Revision of the *Dendrobaena alpina* (Rosa, 1884) species group (Oligochaeta, Lumbricidae) by morphological and molecular methods

Csaba Csuzdi [1], Adriana Antonia Pop [2, 3, 4], Victor V. Pop [2], Michael Wink [4] and András Zicsi [5]

[1]Department of Zoology, Hungarian Natural History Museum, Baross. 13. H-1088 Budapest, Hungary. csuzdi@zoo.zoo.nhmus.hu
[2]Institute of Biological Research, 48 Republicii Street. POBox 229, 4000015 Cluj-Napoca, Romania. antoniapop2001@yahoo.com and victorvpop@yahoo.com
[3]Technical Univeristy Darmstad, Institute of Zoology, Schnittspahnstrasse 3, 64287 Darmstadt, Germany. pop@bio.tu-darmstadt.de
[4] Ruprecht-Karls University, Institute of Pharmacy and Molecular Biotechnology, Im Neuenheimer Feld 364 , 69120 Heidelberg, Germany. wink@uni-hd.de
[5] Systematic Zoology Research Group of HAS, ELTE University 1117 Budapest, Pázmány P. sétány 1/C, Hungary. zicsi@cerberus.elte.hu

SUMMARY. A molecular phylogeny, based on sequences of 16S rDNA and the mitochondrial cytochrome *c* oxidase subunit I from the *Dendrobaena alpina* species complex was reconstructed. The results clearly indicate the validity of *Dendrobaena clujensis*, and the previous subspecific division of *D. alpina* into *D. alpina alpina* and *D. alpina alteclitellata*. The molecular investigations also revealed that the small unpigmented earthworms from the Apuseni Mts. should be regarded as a distinct, yet undescribed species.

INTRODUCTION

The *Dendrobaena alpina* species group contains some fifteen nominal species including several apparently synonymous ones, as well. It can be characterized by a clitellar position on segments 26, 27-33, 34 and tubercles around 30-32. A common feature of these species is the modification of the vascular system with disappearance of the extraoesophageal vessel and frequently the reduction of the last pair of hearts from segment 11. These species show a great variability regarding the number of vesicles, the position of the spermathecal pores, and the pigmentation.

On the basis of the arrangement of the nephridial pores two distinct subgroups can be distinguished. To the first belong the species with irregularly alternating nephropores between setal line *b* and above setal line *d* (*D. alpina* and allies), and the second being characterized by a regularly alternating nephropore system (*D. attemsi* and allies).

In the previous records from Romania (Pop, 1948, 1964, V. V. Pop, 1971, 1972, 1997) all the specimens with spermathecal pores close to the mid-dorsal line were relegated to the species *D. alpina*.

V.V. Pop (1972) emphasized that this species in the Retezat Mts. is represented by two "morphotypes", namely: a smaller one with trapezoidal clitellum on segments ½ 28-½/ 34 and a larger one with parallel clitellum on segments 26, 27-33. A re-examination of the *D. alpina* material from the Retezat Mts. housed in the Institute of Biological Research, Cluj-Napoca, revealed that these two morphotypes belong to different sub-groups of the *D. alpina* species complex. While the larger specimens with parallel clitellum-edges possess irregularly alternating nephropores and represent *D. alpina alpina* (Rosa, 1884), the smaller specimens with trapezoidal clitellum possess regularly alternating nephropores and represent *D. attemsi* (Michaelsen, 1902).

Analysis of a large *D. alpina* material from the Apuseni Mts. revealed that these specimens are unpigmented and possess regularly alternating nephridiopores, a character, which clearly distinguish them from the representatives of *D. alpina* sensu Rosa (1884). According to this character and the trapezoidal form of the clitellum they are similar to *D. attemsi*, but differ from this species by the mid-dorsal opening of spermathecae and the lack of the coiled spermathecal peduncles.

