

Remarks on the molecular phylogeny of the genus *Dendrobaena* (sensu Pop 1941) based on the investigation of 18S rDNA sequences

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SUMMARY. The genus *Dendrobaena* and its close relatives were examined by molecular methods. The small subunit ribosomal DNA (18S rDNA) was amplified and sequenced. Full sequence were aligned and analyzed by molecular phylogeny programs. Our preliminary results revealed that the members of the North American genus *Bimastos* diverge markedly from the Palearctic species *B. syriacus* (Rosa, 1983) furthermore, the genus *Bimastos* itself seems to be polyphyletic. While type species *B. palustris* (Moore, 1893) is nested within the clade formed by the *Dendrodrilus* and *Allolobophoridella* species, *B. tumidus* and other undescribed new *Bimastos* species represent a well-demarcated clade, quite distinct from the rest of the *Dendrobaena* (s.l.) species investigated.

INTRODUCTION

The earthworm genus *Dendrobaena* was erected by Eisen (1873) with type species *Dendrobaena boeckii* Eisen, 1873. Later, Michaelsen (1900) synonymized this species name with *Enterion octaedrum* Savigny, 1826. Furthermore, he regarded *Dendrobaena* as a subgenus of the genus *Helodrilus* Hoffmeister, 1845, with the following diagnosis: “*Setae usually widely paired or distant, pigmentation mainly purple-red. Prostomium epi- sometimes tanylobic. Spermathecae two or three pairs, open in setal line d or rarely c, sometimes lacking. Vesicles three pairs in 9, 11, 12, sometimes with a fourth pair of vesicles in 10, but they are always much smaller than the other three pairs.*”

According to this diagnosis, Michaelsen (1900) classified into this subgenus 19 taxa including the type species *D. octaedra*. It was Svetlov (1924) who elevated the subgenus *Dendrobaena* to a genus rank, and as a result of the ongoing works of the taxonomists, the lumbricid catalogue of Cognetti (1931) listed 46 species belonging to this genus. Unfortunately, the uncertainties in the diagnosis made this

genus quite heterogeneous, and by the early forties, a complete revision (not only of this genus but the whole lumbricid system) was absolutely inevitable.

This tiresome work was accomplished by Pop (1941) who revised the whole lumbricid system and, instead of the previously applied highly variable reproductive characters (vesicles, spermathecae etc.) proposed the use of somatic features such as the pigmentation and the setal arrangement and the type of the prostomium. On the basis of these characters, Pop (1941) created a very simple system containing only six genera representing two larger groups. The first (*Allolobophora* Eisen, 1873; *Eiseniella* Michaelsen, 1900; and *Octolasion* Örley, 1885) was characterized by the lack of porphyrin-based pigmentation and the second (*Dendrobaena*, *Eisenia* Malm, 1877 and *Lumbricus* Linnaeus, 1758) possessing purple-red pigmentation. To the genus *Dendrobaena* he relegated all the species with purple-red pigmentation and widely paired or distant setae. He complemented the description of lumbricids with new anatomic characters such as the structure of the longitudinal musculature. Pop (1941) recognized that the species placed to *Dendrobaena* showed some variability regarding this new character, but having, at that time, information on this new feature only for a limited number of species, he did not venture to create further smaller groups inside the genus.

It was Omodeo (1956) who tried to solve the heterogeneity problem issue inside the genus *Dendrobaena* by introducing a new character, namely the type and segmental location of the calciferous glands. According to this attribution, the *Dendrobaena* species fall into two distinct groups. The first contained the species with calciferous glands open directly into the oesophagus in segment 11, 12, or in one of them (subgenus *Dendrobaena*). The second comprised the species with calciferous glands open via paired calciferous sacs in segment 10 (subgenus *Dendrodrilus* Omodeo, 1956). Omodeo (1956) drew the attention that the subgenus *Dendrobaena* remained still quite heterogeneous.

In the next step, Gates (1975) repositioned the species *D. mammalis* (Savigny, 1826) into his new genus *Satchellius* Gates, 1975 and Zicsi (1978) recognized that the male pore of the *D. platyura* species complex open far beyond the usual position in 15, at the beginning of the clitellum and erected for these, the genus *Fitzingeria* Zicsi, 1978. Furthermore, he transferred the Middle Eastern *D. syriaca* (Rosa, 1893) into the newly revised North American genus *Bimastos* Moore, 1893. But the so restricted genus *Dendrobaena*, containing more than 60 species, remained still heterogeneous, both in terms of pigmentation and the structure of the longitudinal musculature.

