

# An attempt to reconstruct the molecular phylogeny of the genus *Allolobophora* Eisen, 1874 (sensu lato, Pop, 1941) using 16S rDNA and COI sequences (Oligochaeta, Lumbricidae)

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**SUMMARY.** A molecular phylogeny, based on sequences of 16S rDNA and the mitochondrial cytochrome *c* oxidase subunit I from the genus s.l. *Allolobophora* was reconstructed. The results clearly proved the heterogeneous nature of this catch-all genus. Thus, some clearly identifiable clades appearing in most trees might be attributed to different genera, such as *Aporrectodea*, *Proctodrilus*, and *Cernosvitovia*.

## INTRODUCTION

The earthworm genus *Allolobophora* Eisen, 1874 has always been a problematic group for the earthworm taxonomists. The history of this genus is long and complicated by the fact that from the beginning it had no designated type species. The genus *Allolobophora* was established for primitive earthworms, without purple pigment, with closely paired setae, four pairs of seminal vesicles and spermathecal pores in setal line *cd*. Subsequently, species with apparent different characters were included in this genus, and its rank and content underwent several changes (Rosa 1983, Michaelsen 1900, Svetlov 1924).

Pop (1941) carried out a detailed revision of this group. Aiming at defining a species group which was easy to determine, he relegated to genus *Allolobophora* all the terrestrial earthworm species lacking red-violet pigmentation and possessing closely paired setae. Pop (1941, 1948) was fully aware of the fact that the new concept of *Allolobophora* Eisen 1874 emend. Pop 1941, made it a heterogeneous catch-all genus. Moreover, he stressed that this provisional genus should be split into smaller genera when proper new criteria would be discovered. Nevertheless,

due to the scarcity of material and characters used at that time, Pop made no further attempts to subdivide the genus.

Since then, continuous efforts have been made to recognize and delimit monophyletic groups inside this collective genus (Omodeo 1956, Bouché 1972, Gates 1975, Perel 1976, Zicsi 1981 and 1985, Mrcsic & Sapkarev 1988 and finally Qiu & Bouché 1998), but a phylogenetically well-founded solution is still missing. Meantime, the parent genus (*Allolobophora* Eisen 1873 sensu Pop 1941) was split into more than 30 taxa (genera and subgenera) sometimes of doubtful validity (Csuzdi & Zicsi 2003).

In the present molecular phylogeny approach we tried to understand the evolutionary pathways within this group, and test the phylogenetic validity of taxa into which the *Allolobophora* (sensu lato) genus had been split.

## MATERIAL AND METHODS

Fresh material for 30 lumbricid taxa, including 15 species of the *Allolobophora* sensu lato group, and one enchytraeid species have been collected and analysed (Table 1).

The adult earthworm specimens were preserved in 96% ethanol. Fragments of the muscular body wall from behind the clitellar region were cut for analyses, after removing internal organs. As a proof of taxonomic identification, the anterior part of all analysed individuals is kept in 96% ethanol with the Institute of Biological Research Cluj-Napoca. The molecular analyses were carried out in the laboratories of the Institute for Pharmacy and Molecular Biotechnology, Heidelberg, Germany.

The extraction of total genomic DNA was performed using the phenol/chloroform method (Sambrook et al. 1989).

The 16S rDNA fragments were amplified using the primers *16sar* (5'-CGC CTG TTT ATC AAA AAC AT - 3') and *16sbr* (5'- CCG GTY TGA ACT CAG ATC AYG T - 3') (after Palumbi et al. 1991). The amplifications (total volume 50 µl) contained 1 µl DNA and 49 µl PCR-mix, Cycling profile: 1 min at 92 °C, 1 min at 52 °C, and 1 min at 72 °C for 35 cycles with an initial denaturing step at 92 °C for 4 min, and a final extension step at 72 °C for 5 min. Automated sequencing was performed using an ABI Prism Genetic Analyser 3100.

The mitochondrial cytochrome *c* oxidase subunit I sequences (COI) were amplified and sequenced with the universal primers HCO2198 (5'- TAA ACT TCA GGG TGA CCA AAA AAT CA - 3') and LCO1490 (5'- GGT CAA CAA ATC ATA AAG ATA TTG G - 3') (after Folmer et al. 1994), following similar procedures as for the 16S fragments. The DNA sequences were aligned using the Clustal X.81 alignment program (Thompson et al. 1997). Analyses were limited to reliably aligned regions from the data set. Regions that could not be unambiguously aligned were excluded from analysis.

