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Molecular phylogeny of Enchytraeidae (Oligochaeta) indicates separate invasions of the terrestrial environment

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Abstract

Enchytraeidae is a family of soil inhabiting small- to medium-sized oligochaete worms using degradable plant material as a food source and primarily adapted to terrestrial or semi-terrestrial environments. The molecular phylogeny based upon both mitochondrial and nuclear genes indicates early segregations of the two genera *Enchytraeus* and *Lumbricillus* leaving the remaining genera included in this study as a later segregated major monophyletic branch. Extant members of the two former genera dominate in decaying seaweed in the littoral zone along the sea although members of in particular the genus *Enchytraeus* have also invaded other habitats. Historically the littoral zone of the sea is undoubtedly the first terrestrial or semi-terrestrial habitat where dead plant material accumulates to any greater extent and *Enchytraeus* and *Lumbricillus* may represent early successful attempts to exploit this resource. Inland soils probably had to await the emergence of land plants in order to provide a similar food resource and here the major branch of enchytraeid genera diversified into a high number of species in the numerous decomposer networks of this varied environment. A subdivision into the genera *Enchytraeus* and *Lumbricillus* on the one hand and a branch of mainly inland genera on the other is supported by differences in two somewhat neglected morphological features. Firstly, in *Enchytraeus* and *Lumbricillus* the testes are enclosed in a testis sac within which the male cells mature, by one possible exception a unique feature among Oligochaeta, The other enchytraeid genera studied and Oligochaeta in general lack this sac and the male cells mature directly in the cavity of the testicular segment. Secondly, species of *Enchytraeus* and *Lumbricillus* generally have a higher reproductive output than species of the inland terrestrial branch and this may represent an adaptation to the unpredictable littoral zone compared to the more stable nature of inland habitats. In the older literature the genus *Mesenchytraeus* is considered to have a basic position within the entire family but our molecular data do not support this expectation. In Enchytraeidae the nephridia are elaborate organs of a characteristic and constant shape covering species from different genera in a pattern following the molecular phylogeny. Other much used morphological features such as shape of setae, anteclitellar origin of the dorsal vessel and various modifications of the intestine have arisen more than once.

Key words: Enchytraeidae – Oligochaeta – initial littoral adaptation – later terrestrial diversification

Introduction

Phylogeny of the family Enchytraeidae has been given little attention compared with other oligochaete families. The most recent discussion including all known genera date as far back as Černosvitov (1937) where the then accepted genera were assembled in six subfamilies. The suggested subdivision has had limited impact because classification of the subfamilies was not convincing and the discussion included genera with doubtful connection to the family Enchytraeidae. In the 1980th Coates published a series of papers dealing with various aspects of enchytraeid phylogeny based upon morphological observations. Coates (1986, 1987) are focussed upon the genus *Propappus* and conclude that it cannot be retained within the family and a new family, Propappidae, is erected with a suggested sisterelationship to Enchytraeidae. In Coates (1989) the phylogeny of Enchytraeidae is discussed based upon the assumption that a bisetate condition, two setae in each of the four bundles per segment, is the ancestral state in Enchytraeidae, and the discussion mostly includes bisetate species and species without any setae (achaetinous).

Enchytraeid species are also included in more recent studies using DNA data to evaluate the phylogeny of clitellate annelids. Siddall et al. (2001) and Erséus and Källersjö (2004) using nuclear 18S data place the Enchytraidae in a sister group relation to the earthworms. In a quite recent paper, Marotta et al. (2008), by using a combination of morphological and 18S data add Lumbriculidae and leeches to this sister clade thus supporting the use of the two outgroup species chosen in this study. Erséus (2005) review the then known molecular studies and Rousset et al (2008) provide strong support for an 'aquatic' ancestral state of clitellate annelids.

The aim of the present study was to evaluate the intra-familiar phylogeny of Enchytraeidae by using molecular data involving both nuclear and mitochondrial genes covering a total of 4977 bp. The species studied are representatives of well defined species-rich genera from the Northern Hemisphere. *Lumbriculus variegatus* and *Lumbricus terrestris* are used as outgroup species.

