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**APPLYING MOLECULAR METHODS TO RESOLVE
TAXONOMICAL PROBLEMS IN THE FAMILIES LUMBRICIDAE
AND ENCHYTRAEIDAE (ANNELIDA: CLITELLATA)**

Thesis for Ph.D.

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BUDAPEST
2011

1. INTRODUCTION AND OBJECTIVES

The morphological classification within the families Lumbricidae and Enchytraeidae (Annelida: Clitellata) raises several, unsolvable problems. Some characters are undecided to be plesiomorphic or apomorphic, even their homology raise frequently serious issues. Therefore, the validity of the evolutionary lineages is sometimes unambiguous. Moreover, it is often problematic to decide that the presence or absence of certain characters is enough to separate species. The structure of genitalia has a significant relevance in the taxonomy of these taxa, which makes difficult the identification of juvenile specimens.

The phylogenetic analysis of species within genera *Allolobophora*, *Aporrectodea*, *Bimastos*, *Eisenia* and *Dendrobaena* in family Lumbricidae has an emphasised importance, because these genera seem to be morphologically highly heterogeneous, they are mostly considered to be polyphyletic. Several attempts were made since the 80's to revise the family Lumbricidae, but these efforts are based on species of certain geographical regions, but the conclusions concerned the whole family distributed throughout the whole Holarctics. In addition, many new genera were created whose validity is doubtful, moreover the newly described species are often synonymous, which fact complicates further the taxonomy of Lumbricidae.

The majority of the genera within the family Enchytraeidae are considered monophyletic based on morphological as well as molecular data, their relationships are mostly clarified. However, the differentiation of closely related species causes a lot of trouble in the family. The distinction between intra- and interspecific morphological variability sometimes depends on the approaches of taxonomists; no exact border exists between the two category. The genus *Fridericia* contains the largest number of species and a remarkable part of the species like *F. ratzeli*, *F. bulboides*, *F. aurita* are treated as "species complex" due to their significant intraspecific heterogeneity.

The ribosomal genes and spacer sequences between them are frequent objects to resolve phylogenetic and taxonomical issues, because they are universal and conservative and variable regions can be found equally amongst them. The 18S rDNA and the ITS region are widely used in molecular studies. The 18S rDNA or SSU rDNA is a much more conservative locus, it provides reliable resolution mostly on higher taxonomical ranks like family and genus, however it can be applicable at species level in certain families. The sequences of the ITS region is much more variable, ideal for applying at genus, species, subspecies level.

During our investigations, we chose 18S rDNA and the 5,8S rDNA-ITS2 loci to analyze the phylogenetic relationships and the validity of genera within the family Lumbricidae. The whole ITS region was examined in the case of enchytraeid worms to help the morphological differentiation of species.

The aims of the present study were:

Resolving the phylogenetic relationships within the family Lumbricidae applying 18S rDNA and spacer sequences, especially in case of the morphologically heterogeneous *Dendrobaena*, *Allolobophora*, *Aporrectodea*, *Bimastos* and *Eisenia* genera

Evaluation of the morphological characters used in the Lumbricidae family in light of the results of the molecular analyses

Re-examination of species with doubtful validity in the family Enchytraeidae by ITS sequences, like *Buchholzia subterranea*, *Enchytronia christenseni*, *Mesenchytraeus kuehnelti*, *Bryodrilus glandulosus*

The molecular analysis of morphologically heterogeneous species and species-complexes in the genus *Fridericia* (*F. ratzeli*, *F. bulboides*, *F. aurita*, *F. maculatiformis*) using the ITS region

Comparison of the morphological characters applied in the delimitation of enchytraeid species with the results of molecular investigations

2. MATERIALS AND METHODS

37 lumbricid species were examined during our investigations including species mostly from the genera *Dendrobaena*, *Allolobophora*, *Aporrectodea*, *Bimastos*, *Eisenia* as well as a few specimens of *Cernosvitovia*, *Dendrodrilus*, *Helodrilus* and *Proctodrilus*. The samples were collected from the Carpathian Basin and the Balkan-peninsula, and a few specimens originated from North America, Western Europe and the Middle East.

