

Remarks on the molecular phylogeny of Crassiclitellata families using the mitochondrial 16S rDNA gene (Oligochaeta, Opisthopora)

Adriana Antonia Pop^{1,2,4}, Csaba Csuzdi³ and Michael Wink⁴

¹Institute of Biological Research, 48 Republicii Street. POBox 229, 4000015 Cluj-Napoca, Romania, antoniapop2001@yahoo.com

²Technische Universität Darmstadt, Institut für Zoologie, Schnittspahnstrasse 3, 64287 Darmstadt, Germany, pop@bio.tu-darmstadt.de

³Systematic Zoology Research Group of HAS and Department of Zoology, Hungarian Natural History Museum, Baross. Str. 13. H-1088 Budapest, Hungary. csuzdi@zoo.zoo.nhmus.hu

⁴Ruprecht-Karls University, Institute of Pharmacy and Molecular Biotechnology, Im Neuenheimer Feld 364, 69120 Heidelberg, Germany. wink@uni-hd.de

SUMMARY. A molecular phylogeny, based on sequences of 16S rDNA from representatives of different Crassiclitellata families was reconstructed. The results clearly recognised the main classical families, except within the superfamily Megascolecoida. In agreement with a previous molecular analysis (Jamieson et al. 2002) Glososcolecids proved to be the most basal Crassiclitellata taxon, but its sister position with Eudrilidae was not corroborated.

INTRODUCTION

The suborder Crassiclitellata was erected by Jamieson (1988) for the opisthoporans possessing a multilayered clitellum. This taxon, together with Alluroidina and Moniligastrida represents the so-called "megadrile" worms or earthworms.

The phylogeny and classification of the megadrile oligochaetes has long been discussed. After the classical work of Michaelsen (1900), there were several attempts (Stephenson 1930, Gates 1959, Jamieson 1971, Sims 1980, Jamieson 1988, Omodeo 2000) to resolve the evolutionary puzzle of this group and to present a phylogeny-based classification.

In the classical approaches several morphological characters were used to distinguish the family or subfamily level taxa, such as the presence/absence and position (if any) of the muscular gizzards, the calciferous glands, the excretory system, and the attributes of the genital system.

Unfortunately, the number of the reliable characters is quite low as compared to the diversity of the Crassicitellata groups. Therefore, the evaluation of the phylogenetic significance of morphologic characters is very difficult, taking into account possible homoplasies.

This problem was critical in the classification of the superfamily Megadriloidea in which, apart from the prostate apparatus, initially the number of muscular gizzards (Michaelsen, 1900, Stephenson, 1930), later the structure of the excretory system (Gates, 1959, Sims 1966, 1980) were used as defining discriminating characters.

Jamieson (1971) thoroughly criticised these classifications showing that they are based particularly on grades, rather than clades. He argued that the meronephric condition (a “defining” character of Octochaetidae sensu Gates, 1959) was acquired independently in several Australian genera and that the same process was perhaps true for the tubular-racemose structure of prostates (a key distinguishing feature between the families Acanthodrilidae and Megascolecidae). Therefore, he united all these groups into the sole family Megascolecidae, which was further subdivided to Ocnodrilinae, Acanthodrilinae, and Megascolecinae (Jamieson, 1971). However, this system had also several deficiencies. It did not take into account the bulk of Indian “Octochaetoid” genera, and furthermore merged the African and South American Dichogastrids and some typical Australian Megascolecids into the tribe Dichogastrini (subfamily Megascolecidae).

The subsequent general or partial revisions of the Crassicitellata families (Jamieson, 1988; Csuzdi 1996, 2000, Blakemore 2000, 2005) improved the existing system but, because they failed to introduce and use more reliable characters they were unable to solve its inherent problems.

Recently, a new and powerful method has been introduced in systematic studies, namely the use of different marker gene sequences in reconstructing phylogenies.