Materials and methods

Taxa analysed

Representatives of the following genera are used as ingroup taxa: *Enchytraeus, Lumbricillus, Mesenchytraeus, Cernosvitoviella, Cognettia, Henlea, Achaeta, Buchholzia* and *Fridericia.*. These genera are supposed to represent well-defined 'natural' units that have been able to include an increasing number of new species sharing the morphological specializations through which the genus was originally defined. Obvious 'unnatural' groupings such as the nominal genus *Marionina* is deliberately avoided, species referred to this genus lack the combination of features characteristic of genera included in this study and are morphologically quite different indicating distant relationships. All the species included in this study are listed in Table 1 including the cases where combined data from two con-generic species are used in the analysis.

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Table 1. Genbank accession numbers of the sequences included in this study, previously published sequences obtained from Genbank are printed in italics

Species	COI(partim)	COII(partim)	Nadh6	tRNA Gln	tRNA Asn	23sRNA(exp.3)	23sRNA(exp.6)	18sRNA
Lumbricus terrestris	*U24570*	*U24570*	*U24570*	*U24570*	*U24570*	GU453350		*AJ272183*
Lumbriculus variegatus	GU453374	GU453381			GU453402	GU453355	GU453365	*AF209457*
Mesenchytraeus pelicensis	GU453375	GU453382	GU453386	GU453411	GU453400		GU453364	GU453343
'' flavus						GU453351		
Cernosvitoviella atrata	GU453368	GU453385	GU453389	GU453405	GU453396	GU453347	GU453358	GU453338
Lumbricillus lineatus 3x	GU453373	GU453380	GU453394	GU453410	GU453401	GU453354	GU453363	GU453342
Enchytraeus albidus	GU453370	GU453377	GU453391	GU453407	GU453397	GU453353		GU453340
'' capitatus							GU453360	
Cognettia sphagnetorum	GU453369	GU453376				GU453352	GU453359	GU453339
'' glandulosa			GU453390	GU453406				
Henlea nasuta	GU453372	GU453379	GU453393	GU453409	GU453399	GU453349	GU453362	GU453341
Fridericia galba	GU453371	GU453378	GU453392	GU453408	GU453398	GU453348	GU453361	
'' tuberosa								*AF209453*
Buchholzia appendiculata	GU453367	GU453384			GU453395	GU453346		
'' fallax			GU453388	GU453404			GU453357	*AF411895*
Achaeta affinis	GU453366	GU453383	GU453387	GU453403		GU453345	GU453356	GU453344

Lumbricus terrestris and *Lumbriculus variegates* are chosen as outgroup species.

Extraction and amplification

Extraction of DNA followed standard procedures

The mitochondrial genes COI (partim), tRna-Asn and COII (partim) located in succession were amplified by the following overlapping primer sets: 5′-GGTCAACAAATCATAAAGATATTGG (fw), 5′-ACTTCWGGGTGYCCAAAYAATC(rv); 5′-CAGATCGAAAT YTAAATACNTC(fw), 5′-GGGTAGTCWGAATATCGWCGWG G(rv); 5′CATGAYACATAYTATGTAGTAGC(fw), 5′-ATAATTG CTGGWAGAATTGTTCA(rv) and 5′-GGTCAANTAWTATTHC AAGACGC(fw), 5′-GCACCGCAGATTTCTGAGCAGTG (rv). Two additional mt-genes (tRNA-Gln and NADH6) were amplified by 5′CACTTTGGATTCGAAGCCGCTGCC (fw) and 5′CCGTAG TTTCATCAAATGGAGAT (rv). The so-called expansion segments (D3 and D6) of the nuclear 23s-like RNA gene were amplified by D3: 5′-GACCCGTCTTGAAACACGGA (fw); 5′-TCGGAAGGAAC CAGCTACTA (rv) and D6: 5′TAAGGAGTGTGTAACAACTC (fw); 5′AGGGAATATTAACCCTATTC (rv). The nuclear 18s-RNA gene was first amplified by the embracing primers 5′TACCT GGTTGATCCTGCCAGTC′3 (fw); 5′CTTTTACTTCCTAAATG ATC′3 (rv) or 5′GATCCTTCCGCAGGTTCACCTAC′3 (rv) and later refined by intermediary primers 5′GGGAAACCTTGTTACG ACTT′3(fw); 5′GCCATGCATGTCTAAGTCCA′3(rv) and 5′TAC ACGGTGAAGCTGCGAAT′3; 5′CCGAGGATCTCACTAAAC CA′3.

Accession numbers of the genes submitted to Genbank are given in Table 1.