The specimens belonging to the species *D. alpina* themselves also represent two quite distinct groups, as well. The first, distributed in the Southern Carpathians, is medium-sized (4-6 cm) and slightly pigmented, and the second, distributed in the Northern Carpathians, is somewhat larger (6-10 cm) and possess dark red-violet pigmentation. Characters of the first group fit the typical *D. alpina* described by Rosa from the Alps (Rosa, 1844). The second group of *D. alpina* was described by Pop (1938) as a distinct subspecies namely *D. alpina alteclitellata* Pop, 1938, but subsequently rejected and removed to the nominotypical species (Pop, 1964). A similar subspecies was described by Černosvitov (1935) from the Northern part of Eastern Carpathians (Sinevir range) as *Eisenia veneta cognettii* Černosvitov, 1935, which was synonymized to *D. alpina* by Perel (1972).

According to our opinion, these two heavily pigmented populations belong to the same group and deserve a subspecies rank. As the senior available name [*Dendrobaena alpina cognettii* (Černosvitov, 1935)] is a junior homonym of *Dendrobaena cognettii* (Michaelsen, 1903), the other available name *Dendrobaena alpina alteclitellata* Pop, 1938 must be regarded as valid.

MATERIAL AND METHODS

Complex morphological investigations – considering recently used characters, as well – were carried out on the whole *D. alpina* (sensu lato) material of the Institute of Biological Research, Cluj and the Hungarian Natural History Museum, Budapest.

Table 1. Sequenced earthworm specimens

Species	**Locality**	**16S**	**COI**
Dendrobaena clujensis Pop, 1938	330 Romania, Bihar Mts. Marisel. 17.10.2002 Leg. V.V. Pop & A.A Pop	+	+
D. clujensis Pop, 1938	327 Romania, Trascau Mts. 08.10.2002. Leg. V.V. Pop, A.A Pop, A. Pop	–	+
D. clujensis Pop, 1938	438 Romania, Gutin Mts. 01.07.2005. Leg. Cs. Csuzdi & V.V. Pop	+	+
D. clujensis Pop, 1938	434 Romania, Vladeasa, Frantura 1300 m. 29.07.2005. Leg. Cs. Csuzdi	+	+
D. alpina alpina (Rosa, 1884)	330 Romania, Bihar Mts. Marisel. 17.10.2002 Leg. V.V. Pop & A.A Pop	+	+
D. alpina alpina (Rosa, 1884)	386 Romania, Cerna Mts. 22.10.2003. Leg. V.V. Pop	+	–
D. alpina alpina (Rosa, 1884)	436 Romania, Retezat Mts, Rotunta. 30.07.2005. leg. Cs. Csuzdi & V.V. Pop	+	+
D. alpina alpina (Rosa, 1884)	437 Romania, Retezat Mts, Stanuleti. 30.07.2005. leg. Cs. Csuzdi & V.V. Pop	+	+
D. alpina alteclitellata Pop, 1938	440 Romania, Rodna Mts. Borsa. 29.06.2005. Leg. J. Kontschán & D. Murányi	+	–
D. alpina alteclitellata Pop, 1938	441 Ukraine, Sinevir National Park. 07.07.2003. Leg. B. Cser	–	+
D. attemsi (Michaelsen, 1902)	436 Romania, Retezat Mts, Rotunta. 30.07.2005. leg. Cs. Csuzdi & V.V. Pop	+	+
D. attemsi (Michaelsen, 1902)	437 Romania, Retezat Mts, Stanuleti. 30.07.2005. leg. Cs. Csuzdi & V.V. Pop	+	+
D. sp. nov.	433 Romania, Vladeasa, Frantura 1000 m. 29.07.2005. Leg. Cs. Csuzdi & M. Csuzdi	+	+
D. sp. nov.	434 Romania, Vladeasa, Frantura 1300 m. 29.07.2005. Leg. Cs. Csuzdi & M. Csuzdi	+	+
D. sp. nov.	435 Romania, Vladeasa, Rachitele. 29.07.2005. Leg. Cs. Csuzdi & M. Csuzdi	+	–
D. byblica (Rosa, 1893)	314 Romania, Zarand Mts. Corbesti. 14.09.2001. leg. V.V. Pop	+	+
D. cognettii (Michaelsen, 1903)	390 Hungary, Bátaapári. 24.04.2003. Leg. Cs. Csuzdi	+	+
Dendrodrilus r. rubidus (Savigny, 1826)	373 Romania, Deva, Mures River. 22.08.2001. Leg. V.V. Pop	+	+
Dd. r. subrubicundus (Eisen, 1874)	392 USA, MD. Baltimore. 08.11.2002. Leg. Cs. Csuzdi & K. Szlávecz	+	+
Fitzingeria pl. platyura (Fitzinger, 1833)	389 Romania, Cerna Mts. 22.10.2003. Leg. V.V. Pop	+	–
F. pl. depressa (Rosa, 1893)	398 Hungary, Bátaapáti. 24.04.2003. Leg. Cs. Csuzdi	–	+
Enchytraeus albidus Henle, 1837	415 Germany. 11.2003. Leg. & det. J. Römbke.	+	+