The first, and till now, only molecular investigation of Crassicitellata was published by Jamieson et al. (2002). This study focused mainly on the Megascolecoid taxa and apart from corroboration of some previously recognized schemes (such as the relatively basal position of Ocnodrilids towards the Medascolecoids and grouping the true Dichogastrids together with Acanthodrilids, etc.) it revealed some heuristic arrangements, as well. The most interesting novelty was the clade formed by Glossoscolecids and Eudrilids, two groups never associated previously. Unfortunately only three species (one Eudrilid and two Glossoscolecids) were analysed, not enough to prove the close affinities of these families.

The present study aims to evaluate the relatedness among Eudrilidae and Glossoscolecidae families using the mitochondrial 16S rDNA gene for a wider spectrum of species.

Table 1. List of the species newly analyzed and the sequences retrieved from the GeneBank with the accession numbers

Family	Species	Locality
Lumbricidae	<i>Allolobophora robusta</i> Rosa, 1895	Romania, Rachitova, Caras Severin distr. 23.10.2001. Leg. G. Stan
	<i>Cernovitovia opisthocystis</i> (Rosa, 1895)	Romania, Cerna valley, Bedina river. 22.10.2003. Leg. V.V. Pop
	<i>Lumbricus castaneus</i> (Savigny, 1826)	AY885579
	<i>Octodrilus b. bihariensis</i> Pop, 1989	Romania, Bihor Mts, Padis, Ponor 19.10.2000. Leg. A.A.Pop & V.V.Pop
	<i>Octodrilus b. rendzinicola</i> Pop, 1989	Romania, Bihor Mts, Padis, Seaca valley 19.10.2000. Leg. A.A.Pop & V.V.Pop
Glossoscolecidae	<i>Andiorrhinus</i> sp. 1 G2	Venezuela, Sierra Nevada Nat. Park. Paramo La Culata, 3290 m. 01. IV. 2005. Leg. Cs. Csuzdi
	<i>Andiorrhinus</i> sp. 2 G9	Venezuela, Sierra Nevada Nat. Park. Loma Redonda, 4050 m. 06. IV. 2005. Leg. S. Orbán
	<i>Glossoscolecidae</i> sp. G7	Venezuela, Sierra Nevada Nat. Park, Mucubajji., 05. IV. 2005. Leg. Cs. Csuzdi
	<i>Onychochaeta windlei</i> (Beddard, 1890)	Venezuela, Altamira. 29. III. 2005. Leg. Cs. Csuzdi
	<i>Pontoscolex corethrurus</i> (Müller, 1857) 96	Kenya, Ngulia Lodge. I. 2004 Leg. S. Mahunka
	<i>Pontoscolex corethrurus</i> (Müller, 1857) 101	Dominica. 2003. Leg. J. Kontschán
	<i>Hyperiodrilus africanus</i> Beddard, 1891	Kenya, Galu. I. 2004I. Leg. S. Mahunka
	<i>Polytoreutus montiskenyae</i> Beddard, 1902	Kenya, Galu. I. 2004I. Leg. S. Mahunka
	<i>Polytoreutus</i> sp.1	Kenya, near to Nairobi. XI. 2004. Leg. Cs. Csuzdi
	<i>Polytoreutus</i> sp.2	Kenya, near to Nairobi. XI. 2004. Leg. Cs. Csuzdi
	<i>Eukerria saltensis</i> (Beddard, 1895)	DQ257297
Ocnerodrilidae	<i>Eukerria saltensis</i> (Beddard, 1895)	USA, MD, Baltimore, Hillsdale. 10. XI. 2002. Leg. Cs. Csuzdi
	<i>Eukerria saltensis</i> (Beddard, 1895)	AF406590
	<i>Eukerria saltensis</i> (Beddard, 1895)	Kenya, near to Nairobi. XI. 2004. Leg. Cs. Csuzdi
	<i>Nematogonia panamaensis</i> (Eiscn, 1900)	