The phylogenetic analyses were conducted with the computer programs PAUP\* version 4.0b10 for 32-bit Microsoft Windows (Swofford 2001) and MEGA version 2.1 (Kumar et al. 2001).

Table 1. List of the species sequenced

Species	Locality	16S	COI
<i>Allolobophora dacica</i> (Pop, 1938)	311 Romania, Cladova, Zarand Mts. 14.09.2001. Leg. V.V. Pop	+	+
<i>Allolobophora leoni</i> Michaelsen, 1891	310 Romania, Timisoara, Bega canal. 13.09.2001. Leg. V.V. Pop	+	+
<i>Allolobophora mehadiensis</i> Rosa, 1895	314 Romania, Corbesti, Zarand Mts. 14.09.2001. Leg. V.V. Pop	+	+
<i>Allolobophora robusta</i> Rosa, 1895	301 Romania, Rachitova, Caras Severin distr. 23.10.2001. Leg. G. Stan	+	+
<i>Allolobophoridaella eiseni</i> (Levinsen, 1884)	398 Hungary, Bataapáti. 24.04. 2003. Leg. Cs. Csuzdi	+	+
<i>Aporrectodea</i> (s.l.) <i>dubiosa</i> (Örley, 1881)	374 Romania, Danube delta, Sulina. 17.07.2000. Leg. A. A. Pop	+	+
<i>Aporrectodea</i> (s.l.) <i>molleri</i> (Rosa, 1889)	445 Portugal, Azores, St. Marie. 2004. Leg. A. Zicsi Jr.	+	+
<i>Aporrectodea caliginosa</i> (Savigny, 1826)	379 Romania, Tureni, Cluj distr. 20.11.2002. Leg. V.V. Pop	+	+
<i>Aporrectodea handlirschi</i> Rosa, 1897	446 Hungary, Mátra Mts. 2005. Leg. Z. Fehér	+	+
<i>Aporrectodea limicola</i> Michaelsen, 1890	391. USA, MD, Baltimore. 08.11.2002. Leg. Cs. Csuzdi & K. Szilávecz	+	+
<i>Aporrectodea longa</i> Ude, 1885	397 Switzerland, Geneva. 10.04.2003. Leg. S. Mahunka	+	-
<i>Aporrectodea rosea</i> (Savigny, 1826)	331 Romania, Cara, Cluj distr. 23.10.2002. Leg. V.V. Pop	+	+
<i>Aporrectodea sineporis</i> (Omodeo, 1952)	398 Hungary, Bataapáti. 24.04.2003. Leg. Cs. Csuzdi	+	+
<i>Bimastos palustris</i> Moore, 1893	390 USA, MD, Jug-Bay. 11.11.2002. Leg. Cs. Csuzdi & K. Szilávecz	+	+
<i>Bimastos tumidus</i> (Eisen, 1874)	391 USA, MD, Baltimore. 08.11.2002. Leg. Cs. Csuzdi & K. Szilávecz	+	+
<i>Cernosvitovia opisthocystis</i> (Rosa, 1895)	386 Romania, Cerna valley, Bedina river. 22.10.2003. Leg. V.V. Pop	+	+
<i>Dendrodriilus r. subrubicundus</i> (Eisen, 1874)	392 USA, MD, Baltimore. 08.11.2002. Leg. Cs. Csuzdi & K. Szilávecz	+	+
<i>Eisenia andrei</i> Bouché, 1972	411 Spain, Vigo. 10.2002. Leg. J. Dominguez	+	+
<i>Eisenia fetida</i> (Savigny, 1826)	373 Romania, Deva. 22.08.2001. Leg. V.V. Pop	+	+
<i>Eisenia lucens</i> Waga, 1857	375 Romania, Bihor Mts. 20.08.2001. Leg. V.V. Pop	+	+
<i>Eisenoides loembergi</i> (Michaelsen, 1894)	390 USA, MD, Jug-Bay. 11.11.2002. Leg. Cs. Csuzdi & K. Szilávecz	+	+
<i>Enchytraeus albidus</i> Henle, 1837	415 Germany. 11. 2003. Leg. & det. J. Römbke	+	+
<i>Octodrilus frivaldszkyyi</i> (Örley, 1885)	364 Romania, Bihor Mts., Padis. 19.10.2000. Leg. A. A. Pop & V.V. Pop	+	+
<i>Octodrilus permagnus</i> V.V. Pop, 1989	234 Romania, Metaliferous Mts. 09.10.2002. Leg. V.V. Pop	+	+
<i>Octodrilus pseudolissaeioides</i> Zicsi, 1994	399 Hungary, Szigetköz, Denkpál. 10.06.2003. Leg. Cs. Csuzdi	+	-
<i>Octodrilus pseudotranspadanus</i> (Zicsi, 1971)	402 Hungary, Murarátka. 18.04.2003. Leg. Cs. Csuzdi	-	+
<i>Octodrilus transpadanus</i> (Rosa, 1884)	310 Romania, Timisoara, Bega canal. 13.09.2001. Leg. V.V. Pop	+	+
<i>Octolasion cyaneum</i> (Savigny, 1826)	409 Finland. 2002. Leg. J. Terhivuo	+	+
<i>Octolasion lacteum</i> (Örley, 1881)	317 Romania, Tureni, Cluj distr. 04.10.2001. Leg. V. Stefan	+	+
<i>Perelia galileana</i> Csuzdi & Pavlicek, 2005	396 Israel, Tel Keshet. 08.01.2003. Leg. T. Pavlicek	-	+
<i>Proctodrilus tuberculatus</i> (Cernosvitov, 1935)	403 Hungary, Szentmargitfalva. 23.10.2003. Leg. Cs. Csuzdi	+	+