For molecular studies, fresh material of 21 samples, representing 11 lumbricid taxa and one enchytraeid species, have been collected and analysed (Table 1). The adult earthworms 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 identifications, 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 of 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 *16 sbr* (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 denaturating at step 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 and 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). All the trees were rooted with *Enchytraeus albidus*.

RESULTS AND DISCUSSION

16S rDNA sequences

Partial sequences for 16S rDNA of 18 populations representing 11 earthworm species or subspecies were obtained. The parsimony analysis of molecular data (385 bp) resulted in 14 most parsimonious (MP) trees with 357 steps in length, consistency index 0.602, homoplasy index 0.397 and retention index 0.655. Out of 385 characters, 239 are constant (62.1%), 52 are variable but parsimony uninformative (13.5%) and 94 are parsimony informative (24.4%).

The topology of the MP bootstrap consensus tree (Fig. 1.) agrees well with the morphological groupings of the *D. alpina* species group. The four samples of the *D. alpina alpina* form a clear monophyletic group together with the samples of *D. alpina alteclitellata* and *D. clujensis*, with moderate bootstrap support (50%). Within this clade

each taxon (*D. alpina alpina*, *D. a. alteclitellata,* and *D. clujensis*) seems to be monophyletic, with a quite remarkable support. It is interesting that *D. alpina alteclitellata* is nested with *D. clujensis,* but in the same time the monophyly of *D. clujensis* is apparent (bootstrap support 87%). This grouping is also supported by morphological features, both species being intensively red pigmented as opposed to *D. alpina alpina,* which is only slightly pigmented.