Table 2. Approximate length of the genes included in this study and their position in the alignment

Gene	Position
Mitochondrial:	
COI (partim)	1–1491
COII (partim)	1492–2073
NaDH6	2074–2547
tRNA ASN	2548–2620
tRNA GLN	2621–2698
Nuclear:	
23S RNA 3D	2699–2878
23S RNA 6D	2879–3147
18S RNA	3148–4977

Sequencing

Initially sequences were analysed using the classical radio-labelling of di-deoxynucleotide and exposure of a sensitive film, in the later phases the products were analysed in an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Hitachi).

Analysis

Non-coding sequences were aligned by Clustal X while coding sequences were aligned by eye. Positions of individual genes in our alignment are shown in Table 2. The entire data set was analysed in MrBayes v3.01 (Huelsenbeck and Ronquist 2001; Huelsenbeck et al. 2002) using Bayesian inference of phylogeny. Mr Modeltest 2.2 was used to select MrBayes settings for the best fit model: GTR + I + G (Posada and Grandall 1998). Monte Carlo chain length was 4 000 000 generations.

Results

The result of the Bayesian analysis is shown in Fig. 1. *Enchytraeus* sp. appears as sister group to the other in-group species studied. Among the latter again a single species, *Lumbricillus lineatus,* turn up as sister group to a strongly supported monophyletic group comprising the remaining taxa. Thus the molecular data indicate the segregation of the two genera *Lumbricillus* and *Enchytraeus* from a major later diversifying branch including the remaining enchytraeid genera of this study.

Within this major grouping a strong bipartition is revealed with one branch comprising members of the genera *Mesenchytraeus* and *Cernosvitoviella* and another more diverse lineage containing representatives of genera that dominate most terrestrial habitats on the northern Hemisphere. Once again these are distributed among two branches, one which embraces *Henlea nasuta* and *Cognettia* sp. and another consisting of *Achaeta affinis, Fridericia* sp. and *Buchholzia* sp. with the *Achaeta* species appearing as sister-related to the two latter.

Discussion

In the older literature (Michaelsen 1916; Stephenson 1930; Ĉernosvitov 1937; Brinkhurst and Jamieson 1971) it is generally agreed that the Enchytraeidae are classified with some mostly aquatic families such as Tubificidae, Naididae

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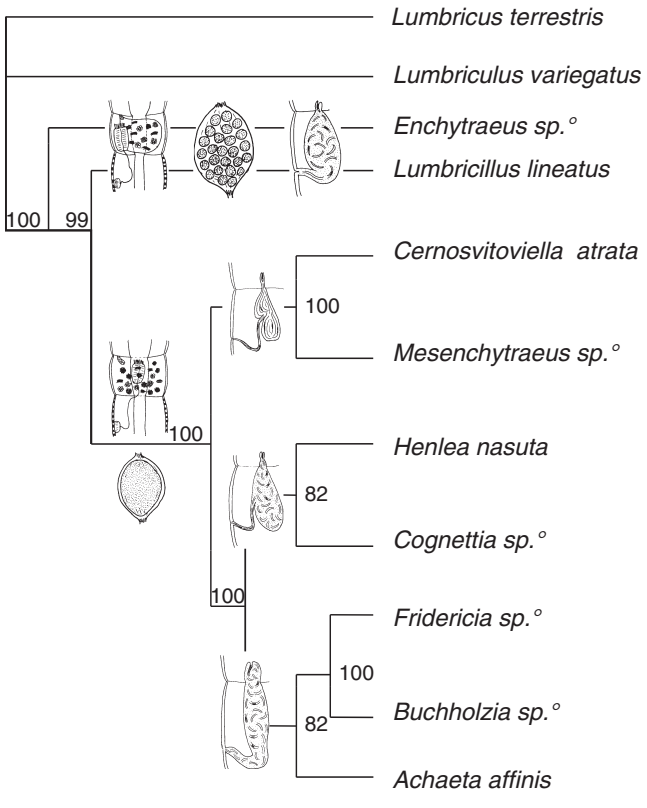

