the form of the species gave the following results. Two variants of internal structure were clearly identified in the reproductive system, that is, two pairs of well-developed spermathecae being present or spermathecae being completely absent

(Fig. 4); same goes with three pairs and two pairs of seminal vesicles, respectively. More diverse variations were observed for the position of the clitellum and tubercula pubertatis (Table 2): the greatest diversity of the clitellum position was noted in the sample from the European part of Russia (6 variants), and the smallest diversity was found in the sample from Belarus (2 variants). When analysing tubercula pubertatis, their visibility was assessed as not visible, barely visible, and visible, as well as their position. In the samples from the European part of Russia and the North-Western Caucasus, four variants of tubercula pubertatis structure were identified, whereas the samples from Western Siberia and Belarus showed three such variants (Table 2). There are five different variants in total.



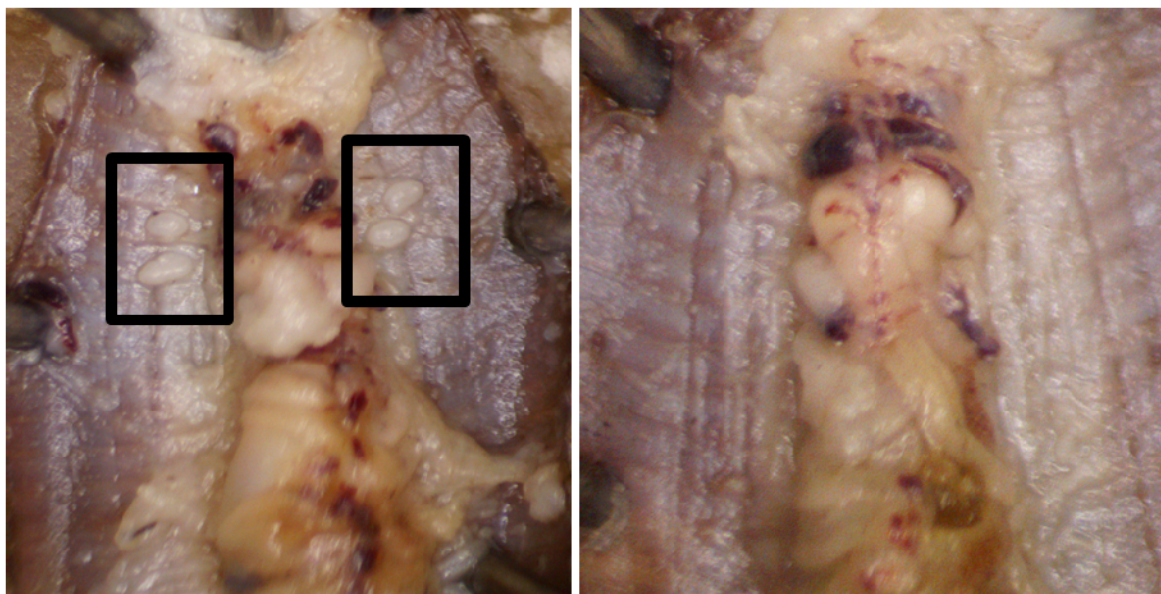


Fig. 4. Dissected specimens of *D. rubidus* (*B. rubidus*). Left: two pairs of spermathecae; Right: complete absence of spermathecae

### Quantitative features

When analysing quantitative features, we also compared the samples in order to identify some differences in dimensions and weight. We found that, in general, there are no statistically significant differences among samples collected in different regions. The only exception was the sample from Belarus that showed worms significantly differing in body length from those collected in Western Siberia and the North-Western Caucasus, and differing in clitellum width and weight from the worms collected in Western Siberia (Table 3). The remaining features in the Belarus sample are similar to those collected in other regions.

When comparing the morphological data, we identified three forms of the species in the studied samples, *D. r. tenuis*, *D. r. rubidus* and *D. r. subrubicundus*.