Acanthodriidae	<i>Diplocardia invecta</i> Gates, 1955	DQ257311
	<i>Diplocardia eiseni</i> Michaelsen, 1894	DQ257310
	<i>Diplocardia komareki</i> Gates, 1977 1.	DQ247768
	<i>Diplocardia komareki</i> Gates, 1977 2.	DQ257314
	<i>Diplotrema acropetra</i> Jamieson, 1997	AF406568
	<i>Diplotrema</i> sp.	AF406570
Benhamiinae	<i>Dichogaster</i> sp.	AF406571
	<i>Dichogaster saliens</i> (Beddard, 1893)	AF406573
Octochaetinae	<i>Octochaetus altanmoui</i> (Jamieson, 1997)	AF406569
Megascolecidae	<i>Amynthas aspergillum</i> (Perrier, 1872)	AY960826
	<i>Amynthas gracilis</i> (Kinberg, 1867)	AY960828
	<i>Amynthas hilgendorfi</i> (Michaelsen, 1892)	USA, MD, Baltimore, Loch Raven. 07. XI. 2002. Leg. Cs. Csuzdi
	<i>Amynthas robustus</i> (Perrier, 1872)	AY960829
	<i>Digaster lingi</i> Jamieson, 1995	AF406583
	<i>Didymogaster sylvaticus</i> Fletcher, 1886	AF406575
	<i>Diporochaeta phalacra</i> Michaelsen, 1916	AF406577
	<i>Diporochaeta nashi</i> Jamieson, 1976	AF406561
	<i>Diporochaeta</i> sp.	AF406564
	<i>Metaphire glareosa</i> Tsai, C. Tsai, S. & Liaw, 2000	AY960816
	<i>Metaphire yuhsii</i> (Tsai, 1964)	AY960812
	<i>Pondodrilus litoralis</i> (Grube, 1885)	AF406586
	<i>Reflachtodrilus sigillatus</i> ((Michaelsen, 1916)	AF406588
Enchytraeidae	<i>Fridericia tuberosa</i> Rota, 1995	AY340457
	<i>Buchholzia fallax</i> Michaelsen, 1887	AY885581
	<i>Enchytraeus albidus</i> Henle, 1837	Germany. XI. 2003. Leg. & det. J. Römcke

MATERIAL AND METHODS

Fresh material for 17 earthworm and one enchytraeid species were collected and analysed. In addition to the newly sequenced specimens, the 26 16S rDNA sequences were downloaded from the GeneBank (Table 1).

The presently analysed 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 Victor V. Pop Earthworm Collection at the Institute of Biological Research Cluj-Napoca, Romania. 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 *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 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 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.

Phylogenetic reconstruction was performed by applying maximum parsimony (MP), minimum evolution (ME), and maximum likelihood (ML) methods using in parallel the PAUP* 4.10b (Swofford 2000), and Treefinder (Job 2005) software respectively. For the MP trees 1000 bootstrap replicates were analyzed by heuristic method with tree-bisection-reconnection (TBR) branch-swapping algorithm implemented in the PAUP program. For the ME trees the Kimura-2 parameter was chosen with 100 bootstrap replicates. In the maximum likelihood analyses, the HKY substitution model was selected and the analysis was carried out with 1000 pseudoreplicates.

RESULTS AND DISCUSSION

Partial sequences for 16S rDNA of 37 earthworm species were analysed. Out of the 411 characters, 175 were constant (42.6%), 53 were variable but parsimony uninformative (12.9%) and 183 were parsimony informative (44.5%). The MP bootstrap analysis resulted in a tree of 1154 steps, consistency index 0.351, homoplasy index 0.648 and retention index 0.534 (Fig. 1.).