The first was Csuzdi (1984) who carried out a detailed revision of all the species of the genus *Dendrobaena* and recognized that the species *D. ruffoi* Zicsi, 1970 and *D. osellai* Zicsi, 1970 are congeneric with *D. calarensis* (Tetry, 1944) and belong to *Kritodrilus* Bouché, 1972. Furthermore, he distinguished four species groups:

- A. *octaedra* group: Small or middle-sized worms. The extraoesophageal vessels are missing and the last pairs of hearts are also frequently lacking. Musculature pinnate and the well-developed calciferous glands open directly in 11, 12.
- B. *schmidti* group: Middle-sized or large worms. Last pair of hearts in 11 and extraoesophageal in 12. Male pores with great glandular atrium usually intruding into the neighbouring segments. Musculature pinnate, calciferous glands open directly in 11, 12, but well-developed usually only in one of them.
- C. *byblica* group: Small or middle-sized worms. Last pair of hearts in 11 and extraoesophageal in 12. Male pores always small, hardly visible. Musculature pinnate, calciferous glands are usually small, open directly in 11, 12.
- D. *veneta* group: Middle-sized or large worms. Last pair of hearts in 11 and extraoesophageal in 12. Male pore usually with glandular atrium. Musculature fasciculate, calciferous glands open directly in 1/210-11, 12 but without remarkable dilatation.

Some species represented such uncial characteristics, that it was impossible to relegate them in a species group (*D. auriculata* (Rosa, 1897), *D. cognettii* (Michaelsen, 1903), *D. mrazeki* (Cernosvitov, 1935), *D. alvaradoi* Moreno, Diaz Cosin & Jesus, 1982).

The recent detailed taxonomic revisions of the family Lumbricidae (Mrsic 1991, Qiu & Bouché 1998) have left the genus *Dendrobaena* untouched, with the remarks that the genus presently contains more than 100 species and urgently needs revision.

Since the morphological investigations failed to produce a well-established division of this group into monophyletic units we tried to solve the problem with the presently more and more recognized molecular methods.

The small subunit ribosomal DNA (18S rDNA) was in the centre of our interest, since it is a universally occurring molecular phylogenetic marker. Because of its moderate conservatism, it is thought to be equally useful to reveal taxonomic relationships in lower (Bargues and Mas-Coma 1997,) and higher levels, as well. (Apakupakul et al. 1999). However, several works report that the considerably quick radiation renders the use of 18S rDNA more difficult (Winnepenninckx et al. 1996), and ; other studies mentioned its moderately high conservatism that prevents resolving the phylogenetic branches in taxa with lower level (Dumont et al. 2005). On the contrary, Erseus et al. (2000) applied successfully the 18S rDNA as a phylogenetic marker in Tubificidae to confirm that Tubificinae and Limnodrilinae are monophyletic taxa, whereas Rhyacodrilinae and Phallo-drilinae are not.

Several authors combine the nuclear 18S rDNA and mitochondrial genes data in the phylogenetic inference (Apakupakul et al. 1999, Gelder and Sidall 2001, Sjölin et al. 2005, Wiklund et al. 2005), but these works concentrate

mostly on aquatic annelides. Martin et al. (2000) analyzed the sequences of the 18S rDNA parallel with mtCOI sequences by many species from various families within Clitellata. Analyzed separately, the two genes did not resolve the relationships among Clitellata taxa, but the consensus tree was congruent with the morphological observations. Jamieson et al. (2002) analyzed the molecular evolution of annelids, including Lumbricidae. They concentrated on resolving the family or higher level evolution of the group and have not tried to infer the within family pattern of Lumbricidae. (Nota trad.: to infer= 1. deduce or conclude from facts and reasoning. 2. imply, suggest. Probabil autorul intentioneaza sa sugereze: to interfere with sau in the family pattern of Lumbricidae?)

Until now, only a single paper, Pop et al. (2003), dealt with the molecular phylogeny of lumbricid earthworms by examining three genes, namely the nuclear 18S rDNA, the mitochondrial 16S rDNA and the cytochrome c oxidase (COI). They found that mitochondrial genes have a higher informative value than the nuclear 18S rDNA that were investigated only in three species. The phylogenetic trees of 16S rDNA and the COI sequences did not show congruent results. The *Octodrilus* species formed a distinct branch on the COI tree but in the 16S tree they were scattered along the tree. The position of the related *Dendrobaena byblica* and *Dendrodrilus rubidus* was more plausible in the COI tree than in the 16S rDNA tree. The position and the branching of *Lumbricus* and *Eisenia* species were in agreement with the classical concept.