## RESULTS AND DISCUSSION

### 16S rDNA sequences

Partial sequences for 16S rDNA of 28 earthworm species were obtained. Out of the 386 characters, 250 were constant (67.8%), 64 were variable but parsimony uninformative (16.6%) and 72 were parsimony informative (18.6%). The parsimony bootstrap analysis was carried out with two options, namely by tree-bisection-reconnection (TBR) and subtree-pruning-regrafting (SPR) methods.

**The TBR bootstrap analysis** resulted in a tree (Fig. 1) with 452 steps, consistency index 0.445, homoplasy index 0.555, and retention index 0.435. The topology of the tree shows eight more or less distinct clades.

The first clade is formed by the *Octodrilus* species with *Octolasion* as sister group. This clade has a moderate bootstrap support (36%), but the two inside clade proved to be clearly monophyletic (70% and 98 % bootstrap support, respectively).

The second and third clades, branching off by polytomy, comprise all species attributed to the *Aporrectodea* genus, but with practically no bootstrap support. Interestingly, *Allolobophora leoni*, the type species of the genus *Pannoniona* Msršić & Sapkarev, 1988, is nested within the third clade along with *Aporrectodea dubiosa* and *Ap. molleri*, both relegated to *Heraclescolex* Qiu & Bouché, 1998.

The fourth clade contains only *Proctodrilus tuberculatus*, the sole member representing this genus in our analysis. The very low bootstrap support might be due in this case to the lacking of other species of the genus.

The branching pattern of the fifth clade (*Dendrodrilus*, *Bimastos* and *Allolobophora-ridella* species) and the sixth clade (*Eisenia* spp) is quite similar to the pattern obtained for the 18S rDNA analysis of the *Dendrobaena sensu lato* group (Cech & Csuzdi, 2006).

A very remarkable group, clustering far from the *Allolobophora* (sensu lato) species, is formed by four Central European endemics, namely *Allolobophora mehadiensis*, *A. robusta*, *A. dacica*, and *Cernosvitovia opisthocystis*. This grouping questions the validity of the genera *Serbiona* Msršić & Sapkarev, 1988 (erected for *A. robusta*, *A. mehadiensis*, and some ten related species), *Cernosvitovia* Omodeo, 1956 (including also *C. opisthocystis*) and *Karpatodinariona* Msršić & Sapkarev, 1988 (including also *A. dacica*).

The last clade, placed in a quite basal position, contains the North American species *Eisenoides loennbergi*.

**The SPR bootstrap analysis** resulted in a tree (Fig. 2) of 461 steps, consistency index 0.436, homoplasy index 0.564 and retention index 0.414. A branching pattern similar to the TBR tree is observed. The *Aporrectodea* group is also present here, but without *Ap. Limicola*, which is shifted to a far basal position. The other groups appear with the same internal topology but with different external linkages.