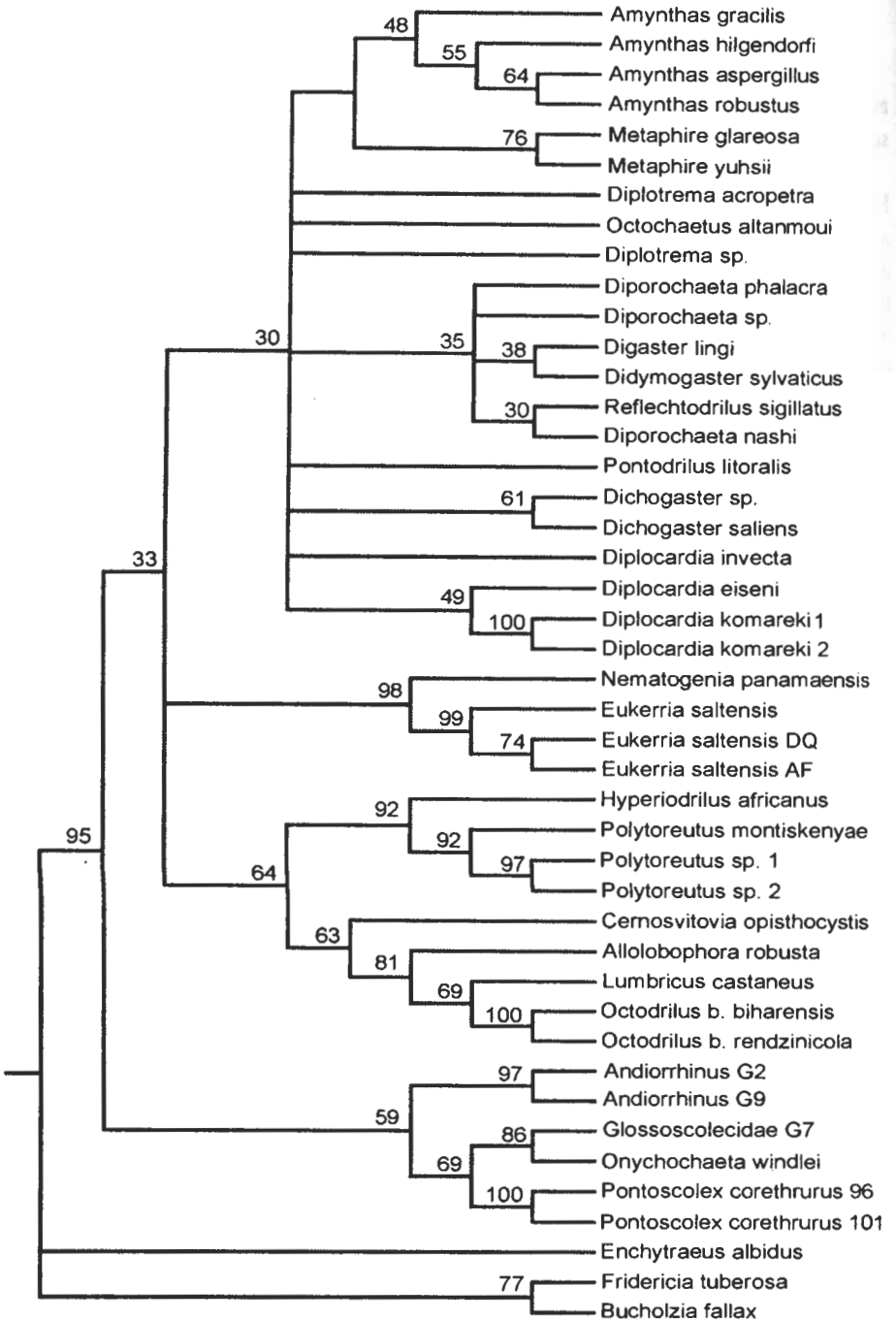


Fig. 1. Maximum parsimony bootstrap tree based on 16S rDNA analysis. Bootstrap support lower than 30% not shown

The topology of the tree shows four more or less distinct clades with variable bootstrap support. The largest and most apical clades consist of the Megascolecoidea taxa, but with only 30 % bootstrap support. Within this clade the “Pheretimoid” group is clearly distinct with high support (94%) and both *Amynthas* and *Methaphire* seem to be monophyletic (with 48% and 75% bootstrap support, respectively). The four North American *Diplocardia* species represent a relatively separated, but unresolved group (together with the clearly detached Dichogastrids) on the basal part of the clade. The other taxa are practically inseparable from each other.