MATERIAL AND METHODS

Earthworm specimens for the molecular studies were fixed in 96% ethanol. A small piece was cut from the caudal part of the muscular tissue for DNA isolation in case of each individual. The genomic DNA was extracted using the Dneasy[®] Tissue Isolation Kit (Qiagen, Germany) according to the manufacturer's protocol. The DNA isolated was checked in 1% agarose gel with the point of reference molecular marker (Lambda DNA/EcoRI+HindIII Marker, 3, Fermentas, Canada) and stored at -20°C.

The gene of 18S rDNA region was amplified via polymerase chain reaction (PCR) [reaction-mix: 5 µl of 10X PCR buffer with (NH₄)₂SO₄ (Fermentas), 4-6.5 mM MgCl₂ (Fermentas), 1 ng/µl genomic DNA, 0,2 mM dNTPs 3,25 µM of each primer (18F35 and 18R1779), 0,02 U/µl Taq DNA Polymarase (Fermantas)]. The primers applied were 18F35 and 18R1779 (Table 1.) annealing to the conservative ends of the 18S rDNA (Struck et al. 2002). The PCR protocol was as follows: initial denaturation 98°C for 5 min and than 35 cycles with 1 min 94°C, 1 min 50°C, 2 min 72°C, and 10 min at

72°C for final extension. The PCR products were tested by agarose gel electrophoresis and purified by PCR-M™ Clean Up System (Viogene, Taiwan). The purified PCR products were sequenced using BigDye v.3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The temperature conditions of the cycle sequencing and the set up of the reaction mix follow the manufacturers' instructions. Six inner primers (Struck et al. 2002) were used for the sequencing (Table 1), which effect approximately 600bps long fragments. The products of the cycle sequencing were purified by ethanol precipitation. The samples precipitated were dissolved in formamide (Promega, USA) or Template Suppressor Reagent Buffer (Applied Biosystems, USA). The sequences were determined with the automatic ABI PRISM 310 Genetic Analyser (Perkin Elmer, USA). All sequences were checked manually. The full sequences were compiled based on the overlapping sections of the fragments using the software Bioedit. Only partial sequences were determined by several samples (see Table 3).

Table 1. Primers used in this study for the 18S rDNA amplification and cycle sequencing

Name	Sequence (5'-3')	Position	Direction
18F35	TCT CAA AGA TTA AGC CAT GCA	35-55	Forward
18R339	CCC TCT CCG GAA TCG AAC CCT GAT	376-399	Reverse
18R772	CTC TAA TTT TTT CAA AGT AAA	751-772	Reverse
18R925	GAT CCA AGA ATT TCA CCT CT	906-925	Reverse
18F997	TTC GAA GAC GAT CAG ATA CCG	997-1017	Forward
18R1256	AGC TCT CAA TCT GTC AAT CCT	1236-1256	Reverse
18F1435	AGG TCT GTG ATG CCC TTA GAT	1435-1455	Forward
18R1779	TGT TAC GAC TTT TAC TTC CTC TA	1757-1779	Reverse

18S rDNA of twenty earthworm species were newly sequenced and the data obtained were complemented by several sequences from the GenBank database (Table 2). The sequences were aligned to each other by ClustalW running the program Mega3 (Kumar et al., 2004). The parameters of the aligning were the following: gap opening penalty: 15, gap extension penalty: 6.66 by the pairwise and multiple parameters, too. DNA weight matrix: IUB, transition weight 0,5.