**The Minimum Evolution (ME)**, a distance based analysis of the 16S rDNA, produced a tree (Fig. 3) of 458 steps, consistency index 0.439, homoplasy index 0.561 and retention index 0.421. The branching pattern concerning the identified groups- is almost identical

to the parsimony trees, but their relative distances are nevertheless different. The only remarkable difference in the inner structure of the clades is the unresolved pattern of the *Eisenia* species.

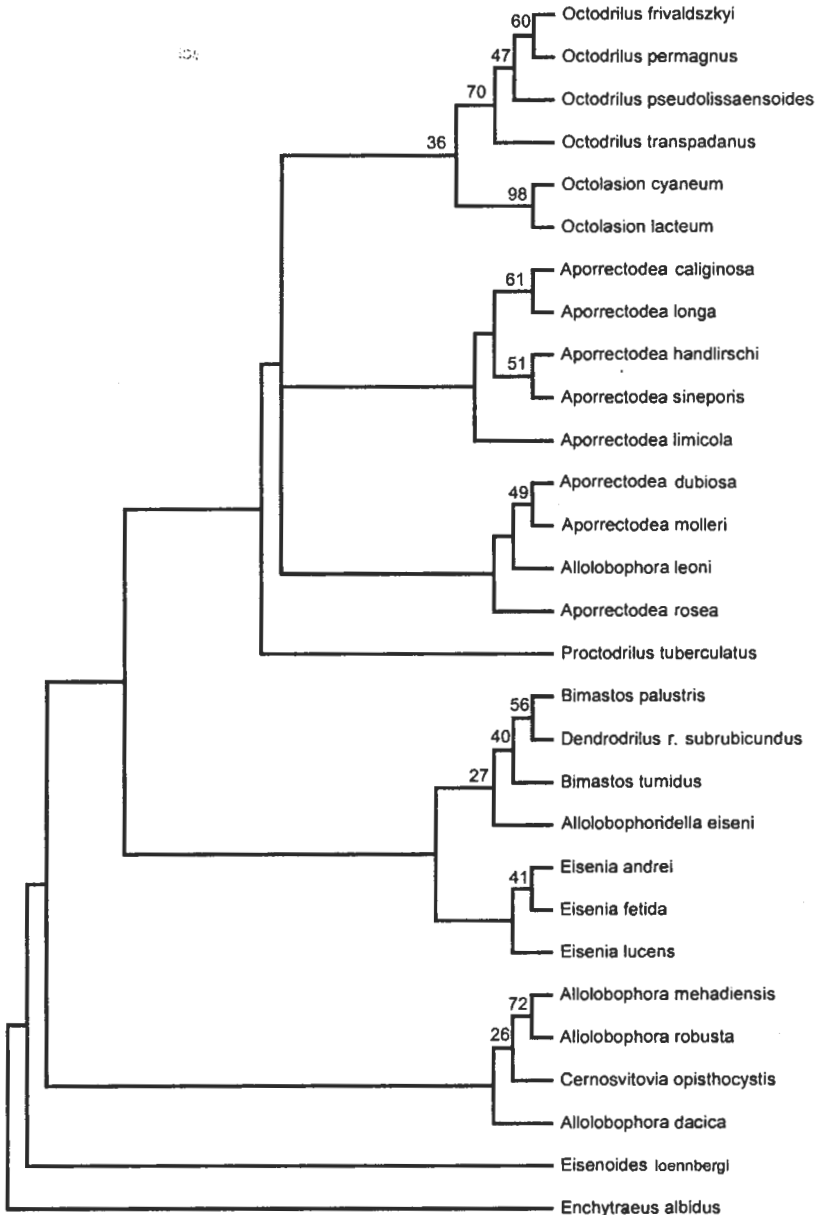


Fig.1. Maximum parsimony bootstrap tree with TBR method based on 16S rDNA analysis