The Ocnetrodrilidae species branch off, with highly supported monophyly (98%), immediately near the Megascolecoidea clade. This relative position supports the previous hypothesis that the superfamily Ocnetrodriloidea might be the plesiomorphic sister group of Megascolecoidea (Jamieson et al., 2002).

The third group is formed by the highly monophyletic Eudrilidae and the somewhat less supported Lumbricidae (with 92% and 63% bootstrap support, respectively). This clade is highly heuristic and clearly contradicts the previous reconstructions either by morphological characters (Jamieson, 1988) or molecular inference (Jamieson et. al 2002); but in the latter the family Eudrilidae was represented only by the peregrine *Eudrilus eugeniae* (Kinberg, 1867). Therefore, its sister group position with Glossoscoecidae might have been due to the biased sampling.

The fourth and the most basal ingroup clade consists of the representatives of the family Glossoscolecidae. Its basal position to the other Crassicitellata families has yet been indicated in the previous study (Jamieson et al., 2002).

The ME tree (Fig. 2) obtained in the bootstrap analysis is somewhat longer, and showing a bit lower index values (length = 1164 steps, CI = 0.349, HI = 0.688, RI = 0.528), but its general topology is similar to the MP trees. Nevertheless, some slight differences exist mainly in the arrangement of the Megascolecoidea taxa. The North American *Diplocardia* species form a basal monophyletic clade (but with only a quite low bootstrap support) and the two holoic *Diplotrema* species branch off together with the meroic *Octochaetus altanmoui* forming a moderately supported Australian “Acanthodrilid” clade. The two *Dichogaster* species stand along with *Pontodrilus litoralis* and seemingly form a basal clade to the “non phaterimoid” megascolecids; but this clade has almost no credible support (bootstrap value as low as 36%).