### Molecular-genetic analysis

We sequenced a fragment of the *cox1* gene for 42 *D. rubidus* specimens. A total of 28 sequences were obtained for *D. r. tenuis*, 13 for *D. r. subrubicundus* and 7 for *D. r. rubidus*. It should be noted that degenerate positions were found in a large number of sequences, i.e. the overlap of peaks corresponding to different nucleotides on chromatograms. Therefore, it would be more correct to use the term “variant” instead of “haplotype” for obtained sequences. 27 unique variants were found for *D. r. tenuis*, 10 for *D. r. subrubicundus* and 7 for *D. r. rubidus*.

We developed a phylogenetic tree using the *cox1* variants, that we obtained, as well as 22 unique sequences obtained from GenBank (Fig. 5).

All sequences of *Dendrodrilus rubidus* (*Bimastos rubidus*), regardless of the form, constituted a single branch without any subdivision within. None of the forms made separate groups. The average value of genetic distance within *D. rubidus* (*B. rubidus*) (p-distance) was 1.7 %.

### Discussion

**Morphological differences of earthworm individuals collected in various regions.** According to the results of morphometric analysis, *Dendrodrilus rubidus* (*Bimastos rubidus*) showed no polymorphism in dimensions or weight. Earthworms in samples from different regions of Russia do not differ significantly by any quantitative feature, while few significant differences in body length and weight in worms from the Belarus sample may be due to the specific ecology of their habitats (e.g., high humidity, sufficient food), which also may have affected their size and weight. Similar reasons might be true for the differences in pigmentation found in worms from Western Siberia, since it may directly depend on food source, in particular the litter composition (Kim-Kashmenskaya, verbal report).

The most variable signs included the position of the clitellum and tubercula pubertatis. Still, even with such a wide range of variations, *D. rubidus* (*B. rubidus*) can be clearly distinguished from all other species based on external morphological features; specifically, the location of setae (slightly converged) makes it possible to identify the species even in juvenile worms (especially those collected in Russia and Belarus). It is possible to reliably determine the form of the species only in adult individuals, based on the combination of said external features and internal structure.