Table 2. List of the earthworm species analyzed

Species	Locality
<i>Allolobophoridella eiseni</i> Levinsen, 1884	Hungary, Bátaapáti. 13. VI. 2002. Leg. Cs. Csuzdi
<i>Bimastos palustris</i> Moore, 1893	USA, MD, Jug Bay. 27. IV. 2001. Leg. Cs. Csuzdi
<i>Bimastos</i> sp. nov.	USA, MD, Jug Bay. 12. VI. 2003. Leg. K. Szlávecz
<i>Bimastos</i> sp. nov.	USA, MD, jug Bay 2005. leg. K. Szlávecz
<i>Bimastos syriacus</i> (Rosa, 1893)	Israel, Nahal Tabor. 21.II. 2004. Leg. T. Pavliček
<i>Bimastos syriacus</i> (Rosa, 1893)	Israel, Mt. Carmel, Muchraka. 22. XI. 2003. Leg. T. Pavlicek
<i>Bimastos tumidus</i> (Eisen, 1874)	USA, MD, Baltimore, Cross Keys. 13. XI. 2002. Leg. Cs. Csuzdi
<i>Dendrobaena auriculata</i> (Rosa, 1897)	Hungary, Vértes Mts. 09. IV. 2002. Leg. J. Kontschán
<i>Dendrobaena clujensis</i> Pop, 1938	AJ272527 Pop
<i>Dendrobaena clujensis</i> Pop, 1938	Romania, Transsylvania, Stana 01. VIII. 2003. Leg. Cs. Csuzdi
<i>Dendrobaena cognetti</i> (Michaelsen, 1903)	Hungary, Szentmargitfalva. 10. IV. 2004. Leg. Cs. Csuzdi
<i>Dendrobaena ganglbaueri</i> (Rosa, 1894)	Hungary, Velem. 10. IV. 2004. Leg. Cs. Csuzdi
<i>Dendrobaena hauseri</i> Zicsi, 1973	Israel, Rehaniya. 12. I. 2005. Leg. T. Pavlicek
<i>Dendrobaena octaedra</i> (Savigny, 1826)	Romania, Transsylvania, Hargita Mts. 30. VII. 2003. Leg. Cs. Csuzdi
<i>Dendrobaena samarigera</i> (Rosa, 1893)	Israel, Tel Keshet. 08. I. 2003. Leg. T. Pavliček
<i>Dendrodrilus rubidus</i> (Savigny, 1826)	USA, MD, Baltimore, Cross Keys. 13. XI. 2002. Leg. Cs. Csuzdi
<i>Dendrodrilus subrubicundus</i> (Eisen, 1874)	Romania, Transsylvania, Vladeasa Mts. 01. VIII. 2003. Leg. Cs. Csuzdi
<i>Dendrodrilus subrubicundus</i> (Eisen, 1874)	USA, MD, Baltimore, JHU Campus. 08. XI. 2002. Leg. Cs. Csuzdi
<i>Eisenia andrei</i> Bouché, 1972	AY365460 Erseus
* <i>Eisenia fetida</i> (Savigny, 1826)	Portugal, Azores, Sta. Maria. VI. 2004. Leg. A. Zicsi Jr.
<i>Eisenia fetida</i> (Savigny, 1826)	AB076887, Winnepenninckx
<i>Eisenia fetida</i> (Savigny, 1826)	X79872 Hiraishi
<i>Eisenia lucens</i> Waga, 1857	Romania, Transsylvania, Hargita Mts. 30. VII. 2003. Leg. Cs. Csuzdi
<i>Eisenia spelaea</i> (Rosa, 1901)	Croatia, Papuk Mts. 24. X. 2004. Leg. D. Murányi
<i>Eisenoides loennbergi</i> (Michaelsen, 1894)	USA, MD, Baltimore, Oregon Ridge. 09. XI. 2002. Leg. Cs. Csuzdi
<i>Fitzingeria pl. depressa</i> (Rosa, 1893)	Hungary, Bátaapáti. 03. VI. 2005. Leg. Cs. Csuzdi
* <i>Fitzingeria pl. platyura</i> (Fitzinger, 1833)	Hungary, Püski. 23. VI. 2004. Leg. S. Mahunka

* Partial sequences

Table 3. Nucleotide composition of the taxa investigated

Species	T(U)	C	A	G
<i>Allolobophoroidella eiseni</i>	24.3	23.4	24.5	27.9
<i>Bimastos palustris</i> USA	24.2	23.3	24.5	27.9
<i>Bimastos</i> sp. nov. USA 2003	24.1	23.4	24.7	27.8
<i>Bimastos</i> sp. nov. USA 2003	24.1	23.4	24.7	27.8
<i>Bimastos syriacus</i> Mt. Carmel	24.2	23.5	24.4	27.9
<i>Bimastos syriacus</i> Nahal Tabor	24.1	23.4	24.5	28.0
<i>Bimastos tumidus</i> USA	24.2	23.4	24.4	27.9
<i>Dendrobaena auriculata</i>	24.3	23.4	24.4	28.0
<i>Dendrobaena clujensis</i> Pop	24.1	23.5	24.5	27.9
<i>Dendrobaena clujensis</i> Sztana	24.2	23.4	24.5	27.9
<i>Dendrobaena ganglbauri</i>	24.1	23.5	24.5	27.9
<i>Dendrobaena hauseri</i> Raheniya	24.2	23.4	24.5	27.9
<i>Dendrobaena octaedra</i> Hargita	24.2	23.4	24.4	28.0
<i>Dendrobaena samarigera</i> Tel Keshet	24.1	23.5	24.3	28.1
<i>Dendrodilus subrudicundus</i> USA	24.2	23.3	24.5	28.0
<i>Dendrodilus rubidus</i> USA	24.3	23.4	24.5	27.8
<i>Dendrodilus subrudicundus</i> Transsylvania	24.3	23.3	24.5	27.9
<i>Denrobaena cognetti</i>	24.5	22.9	24.5	28.1
<i>Eisenia andrei</i> Erseus	24.2	23.3	24.5	27.9
<i>Eisenia fetida</i> Hiraishi	24.3	23.4	24.5	27.8
<i>Eisenia fetida</i> partial Portugal	24.5	23.7	23.8	28.0
<i>Eisenia fetida</i> Winnepenninckx	24.4	23.4	24.5	27.8
<i>Eisenia lucens</i> Hargita	24.2	23.4	24.5	27.9
<i>Eisenia spelaea</i>	24.3	23.3	24.5	27.9
<i>Eisenoides loennbergi</i> USA	24.1	23.4	24.5	28.0
<i>Fitzingeria</i> pl. <i>depressa</i>	24.2	23.4	24.5	27.9
<i>Fitzingeria</i> pl. <i>platyura</i> partial	24.6	24.7	23.5	27.2