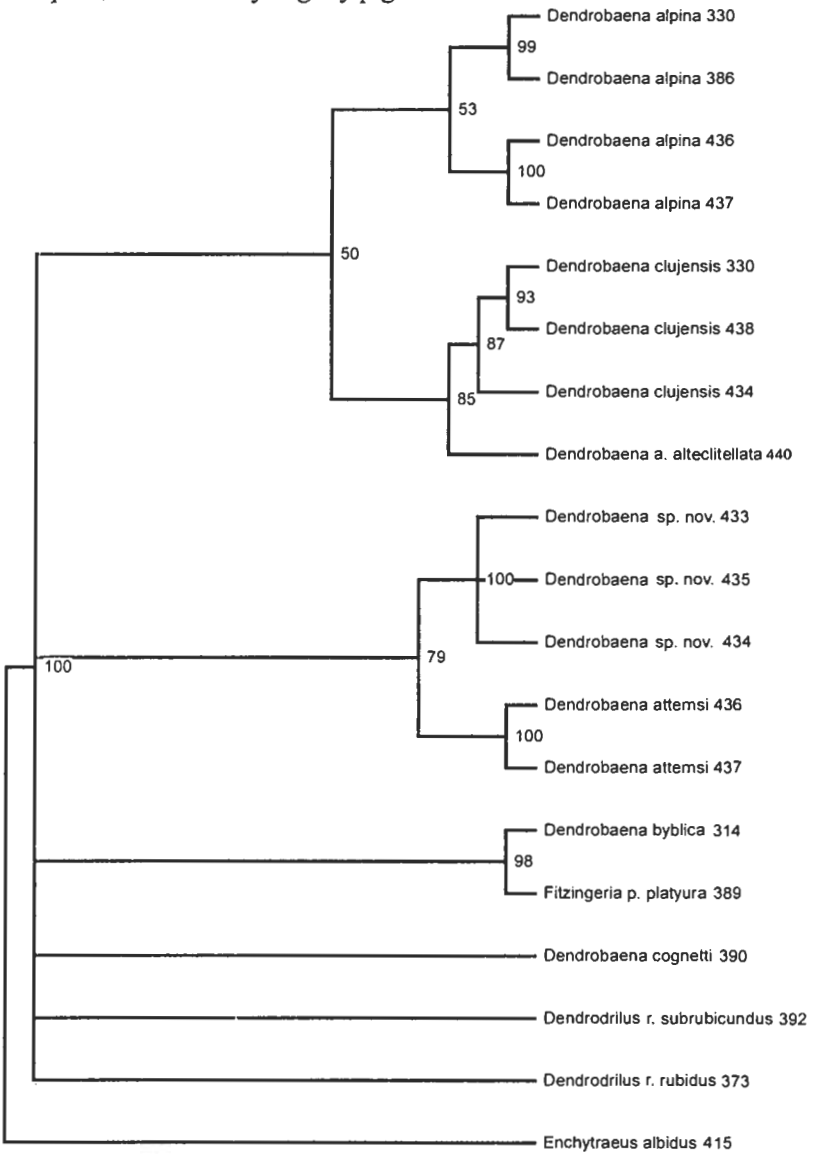

Fig. 1. Maximum parsimony bootstrap tree based on 16S rDNA analysis. Bootstrap support lower than 50% not shown.

Another monophyletic clade, with a high support (79%), is formed by *D. attemsi* and the new species previously relegated to *D. alpina*. However, both species are clearly distinct (with bootstrap support 100 % in each case). All members of this second cluster are characterized by the same regularly alternating pattern of nephridiopores, small size, and trapezoidal aspect of the clitellum.

The Neighbour joining (NJ) analysis of the 16S rDNA shows quite similar branching patterns. The bootstrap consensus tree (Fig. 2.) shows high values for all the recognised subgroups of the *D. alpina* species group. Both *D. attemsi* and the new species are supported by 100% bootstrap values and the clade itself containing these species is very robust (84% bootstrap support). The populations of the nominal subspecies of *D. alpina* join with 94% support and *D. clujensis* with 80%. The moderate support of the sister position of *D. a. alpina* and the clade formed by *D. clujensis* is probably due to the uncertain position of *D. a. alteclitellata*. To clarify the real affinities of this subspecies, samples from more populations are needed.

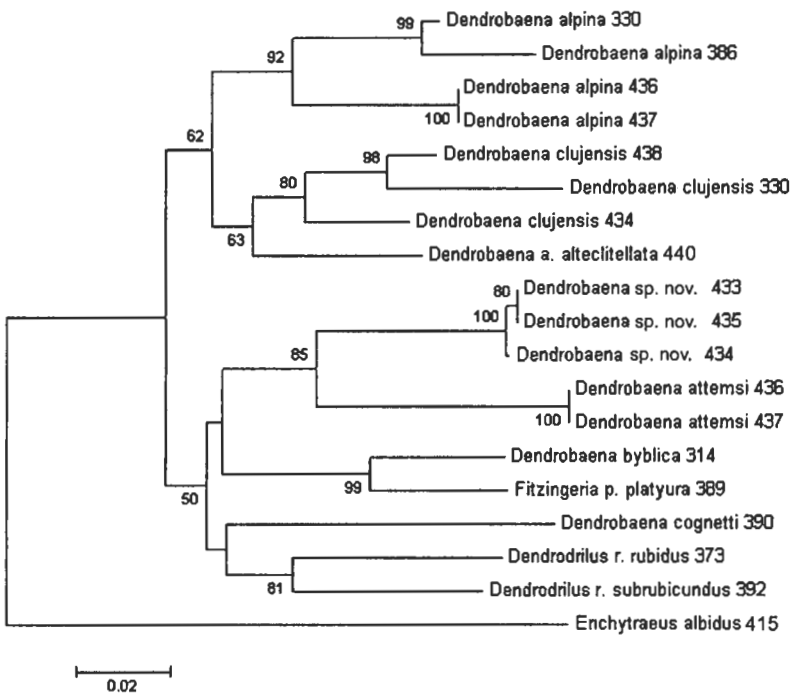

Figure 2. Neighbor Joining bootstrap tree based on 16S rDNA analysis. Bootstrap support lower than 50% not shown