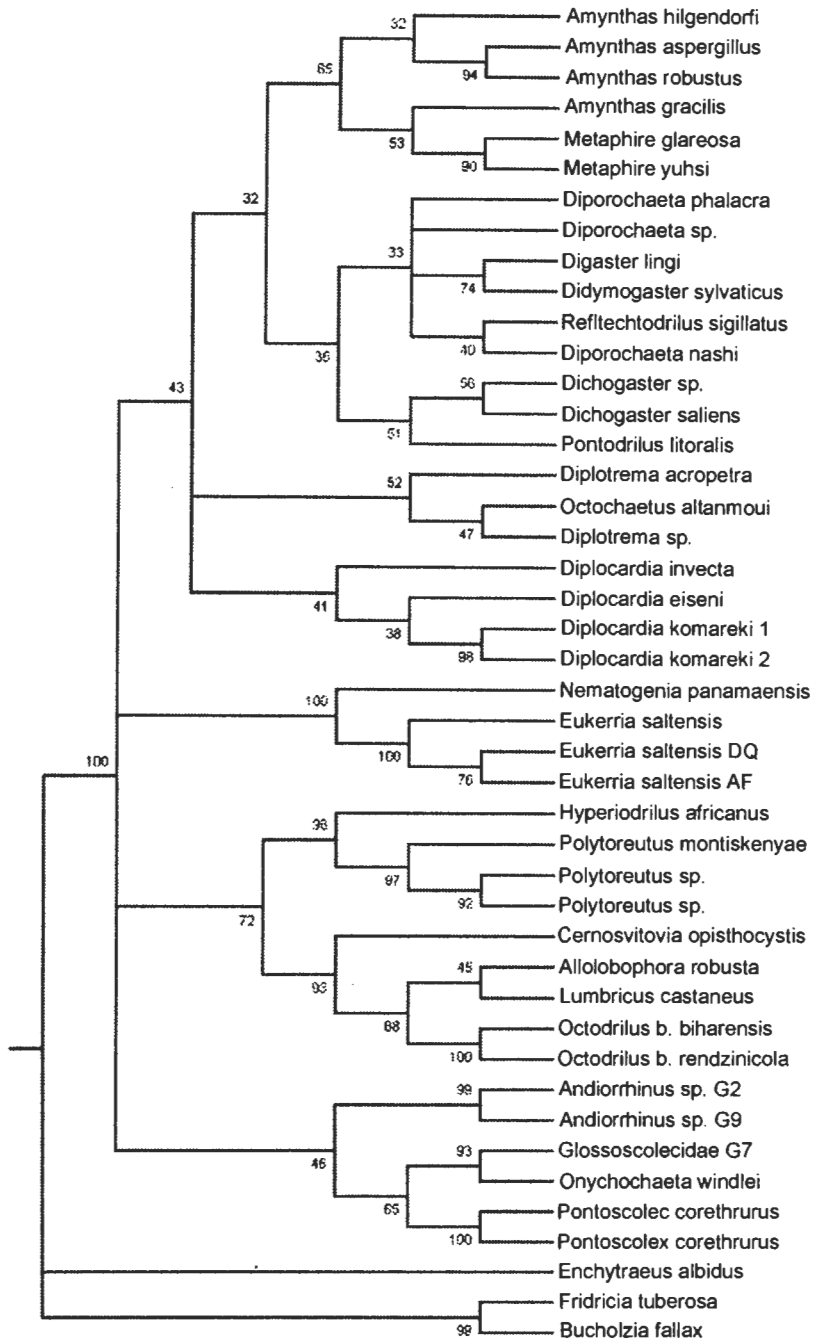


Fig. 2. Minimum Evolution bootstrap tree based on 16S rDNA analysis. Bootstrap support lower than 30% not shown.