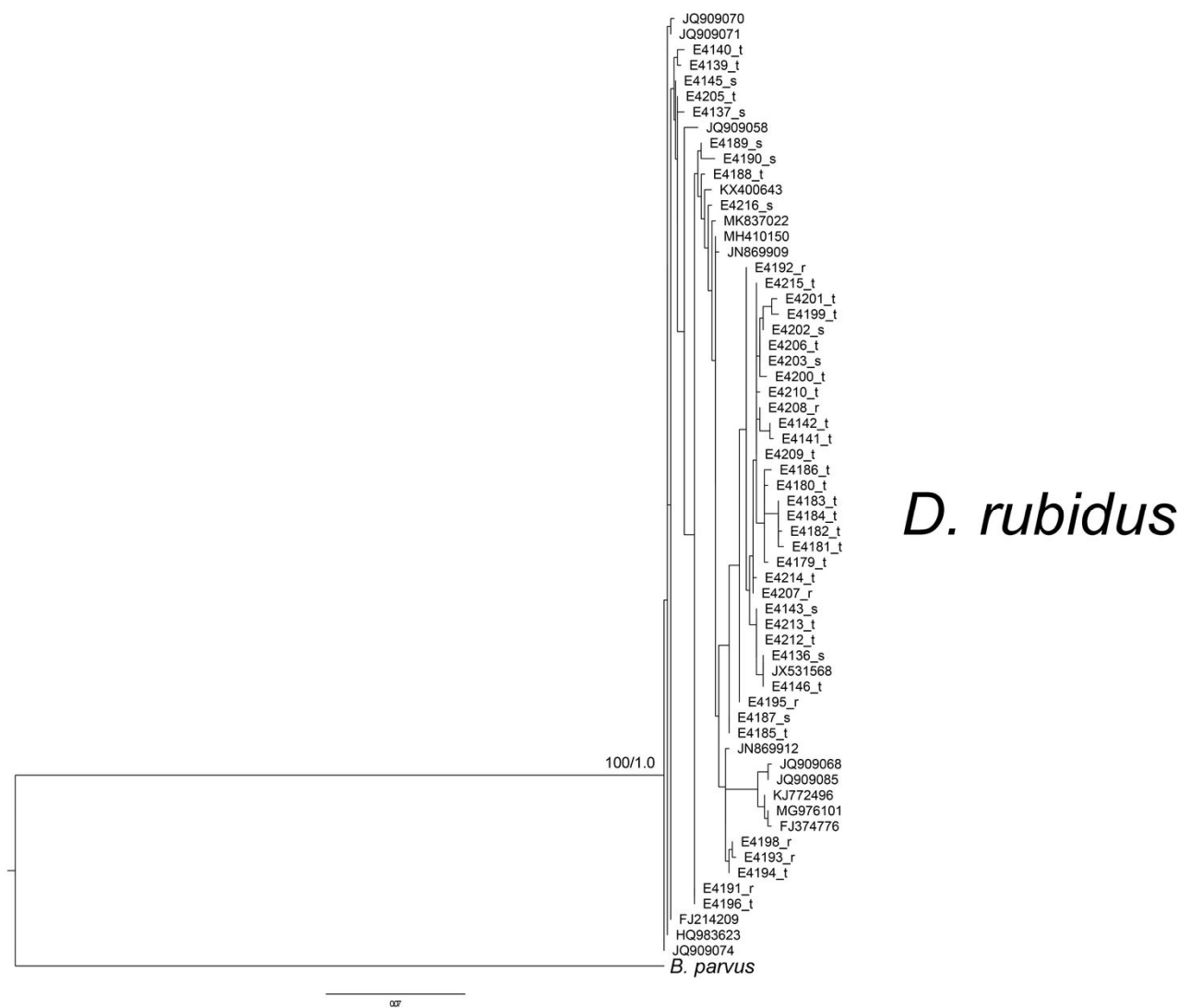


Fig. 5. Phylogenetic tree built based on sequences of *cox1* of *D. rubidus* using the maximum likelihood method.

Key: E#### means sequences obtained; \_r means *D. r. rubidus*, \_t means *D. r. tenuis*; \_s means *D. r. subrubicundus*. Numbers above the branches indicate bootstrap support for the maximum likelihood method / Bayesian a posteriori probabilities

The forms *D. r. tenuis*, *D. r. rubidus* and *D. r. subrubicundus* are widespread and ubiquitous, while *D. r. norvegicus* is endemic to Norway, Sweden and Greenland [15]. Previously only two forms were believed to live in Russia and Belarus, *D. r. tenuis* and *D. r. subrubicundus* [12, 18, 19]. However, taking into account the modern understanding, our study identified three forms of the species (Table 4), assuming some deviations from existing diagnostics. For example, since spermathecae (usually empty) are extremely rare in *D. r. rubidus*, we classified earthworms with developed and filled spermathecae as *D. r. subrubicundus*, even if they lacked tubercula pubertatis or the latter occupied segments 29–30 (Table 4). The majority of field guides, however, reported the position of tubercula pubertatis in this form in segments 28–30 [13, 16, 25]. According to diagnoses, *D. r. rubidus* is also characterised by barely visible tubercula in segments 29–30 [16]. But ap-