COI gene sequences

Partial sequences for cytochrome *c* oxidase subunit I of 17 populations representing 11 earthworm species or subspecies were obtained. The Maximum parsimony analysis of molecular data (626 bp) resulted in one most parsimonious (MP) tree of 907 steps

length, consistency index 0.456, homoplasy index 0.543, and retention index 0.532. Out of 626 characters, 346 are constant (55.3%), 40 are variable but parsimony uninformative (6.4%), and 222 are parsimony informative (35.5%).

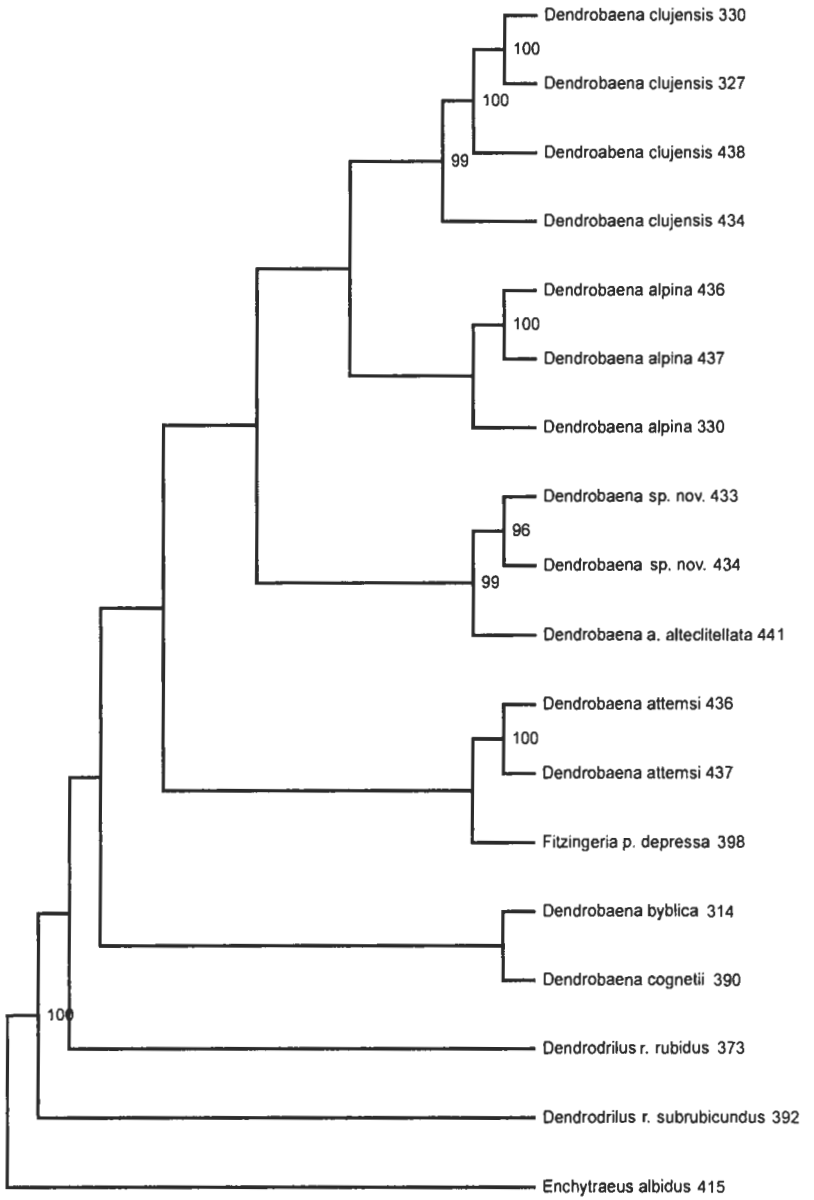

Figure 3. Maximum parsimony bootstrap tree based on COI analysis. Bootstrap support lower than 50% not shown.