72 specimens of 19 species (2 *Bryodrilus*, 4 *Buchholzia*, 3 *Enchytronia*, 8 *Fridericia*, 2 *Mesenchytraeus*) were investigated in the family Enchytraeidae, mostly from Hungarian collection sites, some species were represented by individuals from other European countries (Germany, Italy, Estonia) as well.

The earthworms were preserved in 96% ethanol until the molecular studies; a dissected part of their body wall was used for DNA extraction. The enchytraeid worms were stored frozen; the DNA was isolated from whole specimens.

Two loci were amplified to analyse the phylogenetic relationships among the earthworms, the conservative 18S rDNA, and the variable 5,8S rDNA-ITS2 section. The whole ITS region were amplified in case of Enchytraeidae. The PCR products were sequenced from both directions.

The sequenced fragments were aligned along the overlapping parts, then the whole sequences of the species were aligned by Clustal W. Maximum likelihood (ML), neighbour-joining (NJ) and Bayesian inference (BI) algorithms were applied to generate phylogenetic trees. Pairwise distance values were calculated to determine the genetic distances of enchytraeid worms (using Tamura-Nei distance).

3. RESULTS

18S rDNA proved to be much more conservative as it was expected, therefore it was inappropriate to conclude phylogenetic observations, except for a few taxa. The locus is not suitable itself to analyse the phylogenetics of earthworms. In contrast, the spacer sequences gave us higher resolution due to their higher variability, the results were mostly in accordance with morphological and biogeographical facts.

The sequence analyses of the family Lumbricidae supported the monophyly of the genus *Eisenia* despite the heterogeneity observed in the longitudinal musculature. The North-American *Bimastos* species, the peregrine *Dendrodrilus* spp. and *Allolobophora eiseni* formed a monophyletic clade, but the Middle Eastern *Bimastos syriacus* was excluded. The heterogeneity of the genera *Allolobophora* and *Aporrectodea* was confirmed, however all the species of both genera were united in a single group except *Ap. sineporis* and *Ap. handlirschi*. The genus *Dendrobaena* proved also to be heterogeneous, but the relationship between the species of Central Europe and the Balkan Peninsula was demonstrable. The molecular data did not support the validity of *Cernosvitovia*, *Helodrilus* and *Proctodrilus*.

The sequence analyses shed light upon the usability of the taxonomical characters applied in the family Lumbricidae. The setal ratio, the presence or absence of pigmentation and the anatomy of the calciferous glands proved to be stable throughout the family, however the nephridial bladders, the structure of the longitudinal musculature and the position of the male pores are not reliable characters to define genera.

The ITS region was an informative locus to resolve the taxonomical problems of enchytraeid worms. Its variability was suitable to separate morphologically indistinguishable species, to analyse heterogeneous species complexes, and to support the monophyly the enchytraeid genera.

The validity of the species *Buchholzia subterranea*, *Enchytronia christenseni* and the undescribed *Enchytronia sp. nov* was supported by the molecular data, but *Bryodrillus ehlersi* and *Mesenchytraeus kuehneli* need revision on the bases of our results.

The genus *Fridericia* had several taxonomical issues to solve. It was proved, that significant genetic distances separate the species *F. eiseni*, *F. crassiductata* from *F. ratzeli*, latter species included previously also the individuals with characteristics of *F. eiseni* and *F. crassiductata*. Similarly, *F. schmelzi* and *F. semisetosa* differed from *F. bulboides* in the nucleotide sequences, all three species have the same spermatheca structure. Four morphological variants of *F. aurita* were found, and all of them were found to be separated from each other based upon sequence analyses. Several specimens of *F. maculatiformis* were collected with five pairs of praeclitellar nephridia. Our molecular studies showed remarkable genetic distance between the two variants.

According to our molecular investigations, a supportable standpoint is within the genus *Fridericia* that the most important taxonomic character on species level is the structure of the spermatheca, but the examination of other morphological characters (eg. number of praeclitellar nephridia, setae, oesophageal appendages, position of chylus cells, bursal slit etc.) can equally be important as possible indicators in separation of species.