parently, it also is possible to classify as *D. r. rubidus* the worms with varying tubercula pubertatis visibility in segments 28–30 as well as those with absent spermathecae [11], which was observed in samples collected in the North-Western Caucasus and the European part of Russia (Table 4). According to existing diagnoses, *D. r. tenuis* can be identified most accurately; its crucial feature is complete absence of both tubercula pubertatis and spermathecae [16]. The observed clitellum position variations in *D. r. tenuis* are consistent with the diagnoses too. Besides, in many adult earthworms, the swollen clitellum during the mating season may partially ‘extend’ to 1/n part of neighbouring segments (Kim-Kashmenskaya, verbal report). The *D. r. tenuis* form is predominant in some samples from the European part of Russia, the North-Western Caucasus and the Republic of Belarus; the sample from the Caucasus is the only one where *D. r. subrubicundus* was completely absent;



the *D. r. rubidus* form was found in various amounts in all the samples studied. It is noteworthy

that the sample from Western Siberia showed an almost equal proportion of all forms (Table 4).

Table 4

Forms of *D. rubidus* identified on the basis of the main diagnostic features

Position of clitellum	Tubercula pubertatis	Spermathecae	Number of individuals	Form of <i>D. rubidus</i>
<b>European part of the Russian Federation</b>				
25–30	not visible	absent	1	<i>D. r. tenuis</i>
25–31	not visible	absent	4	<i>D. r. tenuis</i>
26–31	not visible	absent	1	<i>D. r. tenuis</i>
27–32	not visible	absent	1	<i>D. r. tenuis</i>
25/26–32*	28–30, barely visible	absent	1	<i>D. r. rubidus</i>
26–31	28–30, barely visible	absent	1	<i>D. r. rubidus</i>
26–31	29–30, barely visible	absent	1	<i>D. r. rubidus</i>
25–31/32**	29–30	2 pairs	1	<i>D. r. subrubicundus</i>
<b>Western Siberia</b>				
25/26–31	not visible	absent	1	<i>D. r. tenuis</i>
26–31	not visible	absent	3	<i>D. r. tenuis</i>
26–31	29–30, barely visible	absent	3	<i>D. r. rubidus</i>
26–31/32	29–30, barely visible	absent	2	<i>D. r. rubidus</i>
26–31	not visible	2 pairs	1	<i>D. r. subrubicundus</i>
25/26–31	29–30, barely visible	2 pairs	1	<i>D. r. subrubicundus</i>
26–31	29–30	2 pairs	1	<i>D. r. subrubicundus</i>
<b>North-Western Caucasus (Russian Federation)</b>				
25–31	not visible	absent	1	<i>D. r. tenuis</i>
25/26–31	not visible	absent	2	<i>D. r. tenuis</i>
26–31	not visible	absent	8	<i>D. r. tenuis</i>
26–31	29–30, barely visible	absent	2	<i>D. r. rubidus</i>
26–32	28–30, barely visible	absent	1	<i>D. r. rubidus</i>
26–31	28–30	absent	1	<i>D. r. rubidus</i>
<b>Republic of Belarus</b>				
25–31	not visible	absent	1	<i>D. r. tenuis</i>
26–31	not visible	absent	7	<i>D. r. tenuis</i>
26–31	29–30, barely visible	absent	5	<i>D. r. rubidus</i>
26–31	29–30, barely visible	2 pairs	3	<i>D. r. subrubicundus</i>

\* – the clitellum partially occupies segment 25

\*\* – the clitellum partially occupies segment 32

Comparing quantitative features in different forms of *D. rubidus* (*B. rubidus*) from different regions does not seem valid in this study. The samples of individual forms made up of general samples are extremely small; thus, the reliability of alleged differences is questionable. At the same time, when analysing general samples and identifying individual forms, several variations of qualitative features were found for each form in each region (Table 4). The largest number of variations (4) in *D. r. tenuis* was noted in the sample from the European part of the Russian Federation, whereas the smallest (2) one was found in Western Siberia and in the Republic of Belarus. The ratio of variations of *D. r. rubidus* is very similar in all regions except for Belarus, where this form is represented by a single variation. The *D. r. subrubicundus* form showed three variations in the sample collected in Western Siberia and one in the samples

collected in the European part of the Russian Federation and Belarus.