The topology of the bootstrap MP tree (Fig. 3) is quite similar to that of the corresponding 16S tree. A main difference exists in the position of *D. alpina alteclitellata,* which branches off together with the new species (99% bootstrap support). This branching pattern also explains the clear sister group position of *D. alpina alpina* and *D. clujensis* (though with only 39% support), as well as the splitting of *D. attemsi* from *Dendrobaena* sp.nov. clade. Another interesting feature of this tree is the position of *Fitzingeria pl. depressa* attached to the *D. attemsi* clade.

The NJ bootstrap consensus tree (Fig. 4) shows a similar topology with very high support of the ingroup clades. Nevertheless, the relatedness of the different clades are less supported or even unsupported as compared with that in 16S tree. This situation confirms the fact that the COI gene is especially a reliable indicator of relatedness at lower taxa levels (at population or subspecies level or among very closely related species) but of less informative significance at higher taxonomic levels.

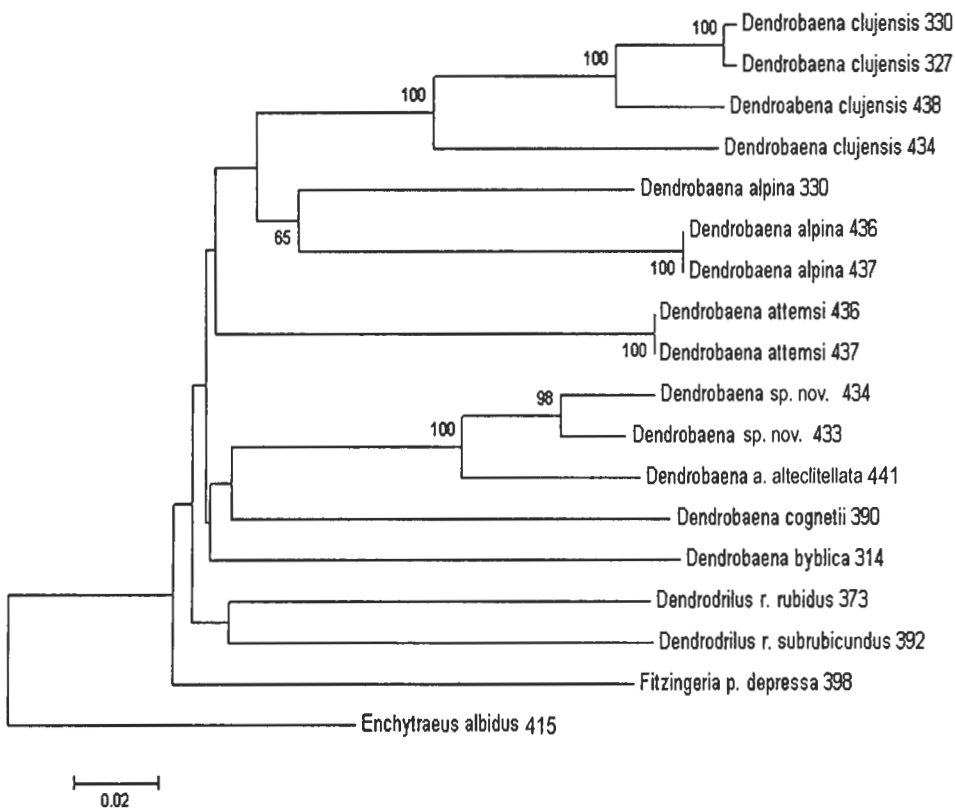

Figure 4. Neighbor Joining bootstrap tree based on COI analysis. Bootstrap support lower than 50% not shown

CONCLUSION

The branching pattern of the *Dendrobaena alpina* species group revealed by molecular phylogenetic investigations confirms its sub-groupings according to the morphological characters, into five distinct taxa:

(i) *D. alpina alpina*, the nominal subspecies occurs in the Alps, the Southern Carpathians and Balkans.

(ii) *D. a. alteclitellata* is distributed in the Eastern and North-Eastern parts of the Carpathian Mountains.