Fig. 1. Bayesian analysis of the molecular data showing posterior probabilities at ingroup nodes. The distribution of some important morphological characteristics such as the structure of the seminal vesicle, number of eggs pr. cocoon and shape of nephridia are inserted into the molecular tree. º) Combined data from two closely related species, see Table 1

and Phreodrilidae in a subgroup of Oligochaeta called Microdrili referring to their moderate size and contrasting another subgroup of oligochaetes, Megadrili, comprising the generally larger terrestrial earthworm families. Consequently, much discussion on the intra-familiar phylogeny has been based on the assumption that Enchytraeidae descended directly from aquatic ancestors as an invasion of terrestrial habitats independent from that which gave rise to the megadrile earthworms. However, very few morphological features support the above sketched relationships and even if terrestrial oligochaetes may ultimately stem from an aquatic ancestor of a microdrile appearance, i.e. Rousset et al (2008), nothing indicates that this is specifically the case for Enchytraeidae, and the view expressed by Černosvitov (1937) that enchytraeid genera with morphological features resembling similar organs in aquatic Microdrili hold an ancestral position within the family is doubtful. Here exemplified by the genera *Mesenchytraeus* and *Cernosvitoviella,* which through the possession of sigmoid setae with nodulus and nephridia with little interstitial tissue from a morphological point of view are supposed to hold an ancestral position, but do in fact join the derived branch of the family comprising most of the genera included in this study. Thus if the molecular phylogeny indicates the true relationship, it remains doubtful whether the above mentioned features represents the ancestral states in Enchytraeidae or are secondary adaptations to the relatively moist environments inhabited by *Mesenchytraeus*. and *Cernosvitoviella* species.

The isolated position of *Enchytraeus* and *Lumbricillus* in the molecular phylogeny may at first sight appear surprising. However, both genera have morphological characteristics in which they differ not only from other enchytraeid genera included in this study but also from Oligochaeta in general, features that may have gained too little attention in phylogenetic discussions. As pointed out by Stephenson (1930) some species of *Enchytraeus* and *Pachydrilus* (now *Lumbricillus*) are unique among enchytraeids in having their testis enclosed in a dense sac (testis sac) within which the male cells mature. As now defined this would seem to be the case in all members of the genera *Enchytraeus* and *Lumbricillus* while in the other enchytraeid genera studied immature male cells are released from the testes and mature freely in the cavity of segment XI (see Fig. 1). Thus in Enchytraeidae the term seminal vesicle covers morphologically quite different structures. In say *Fridericia galba* the testes release immature sperm cells directly into the lumen of segment XI where they mature and the seminal vesicle is bounded anteriorly by septum X/XI and posteriorly by septum XI/XII while in say *Enchytraeus albidus* immature sperm cells are released into a closed sac surrounding the testes and the growing seminal vesicle is a separate structure within the lumen of segment XI. This strong difference is directly seen in the positioning of the so-called sperm funnel in relation to the seminal vesicle. The sperm funnel is the first part of the vas deferens and in most species a conspicuous cylindrical structure located in segment XI and in *Fridericia galba* and other species and genera with the same type of seminal vesicle the sperm funnel is found within the same cavity as the developing sperm cells, i.e. within the seminal vesicle while in *Enchytraeus albidus* and other members of the genera *Enchytraeus* and *Lumbricillus* sperm funnels are found outside this structure. The so-called sperm sac characteristic of the genus *Mesenchytraeus* is a backward directed pouching of septum XI/XII in which sperm cells mature, but since this lumen is a mere extension of segment XI the situation in *Mesenchytraeus* is in principle identical to that described for *Fridericia galba*. According to Stephenson (1930) the latter situation is with minor modifications universal in Oligochaeta indicating that it might be the ancestral condition while *Lumbricillus* and *Enchytraeus* and perhaps the megascolecid genus *Ocnerodrilus* represent a unique specialization.

Another partitioning which organizes enchytraeids in the same two groups as described above and which may have important ecological implications is a strong difference in the number and size of eggs. This is most obvious when medium sized to large species are compared and in both *Enchytraeus* and *Lumbricillus* species the eggs are relatively small compared to the size of the worm and usually 10–20 and occasionally more than 30 eggs mature simultaneously (Christensen 1956). They are laid in the same cocoon and the vast majority develop and hatch out. This is in sharp contrast to species of the other genera such as *Fridericia, Henlea* and *Mesenchytraeus* which produce relatively much larger eggs and normally only one egg is laid in each cocoon (see Fig. 1).

Under optimal conditions sexually mature worms usually deposit one cocoon per day which means that *Enchytraeus* and *Lumbricillus* have a much higher reproductive rate measured in number of offspring than members of the other genera mentioned above (B. Christensen, pers. obs.). On a scale covering the reproductive output in enchytraeids, members of *Enchytraeus* and *Lumbricillus* may be called r-strategists while members of *Fridericia* etc relative to that may be called

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K-strategists and this may be an adaptation to the preferred habitat types of the species in question.