### Genetic differences among forms of *D. rubidus* (*B. rubidus*)

For *D. rubidus* (*B. rubidus*), clearly demarcated variability was identified with variations classified as forms [16], subspecies [13] or species [26]. The revealed variants are sympatric; therefore, classifying them as subspecies should be considered incorrect, since the term ‘subspecies’ is traditionally understood as a geographically isolated group of animals [27].

Can the forms of *D. rubidus* (*B. rubidus*) actually be different species? Csuzdi et al. briefly mentioned that molecular genetic analysis did not confirm the division of *D. rubidus* (*B. rubidus*) into different species [16]. With this study, we

aimed to explore this issue in more detail using molecular genetic analysis of samples containing different forms, and included all published sequences of *D. rubidus* (*B. rubidus*) in the analysis in order to search for hidden variability.

The analysis showed that, despite significant morphological and karyotypic variability, *D. rubidus* (*B. rubidus*) turned out to be one of the few earthworm species with very little genetic variability. The average distance within the sample of *D. rubidus* (*B. rubidus*), including the entire sample from GenBank, was 1.7 % (Fig. 5). Average distances among different geographic regions and within them (Table 5), as well as among the sam-

ples of different forms (Table 6) were equally low. The maximum distance between variants was 4.1 %. Although such values can indicate the presence of distinct species level taxa in some groups, they are actually quite low for earthworms [28–30]. Values as high as 7 % were found even in a single genetic lineage of *E. nordenskioldi* [31]. This, and the fact that we found no split of *D. rubidus* (*B. rubidus*) into reciprocally monophyletic clades, nor any branches corresponding to any of the morphological forms, suggests that there are no distinct taxonomic groups within this species.

Table 5

Average nucleotide p-distances within and between samples of *D. rubidus* different geographic regions. All sequences from GenBank were pooled into a single sample

	North-Western Caucasus	European part of Russia	Belarus	Western Siberia	GenBank
North-Western Caucasus	0.6				
European part of Russia	0.9	0.4			
Belarus	1.6	1.2	1.6		
Western Siberia	1.5	1.1	1.5	0.9	
GenBank	2.7	2.4	2.2	1.8	1.3

Table 6

Average nucleotide p-distances within and between samples of different forms of *D. rubidus*

	<i>tenuis</i>	<i>subrubicundus</i>	<i>rubidus</i>
<i>tenuis</i>	1.2		
<i>subrubicundus</i>	1.6	1.3	
<i>rubidus</i>	1.1	1.3	1.0

Having compared the data of morphological analysis (especially regarding the structure of reproductive systems) and molecular genetic analysis, we can assume that the division of *D. rubidus* (*B. rubidus*) into different sexual and parthenogenetic forms is in the making. Similar phenomenon was described for *Aporrectodea trapezoides* [32], where different populations have variants from fully functional spermatogenesis up to its absence, and the transition to parthenogenesis seems to have occurred several times independently. Considerable chromosome variability of the species is also of great interest as races with 34, 48, 51, 68, 85, 102, and 136 chromosomes have already been reported [11, 17, 33, 34]. Based on the data obtained, we can assume that all these races have formed relatively recently.

Average nucleotide p-distances within and between samples of *D. rubidus* different geographic

regions. All sequences from GenBank were pooled into a single sample.

### Conclusion

The results obtained allow for a conclusion that in Russia and Belarus, *Dendrodrilus rubidus* (*Bimastos rubidus*) is represented by three forms, *D. r. tenuis*, *D. r. rubidus* and *D. r. subrubicundus*, which can be reliably distinguished only after a thorough analysis of internal and external morphological features in adult worms. No significant genetic differences were found between different forms of the species. Therefore, if it is not possible to accurately group earthworms using general soil-zoological, ecological and biogeocenotic studies, the name *Dendrodrilus rubidus* (*Bimastos rubidus*) is recommended to be used for the species without specifying any individual forms in order to avoid error.

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