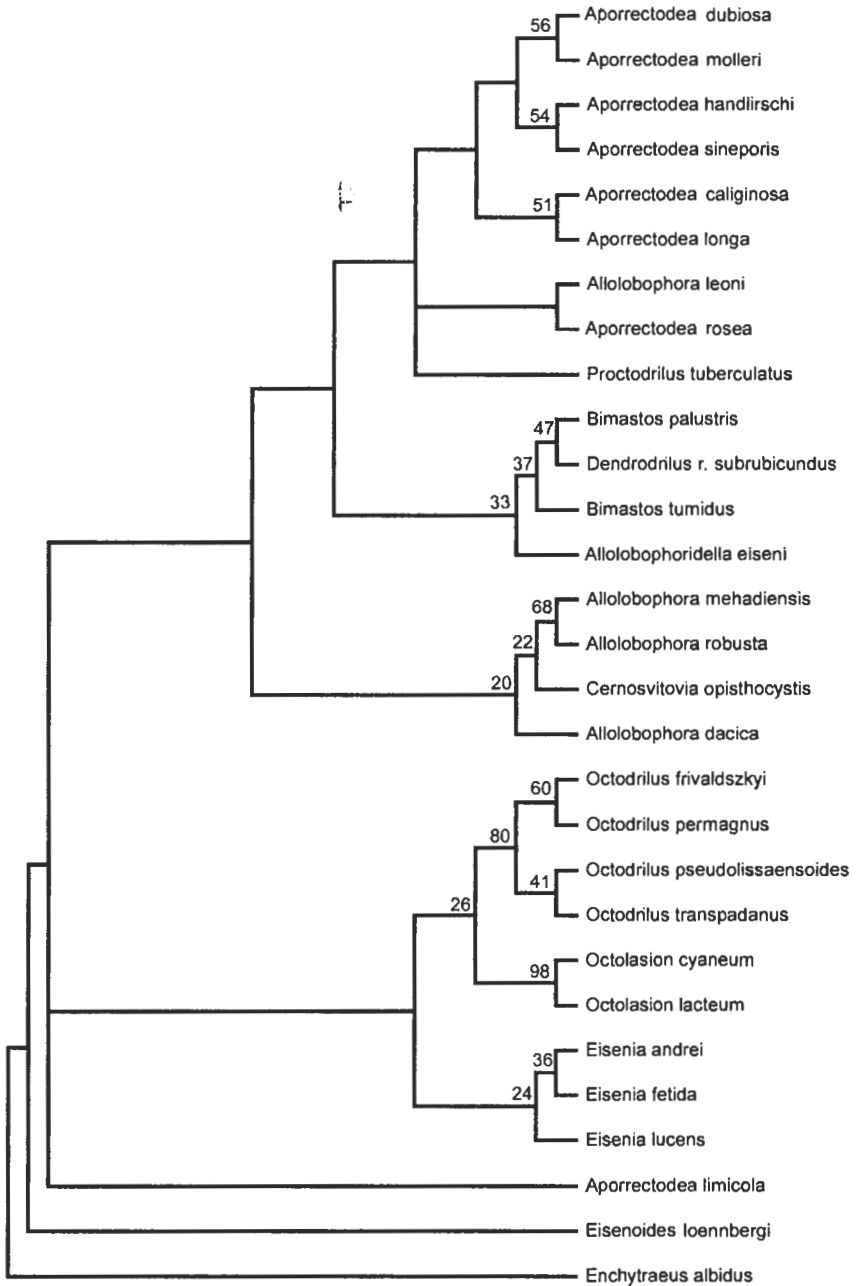


Fig. 2. Maximum parsimony bootstrap tree with SPR method based on 16S rDNA analysis.  
Bootstrap support lower than 20% not shown