(iii) *D. clujensis*, which was questioned by Perel (1972), is highly supported as a distinct species occurring from the Dacian Hills to the Pannonian Lowlands.

(iv) *Dendrobaena* sp.nov. should be erected for a group of small whitish earthworms, endemics in the Apuseni Mts.

(v) *D. attemsi* attributed by Pop and V.V Pop for a long time to *D. octaedra* or *D. alpina*, respectively, is proved to be clearly distinct. It is a widely distributed transaegean mountainous species.

ACKNOWLEDGEMENTS

This study was partly supported by the Hungarian Scientific Research Grant (No. T42745), and partly by the Romanian Scientific Grant CEEx No. 05-D11-82. Our gratitude to all colleagues indicated in Table 1 for collecting valuable earthworm samples, suited for DNA analysis. The third author acknowledges the financial support of the "European Commission's Research Infrastructure Action via the SYNTHESYS Project, GB-TAF-328" for a research visit to the Natural History Museum in London.

REFERENCES

Cech, G., Csuzdi Cs., Márialigeti, K. (in press). Remarks on the molecular phylogeny of the genus Dendrobaena (sensu Pop 1941) based on the investigation of 18S rDNA sequences in: V.V. Pop and A.A. Pop (Eds). Advances in Earthworm Taxonomy II.

Cernosvitov, L. 1935. Monografie československých dešťovek /Monographie des tschechoslovakischen Lumbriciden/. *Archiv pro Přirodovědecký výzkum čech.* 19.: 1-86.

Folmer, O., Black, M., Hoch, W., Lutz, R., Vrijenhoek, R. 1994. DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3: 294-299.

Kumar, S., Tamura, K., Jakobsen, J. C., Nei, X. 2001. MEGA2 : Molecular Evolutionary

Genetics Analysis software, Arizona State Univerisity, Tempe, Arizona, USA.

Mrsič, N. 1991. Monograph on earthworms (Lumbricidae) of the Balkans. *Academia scientiarum et artium Slovenica, Historia naturalis, Ljubljana*, 31, I: 1-355; II: 356-757.

Palumbi, S., Martin, R., Romano A., McMillan,W. O., Stice, L., Grabowski, G. 1991. The simple fool's Guide to PCR, Version 2. University of Hawaii Zoology Department, Honolulu. Manual, second edition. Cold Spring Harbor Laboratory, New York.

Pereli, T. 1972. Species of the genus *Dendrobaena* in the Fauna of the USSR. *Zoologicheseki Zhurnal* 51 (12): 1788-1797.

Pop, V. 1938 Neue Lumbriciden aus Rumänien. *Bul. Soc. Sti. Cluj*, 9: 134-152.

Pop, V. 1948. Lumbricidele din România. *Anal. Acad. Republicii Populare Romane. Sect. Stiinte Geologice, Geografice, Biologice.* Ser. A, Mem. 9: 383-506.

Pop V. 1964. New faunistic and systematic data on the Romanian Lumbricids (Oligochaeta). *Studia Univ. Babes-Bolyai Cluj, Ser.Biol.* 2:106-116.

Pop, V. V. 1972. Contributions to the study of Lumbricids (Oligochaeta) from the Retezat National Park. *Ocrotirea Naturii, Bucharest*, 16: 33-41.

Pop, V. V. 1987. Density and biomass of earthworm synusia in forest ecosystems of the Romanian Carpathians. in: Bonvicini Pagliai A.N. and P.Omodeo (Eds): On Earthworms. Selected Symposia and Monographs. U.Z.I.,2, Muchi, Modena, 183-190.

Pop, V. V. 1997. Earthworm - Vegetation - Soil Relationships in the Romanian Carpathians. *Soil Biology Biochemistry* 29: 223-229.

Rosa, D. 1884. *I Lumbricidi del Piemonte*, Torino, 1-55.

Sambrook J., Fritsch, E., Maniatis, T. 1989. Molecular Flonin. A Laboratory Manual, second edition. Cold Spring Harbor Laboratory, New York.

Swofford, D. L. 2001. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Thompson, J. D., Higgins, D. G., Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence wighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22: 4673-4690.