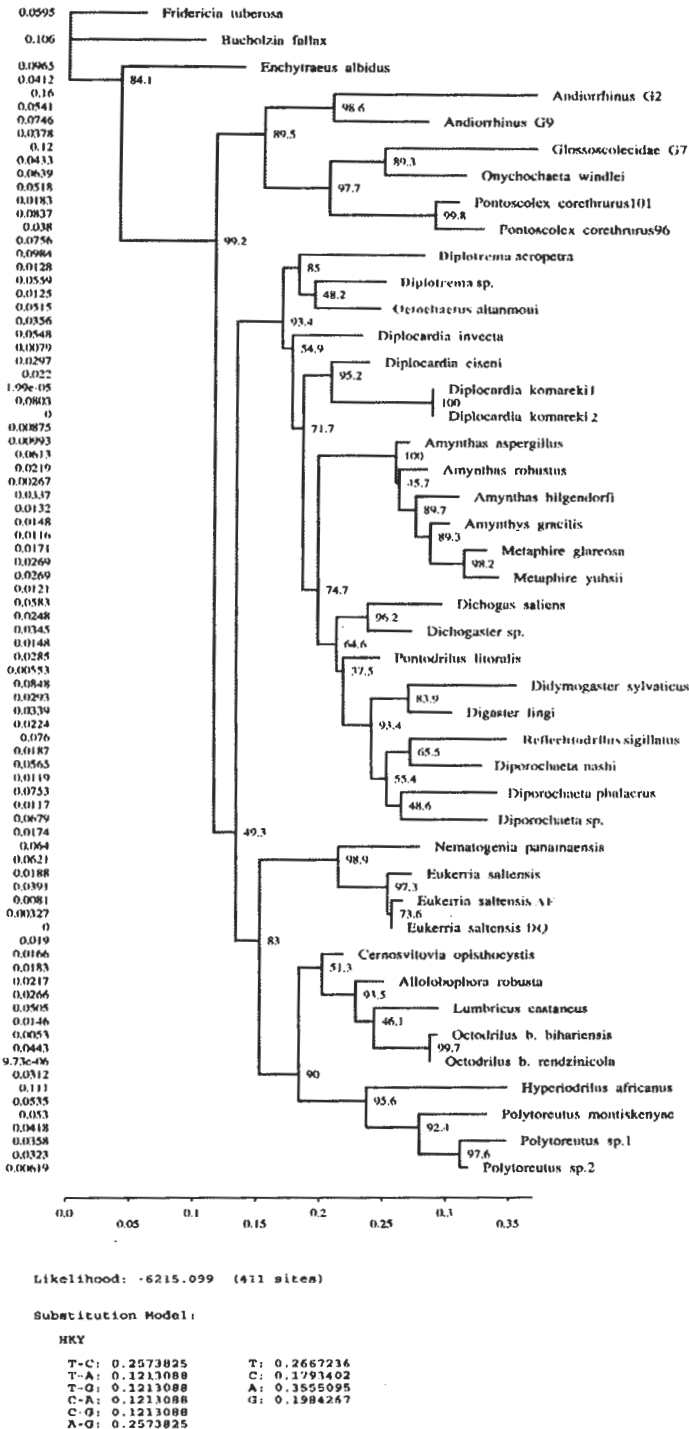


Fig. 3. Maximum likelihood consensus tree based on 16S rDNA analysis

The ML analysis (Fig. 3) recognises almost the same groups as the previous methods, but there are some interesting features in the branching patterns. Interestingly, Ocneroдрilids branch off together with the Lumbricidae+Eudrilidae clade that clearly contradicts the previous reconstructions. Inside the Megascolecoidea clade the "Acanthodrilids" form a basal paraphyletic assemblage mainly due to the ambiguous position of *Diplocardia invecta*. The "Pheretimoid" clade is clearly recognized, but the genus *Amyntas* seems to be paraphyletic here. The two *Dichogaster* as a distinct group join to the other "non Pheretimoid" Megascolecids where *Pontodrilus* represents the most basal taxon.

CONCLUSION

The analysis of the 16S rDNA gene was clearly successful in recognizing the main Crassicitellata families (or superfamilies). The families Glossoscolecidae, Lumbricidae, and Eudrilidae were recognized by each method in all trees (supported by quite high bootstrap values).

The picture is not so clear regarding the superfamily Megascolecoidea. The Megascolecoideid clade is also recognized in all the trees, but with almost no credible support. Furthermore, the inner structure of this clade was quite unresolved. It is interesting that the North American and the Australian species with Acanthodrilid male apparatus were always placed near together in a quite basal position of the Megascolecoidea clade, and merioic *Octochaetus/Neodiploptrema altanmoui* usually branch off together with the holoic *Diploptrema* species. The merioic Dichogastrids, also with Acanthodrilid type male apparatus, constantly form a distinct clade, sometimes together with *Pontodrilus litoralis* or close to it.

This scenario indicates that the Australian "Octochaetid" species evolved locally (as it previously was presumed by Lee (1959) and Jamieson (1971)), but it could not be excluded that Octochaetidae, at least in a restricted sense (containing the Indian taxa), could be still valid.

Due to the biased sampling, we were not able to identify the exact position of Dichogastrids. According to the present data, they seem to form an independent clade close to the Acanthodrilid assemblage.

It seems that the 16S rDNA gene alone is not adequate to reveal more precise phylogenetic relationships at this level. The phylogeny reconstruction could be improved by involving more gene sequences, sensible to finer resolution, and more taxa, especially representatives of the Indian Octochaetids and the African and South American Acanthodrilids and Benhamiids.

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