As stated above species of *Enchytraeus* and *Lumbricillus* are dominant members of the enchytraeid fauna in the littoral zone along the sea, a habitat type which according to conventional ecological thinking favour r-strategists because it is so unpredictable, one day heaven on earth for enchytraeids with an ideal water regime and an abundance of food in the decaying seaweed washed ashore, the next day rough weather may have swept the wrack bed away and more or less destroyed the habitat for a while. In contrast, inland terrestrial habitats are relatively more predictable both with respect to water regime and food supply and such environments are supposed on the enchytraeid reproductive scale to favour K-strategists. Thus the molecular data presented may indicate an early split into two main strategies: one adapting to exploit decaying plant material washed ashore along the sea and another main line primarily adapting to exploit decaying plant material of inland soils.

However, the above scenario is painted with broad strokes and extant members of the genus *Enchytraeus,* in particular, are in fact more diverse in their ecologies. A group of medium sized to large species including the nominal species of the family *Enchytraeus albidus* mainly inhabit the littoral zone, they have the same chromosome number ($n = 21$) and they all reproduce sexually (Christensen 1980). This is in sharp contrast to another group, the *Enchytraeus buchholzi* group, which differ in all these features. The species in question inhabit a wide variety of inland soils, the basic chromosome number is different ($n = 18$) and several cases of polyploidy have been observed even as high as decaploidy (Christensen op.cit.), and in addition to normal sexual reproduction a case of so-called subamphimixis is recorded in which some members of the chromosome complement behave as in a normal sexual species while others follow a course as in mitotic parthenogenesis (Christensen and Jensen 1964). Finally multiplication through transverse fission of the adult body and subsequent regeneration of new anterior and posterior ends is found in other species (Christensen 1964, 1973). This high degree of intra-generic heterogeneity may be in line with the long prehistory of the genus *Enchytraeus* indicated in the molecular phylogeny.

Compared with their supposed aquatic ancestors terrestrial oligochaetes must have required several adaptations among which regulation of the water balance in these occasionally dry environments represents an important challenge to the functioning of the nephridium. This is clearly seen in the Enchytraeidae where the nephridia in most genera are elaborate organs of a characteristic shape and with an extremely narrow nephridial canal embedded in interstitial tissue. The only exceptions are species of the genera *Mesenchytraeus* and *Cernosvitoviella* where the nephridial canal is loosely wound, relatively short and wide and the interstitial tissue is poorly developed and as such resembling the situation in aquatic oligochaete families. In members of *Enchytraeus* and *Lumbricillus* the ciliated nephrostome or funnel is the only anteseptal part of the nephridial canal while the tightly coiled postseptal part embedded in interstitial tissue form a solid ovoid structure and the fairly wide efferent duct has a postero-ventral origin. In species of *Henlea* and *Cognettia* coils of the canal may or may not extend forward into the anteseptal part but in either case the nephrostome is a distinct forward directed structure. The post-septal part is elongate, occasionally somewhat pear-shaped and the long and slender efferent duct usually with an antero-ventral origin. In *Fridericia, Buchholzia* and *Achaeta* the ante-septal part is usually large with dense coils of the canal and in contrast to the former group the nephrostome is completely embedded in the tissue; the postseptale elongate and may gradually extend into a fairly wide efferent duct or the duct may arise as a more distinct structure in a postero-ventral position. Thus, a sub-division into four groups of genera indicated by the molecular data is reflected also in the evolution of the enchytraeid nephridium and this organ thus appears as a fairly conservative structure illustrating the major subdivisions of the family (see Fig. 1).

This contrasts a more diversified pattern seen in other much used taxonomical characters such as shape of setae, origin of dorsal vessel and various specializations of the intestinal canal. If the molecular tree reflects the true relationships functionally identical specializations appear to have arisen independently within different lineages and a closer inspection usually shows that the morphological similarities between these are only superficial.

Irrespective of the ancestral state of the setae (straight or sigmoid) the same type has developed independently and repeatedly within all the major branches: *Lumbricillus* has sigmoid setae while they are straight in *Enchytraeus*; in *Cognettia* they are sigmoid and in *Henlea* straight and finally in *Buchholzia* they are sigmoid but in *Fridericia* straight. A further specialization where the innermost setae in a bundle is shorter than the outer ones is seen in two different lineages namely in members of the genera *Henlea* and *Fridericia* with four or more setae per bundle and again this situation has undoubtedly arisen independently in the two genera and the similarity is only superficial, in *Fridericia* we see a perfect pair wise symmetric arrangement around the mid-axis of the setal bundle while in *Henlea* the setae are not arranged in distinct pairs.