The phylogenetic reconstruction was performed by applying maximum parsimony (MP), maximum evolution (ME), neighbour joining (NJ) and maximum likelihood methods in parallel using the PAUP 4.07b (Swofford 2000), Mega3 (Kumar et al. 2004) and Treefinder (Job 2005) softwares respectively. For the MP trees 100 bootstrap replicates were analyzed with the fast heuristic methods implemented in the PAUP program. For the NJ and ME trees the Kimura-2 parameter was chosen with gamma distributed among-sites substitution rates. In the maximum likelihood analyses the GTR substitution model was chosen and the analysis was carried out with 1000 replicates.

All the trees were rooted using *Dendrobaena cognetti* as outgroup because previous analyses, when several *Allolobophora* sensu lato species were also included, showed a basal position of *D. cognetti* to the other *Dendrobaena* sensu lato species. This is in accordance with the morphological characters since *D. cognetti* possess nephridial bladders (U shaped in the front of the body and bilobate after the clitellum) more similar to the *Allolobophora* (s.l.) species than to any representative of the *Eisenia-Dendrobaena* group.

RESULTS AND DISCUSSION

The small subunit ribosomal DNA (18S rDNA) was amplified and sequenced in twenty three earthworm specimens representing twenty species or subspecies (Table 2). In addition to the newly acquired sequences, the other four were downloaded from the GeneBank database (Table 2). The sequences investigated showed quite low variability

in terms of nucleotide composition (Table 3), and the number of the variable sites was also very small. Across the 27 sequences 78 of 1703 sites are variable in the 18S rDNA gene. This low variability must be responsible for the quite low bootstrap values observed in the analyses. Nevertheless, the phylogenetic reconstructions by the different methods MP, NJ, ME and ML) resulted in quite similar tree topologies and recovered some interesting lineages (Figs. 1-4).