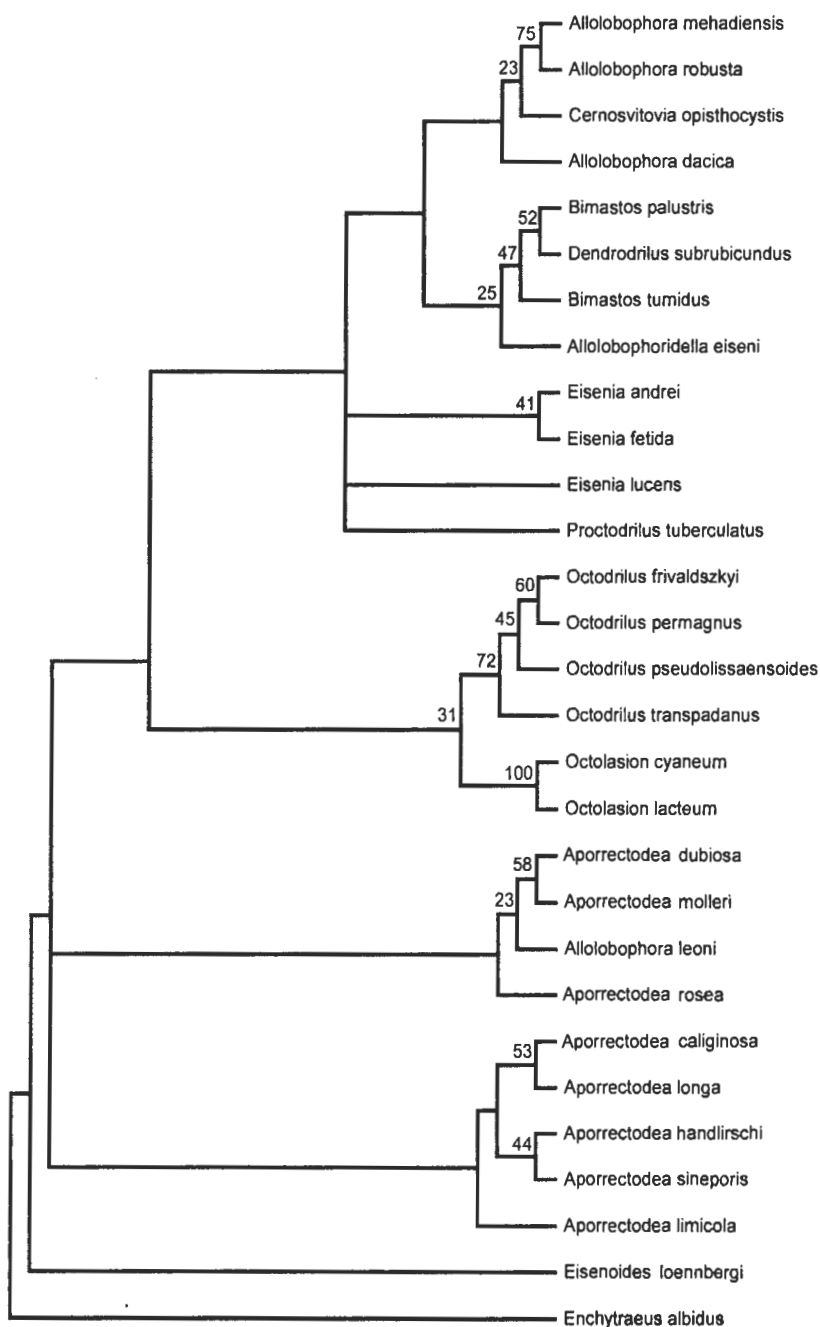


Fig. 3. Minimum Evolution bootstrap tree based on 16S rDNA analysis.  
 Bootstrap support lower than 20% not shown