A somewhat similar situation is seen in species of *Henlea* and *Buchholzia* in the combination of a heart-like ante-clitellar origin of the dorsal vessel and the presence of various appendages in the very same region of the intestine. The ancestral situation in the family is undoubtedly a smooth origin of the dorsal vessel immediately behind the clitellum and absence of intestinal specializations. The presence of these may perhaps improve digestion and the large pulsating heart-like expansion of the dorsal vessel in the nearby segments may secure an efficient blood supply to this physiologically active region of the intestine. The molecular data indicate an independent origin in *Henlea* and *Buchholzia* and a detailed morphological analysis support this. In *Buchholzia* the intestinal appendages are dorsal while in *Henlea* they are mostly of a lateral position. In *Buchholzia* the dorsal vessel arises from the summit of the intestinal appendage while in *Henlea* it arises independently of the appendages from the blood sinus of the intestine itself which usually widens considerably in this region.

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Zusammenfassung

Enchytraeidae sind eine Familie kleiner bis mittelgroßer edaphischer Oligochaeten, welche abbaubares Pflanzenmaterial als Nahrungsquelle

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nutzen und primär an terrestrische oder semiterrestrische Lebensräume angepaßt sind. Die hier vorgestellte**,** auf mitochondrialen und nukleären Genen beruhende molekulare Phylogenie indiziert eine frühe Abtrennung der beiden Gattungen *Enchytraeus* und *Lumbricillus* und beläßt die übrigen der in dieser Studie untersuchten Gattungen als eine später abgezweigte monophyletische Großgruppe. Rezente Glieder der beiden erwähnten Gattungen dominieren in verrottendem Strandanwurf des Meereslitorals, obwohl Arten insbesondere der Gattung *Enchytraeus* auch andere Lebensräume erobert haben. Historisch gesehen ist das Meereslitoral zweifelsohne das erste terrestrische oder semiterrestrische Habitat, in dem totes Pflanzenmaterial in größerem Ausmaß akkumulierte, und *Enchytraeus* und *Lumbricillus* könnten frühe erfolgreiche Versuche der Nutzung dieser Ressource darstellen. Festlandsböden dagegen bedurften wahrscheinlich der Entwicklung von Landpflanzen, um eine vergleichbare Nahrungsquelle zur Verfügung zu stellen, und hier diversifizierte sich der Hauptzweig der Enchytraeidengattungen in viele Arten in den diversen Zersetzernetzen dieses vielgestaltigen Lebensraums. Eine Unterteilung in die Gattungen *Enchytraeus* und *Lumbricillus* auf der einen Seite und einen Zweig mit vorwiegend terrestrischen Gattungen auf der anderen wird durch zwei bislang eher vernachlässigte morphologische Merkmale gestützt. Erstens, bei *Enchytraeus* und *Lumbricillus* wird der Hoden von einem Testis-Sack, in dem die männlichen Zellen reifen, umschlossen. Dies ist mit einer möglichen Ausnahme einzigartig bei Oligochaeten. Bei den übrigen untersuchten Enchytraeidengattungen und bei Oligochaeten generell fehlt dieser Sack, und die männlichen Zellen reifen unmittelbar in der Körperhöhle des Hodensegmentes. Zweitens, die Reproduktionsrate von *Enchytraeus* und *Lumbricillus* ist generell höher als die der Arten des Festlandszweigs. Dies könnte eine Anpassung an die im Vergleich zu Festlandsböden instabileren Bedingungen des Meereslitorals bedeuten. In der älteren Literatur wird der Gattung *Mesenchytraeus* eine basale Position innerhalb der gesamten Familie zugewiesen, aber unsere molekularen Daten stützen diese Annahme nicht. Die Nephridien der Enchytraeiden sind komplexe Organe mit einer charakteristischen und artübergreifend konstanten Form, deren gattungsmäßige Abwandlungen der molekularen Phylogenie entsprechen. Andere oft verwendete morphologische Merkmale wie Borstenform, anteclitellarer Ursprung des Dorsalgefäßes und verschiedene Darmmodifikationen sind mehr als einmal entstanden.

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