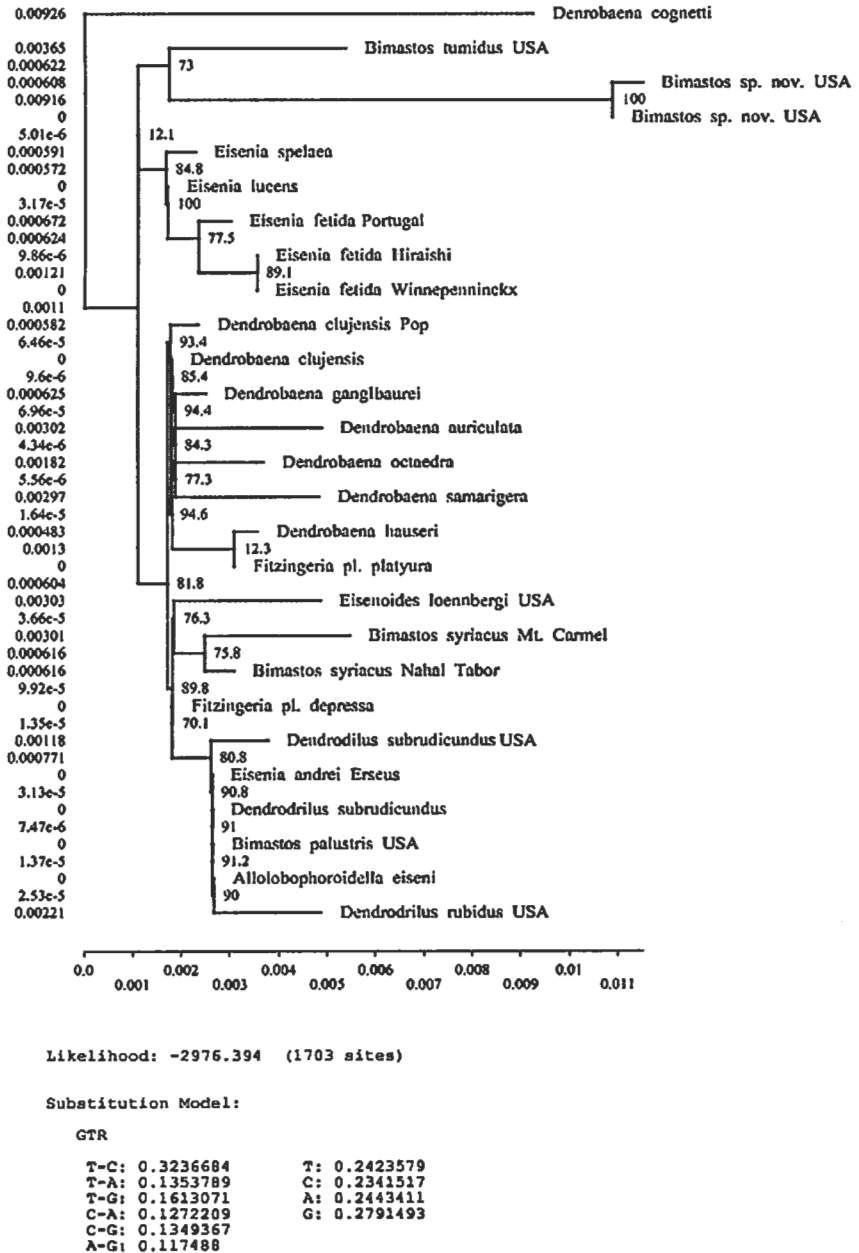


Fig. 1. 18S rDNA Maximum Likelihood consensus tree of 100 pseudoreplicates

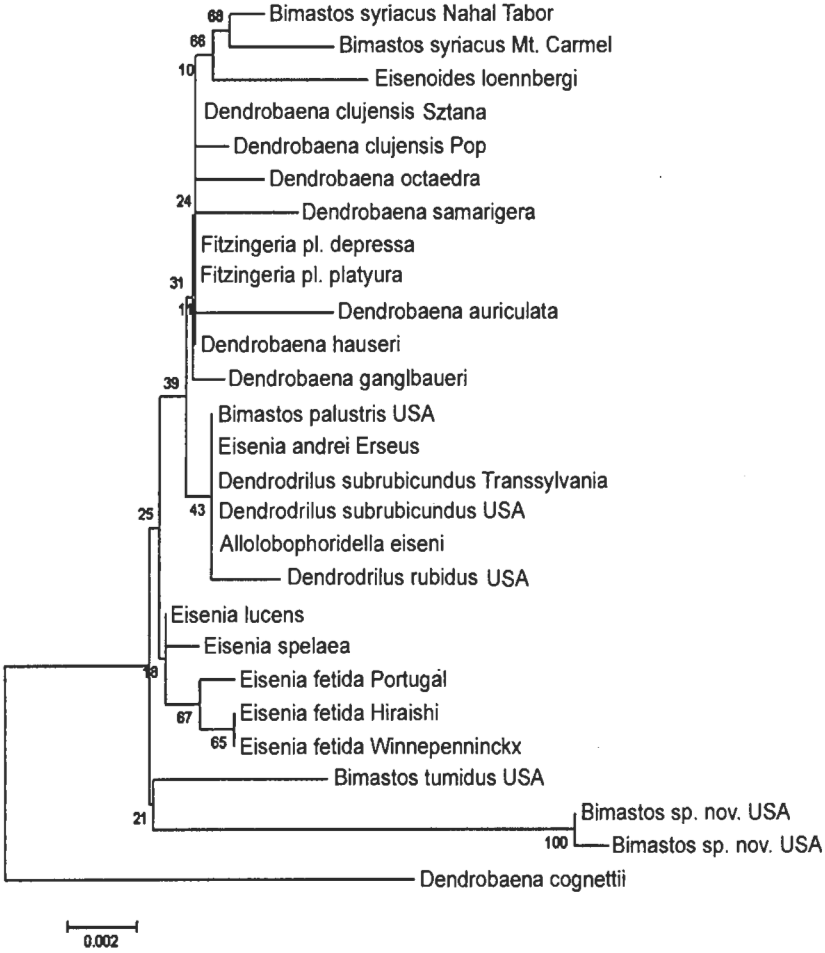


Fig. 2. 18S rDNA Neighbour Joining consensus tree with the bootstrap values of 1000 repetitions