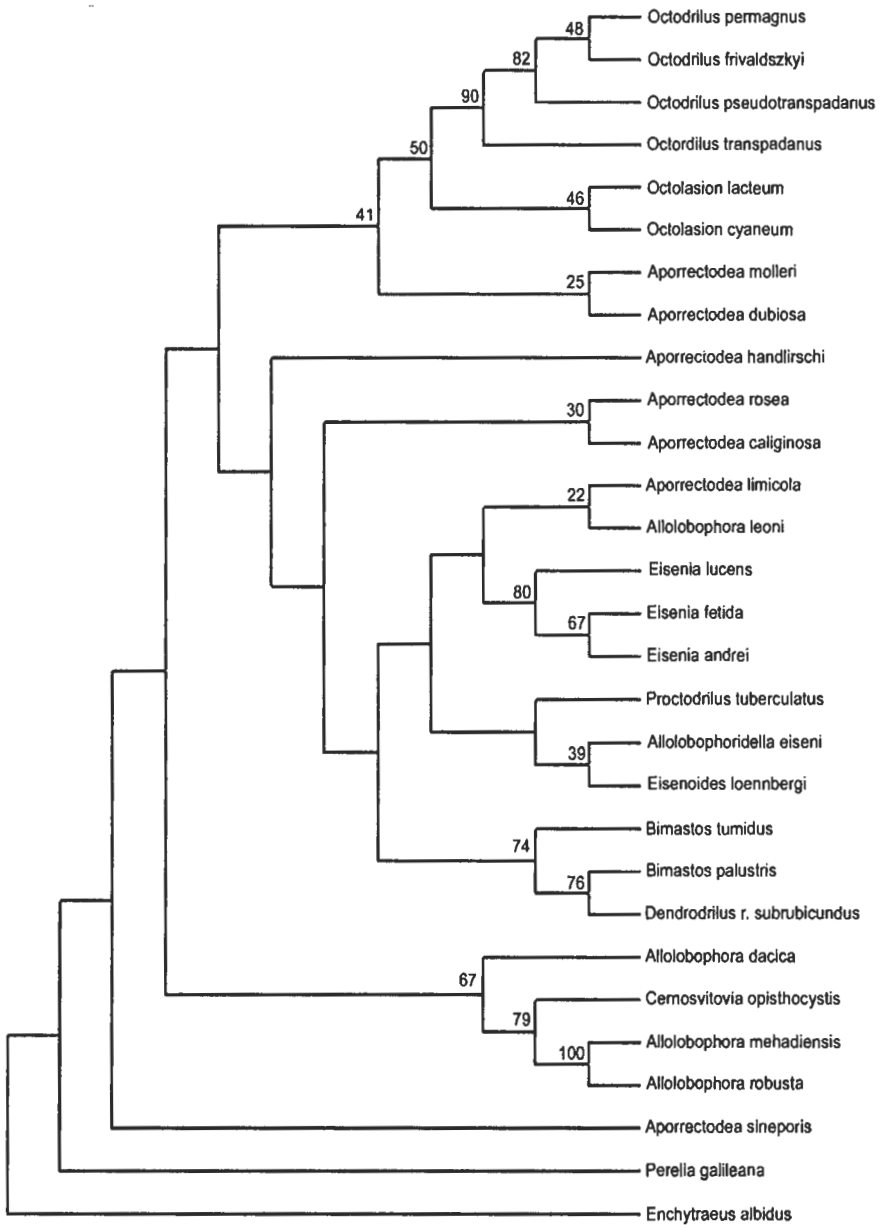


Fig. 4. Maximum parsimony bootstrap tree with TBR method and reweighted character scores based on COI analysis. Bootstrap support lower than 20% not shown

## COI sequences

Partial sequences for cytochrome *c* oxidase subunit I of 28 earthworm species were obtained (Table 1). The parsimony analysis of the 626 characters, from of which 343 were constant (54.8%), 41 were variable but parsimony uninformative (6.5%) and 241 were parsimony informative (38.5%) produced a highly unsettled tree with scattered species position. Therefore, an "a posteriori" weighting of the characters, according to the rescaled consistency index as implemented in the PAUP\* version 4.0b10 software, was performed. The weighted MP tree (Fig. 4) had a length of 1509 steps, consistency index 0.514, homoplasy index 0.493 and retention index 0.423. This measure improved considerably the topology of the tree and made its topology comparable to that of the 16S trees.

The majority of the groups revealed by the 16S analysis are also recovered in the COI tree, sometimes with even higher bootstrap support. Nevertheless, the species attributed to *Aporrectodea* are scattered quite randomly. It is worth to note the separation of *Ap. molleri* and *Ap. dubiosa* from the other *Allolobophora* sensu lato species recalling the genus *Heraclescolex* Qiu & Bouché, 1998. However, the low bootstrap support does not allow their separation in a distinct genus.

## CONCLUSION

The branching pattern of the *Allolobophora* sensu lato group obtained by molecular phylogenetic investigations confirms its highly polyphyletic nature. Nevertheless, inside this catch-all genus some well-defined groups of species can be identified in all trees, such as the *Allolobophora dacica*, *A. mehadiensis*, *A. robusta*, and *Cernosvitovia opisthocystis* quartet present. This group is quite homogeneous, both in terms of morphology and biogeography. The slight differences in the position of male pores or in the form of nephridial bladders do not justify their separation into three distinct genera. Thus, considering the priority rules, all these species could be possibly classified into the genus *Cernosvitovia* Omodeo, 1956.

The bulk of *Aporrectodea* s.l. species was almost always recognizable, but never with unequivocal monophyly. To clarify their relationships further investigations, involving new gene sequences, are needed.

The distinct but different position of *P. tuberculatus* in the all trees supports an independent and valid status of the genus *Proctodrilus*.

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