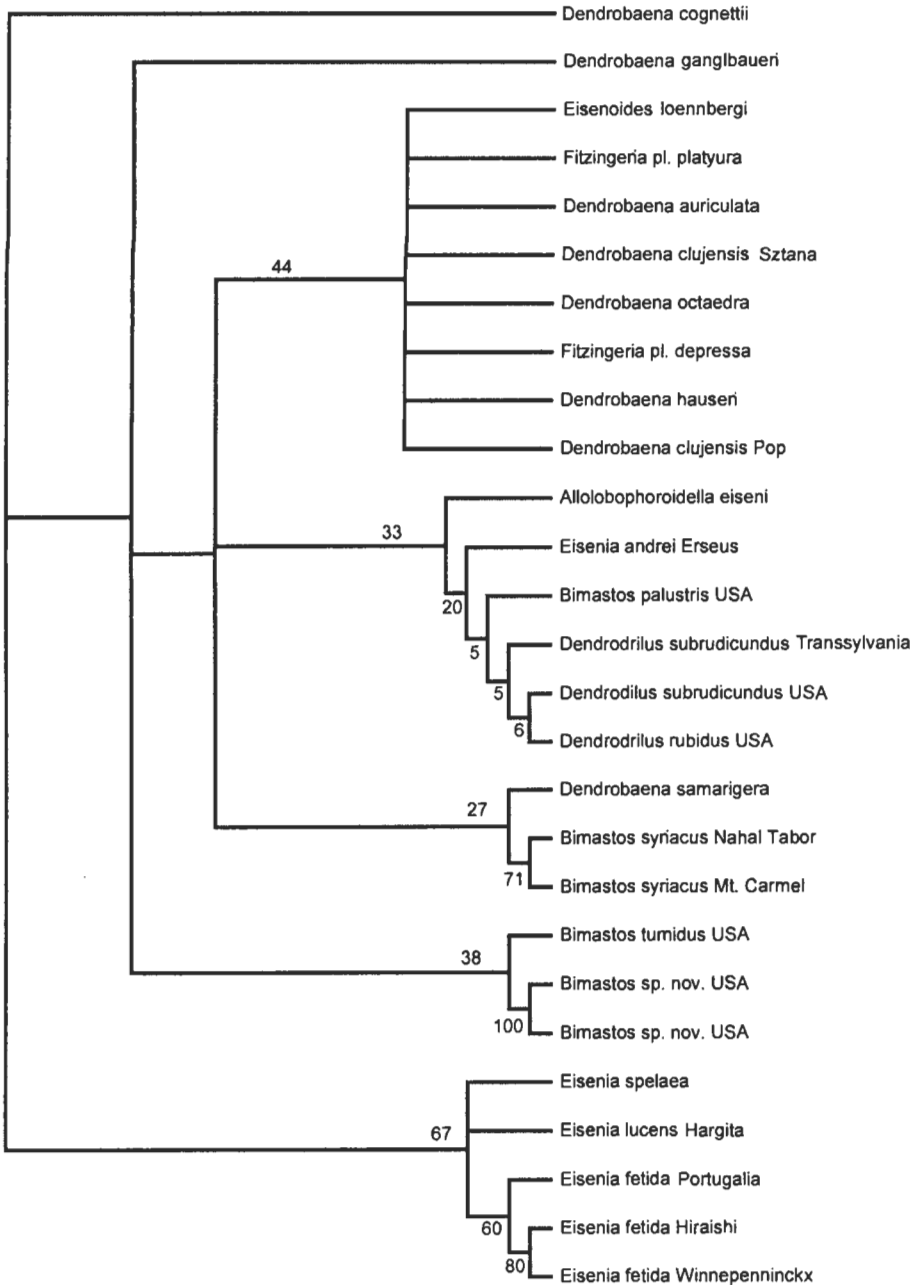


Fig. 3. 18S rDNA Maximum Parsimony consensus tree with the bootstrap values of 100 repetitions

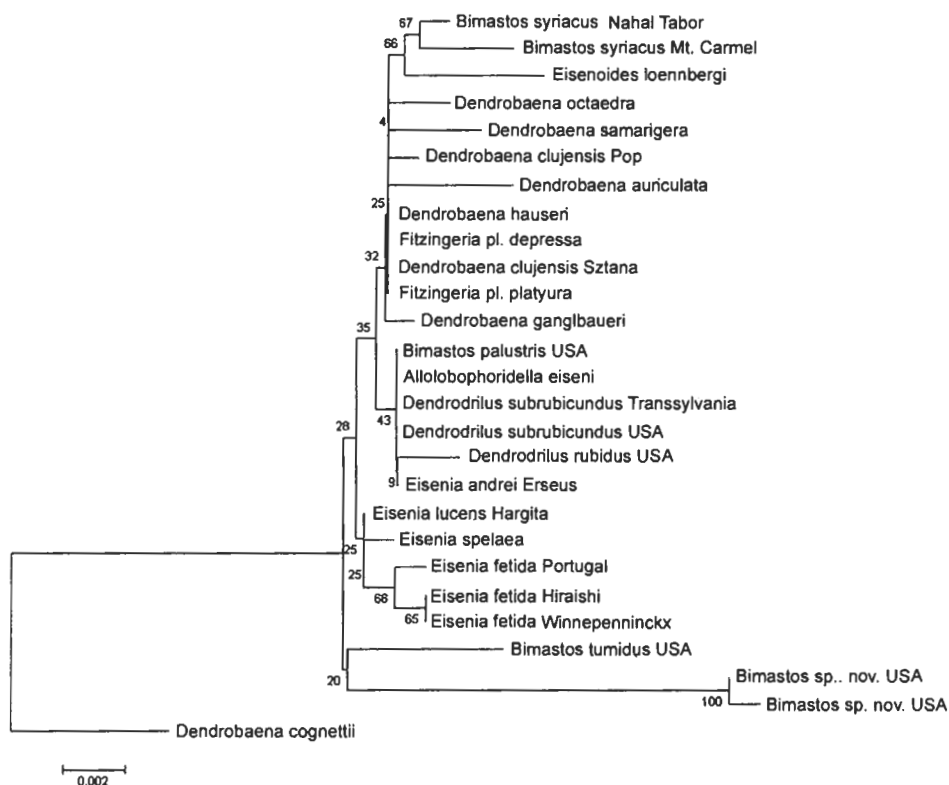


Fig. 4. 18S rDNA Minimum Evolution consensus tree with the bootstrap values of 1000 repetitions

All the four analyses showed the monophyly of the *Eisenia* species but with moderate bootstrap support (highest in case of MP, 67%). It was not evident because the three species analyzed are not uniform morphologically. Whereas *E. fetida* possess pinnate, *E. lucens* and *E. spelaea* show fasciculated longitudinal musculature. This somewhat weakens the views of Pop (1941) and Csuzdi & Zicsi (2003) that the musculature type is an important parameter of the monophyly of a genus.

Another remarkable group is formed by *Bimastos palustris*, *Allolobophoroidella eiseni*, *Dendrodrilus rubidus*, *Dd. subrubicundus* and *E. andrei* (GeneBank data). It appears in all the four trees but with quite low bootstrap support. The placement of *Eisenia andrei* far from *Eisenia fetida* among the *Dendrodrilus* species is interesting. The close proximity of this specimen to *Dd. subrubicundus* is most likely due to an identification error and actually Erseus & Kallersjo (2004) used in their analysis a specimen of *Dd. subrubicundus*. To take *Dd. subrubicundus* as an *E. fetida* / *andrei* specimen is quite easy because in both cases the clitellum and tubercles are almost in the same position. But the most remarkable is the appearance of *B.*

palustris, the type species of the genus *Bimastos*, in this clade. It is clearly contradictory to the genus concept of Zicsi (1981) who united the North American species of *Bimastos* with the Anatolian *Dendrobaena* species possessing circular clitellum, as well. This result draws the attention to the homoplastic nature of this character. Therefore, the Anatolian species with circular clitellum must be relegated to the new genus *Healyella* proposed by Omodeo & Rota (1989). This genus is represented in our analyses by two specimens of *B. syriacus* and forms a separate, advanced clade among the unresolved *Dendrobaena* species. However, the position of the *Eisenoides loennbergi* as sister to the two *B. syriacus* specimens requires more analyses.

The close affinity of *B. palustris*, *Ai. eiseni* and the *Dendrodrilus* species is supported also by morphological characters. All the three species possess U-shaped proclinate nephridial bladders and calciferous diverticula in segment 10. The differences lies in the setal arrangement and in the type of the musculature. *B. palustris* and *Ai. eiseni* have strictly paired setae, but while *B. palustris* possesses pinnate musculature, *Ai. eiseni* shows typical fasciculated musculature. The *Dendrodrilus* species possess widely paired setae and transitory musculature.

Surprisingly, the two other North American *Bimastos* species newer nested within this clade. They always form a separate lineage close to the root that raise the question of polyphyly even of the restricted *Bimastos*.

The rest of the taxa form a highly unresolved clade involving all *Dendrobaena* and the two *Fitzingeria* species. The moderate bootstrap support of the recognized clades emphasise the limitations of using the 18S rDNA genes in the reconstruction of earthworm phylogeny at genus level. It is considered that, for more robust phylogenetic conclusions, several other, more sensitive genes should be investigated.

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