4. DISCUSSION

The observed conservativity of 18S rDNA limited its applicability to determine phylogenetic relationships within the Lumbricidae family. However, the sequences of spacer regions were adaptable to resolve closer connections. The results are often in correspondence with the classical morphological and biogeographical observations.

The monophyly of genus *Eisenia* was not obvious in the light of morphology, because *E. fetida* possesses pinnate longitudinal musculature, while *E. spelaea* and *E. lucens* have fasciculate musculature. Our result was supported by other authors using mitochondrial sequences as well. The 3 North American *Bimastos* species formed a single clade as it was expected, but their relationship with the *Dendrodrilus* species needs an explanation. All of those species have U-shaped “proclinate” nephridial bladders, moreover the calciferous glands have diverticula in the 10th segment. There are slight differences in the setal ratio and the structure of longitudinal musculature, but the latter showed heterogeneity in the genus *Eisenia* too. *Ai. eiseni* is similar to the *Bimastos* species in several characters, like closely paired setae and purple pigmentation. The possible connection to the genus *Bimastos* was mentioned formerly based upon mitochondrial sequences. The 3 *Bimastos* species have a nearctic distribution, while the *Dendrodrilus* species and *Ai. eiseni* are known to be dispersed through the whole temperate world, therefore their nearctic origin cannot be excluded. The monophyletic clade of *Allolobophora* and *Aporrectodea* species recalls Pop’s genus conception from 1941, the included species have closely paired setae and no pigments. Only *Ap. sineporis* and *Ap. handlirschi* are standing apart, but these species show a slight purple pigmentation, thus Omodeo (1956) created the genus *Eiseniona* to accommodate them. The observed monophyly of the majority of the analysed *Dendrobaena* species can be supported by morphological data, they possess the synapomorphic characters of genus the *Dendrobaena*, like purple pigmentation, wide setal ratio, sausage-shaped nephridial bladders and diverticula of calciferous glands in 11, 12. The clade is uniform biogeographically as well, it involves Central European and Balkan species. The excluded *D. cognettii* shows unique features not only within the genus *Dendrobaena*, but in the family Lumbricidae; it can be characterised by purple pigmentation and wide setal ratio, however it has bilobate nephridial bladders and lacks receptaculum seminis. *D. ganglbaueri* was excluded too, which is supported by numerous discriminative characters like the forward position of clitellum and tubercula pubertatis, the lack of calciferous diverticula and the small, hardly recognisable male pores.

The examined enchytraeid genera formed monophyletic groups in every case which is in agreement with the morphological genus conceptions and former molecular investigations.

The validity of the 3 *Enchytronia* species was not without doubts as *E. christenseni* was synonymised with *E. parva* recently, because of the separated spermathecae of *E. christenseni* proved insufficient to make distinction between the two species. The ITS sequences denies the justification of the above fact, the two species showed significant genetic distance from each other, moreover additional morphological features support the validity of *E. christenseni* like the variance in the number of preclitellar nephridia and the morphology of intestinal diverticula. Similarly, *E. sp. nov.* was separated clearly and the presence of lateral chaetae in every segments confirms the differentiation of the new species. Three species of *Buchholzia* (*B. fallax*, *B. simplex*, *B. appendiculata*) were considered as valid species, but the original description of *B. subterranea* was imperfect and it resembles *B. simplex* heavily. The sequence analysis of ITS region supported the species *B. subterranea* and the validity of the three other *Buchholzia* species.

Mesenchytraeus kuehnelti and *Bryodrilus glandulosus* differed in one relevant character from the description of *M. pelicensis* and *Br. ehlersi*. Both newly described species originated from a closed relictum area namely *Sphagnum*-bogs, which made probable the isolated speciation. Regarding the species *M. kuehnelti* 4 atrial glands are attached to the atrium which is the only difference compared to *M. pelicensis*, however it does not appear in the description of *M. pelicensis*. *Br. glandulosus* had glands at the orifice of the spermathecal duct. The original description of *Br. ehlersi* does not mention these glands. The recently collected *Mesenchytraeus* and *Bryodrilus* samples showed these discriminative features. Specimens were collected from other European countries as well, where the senior species are widespread, those specimens were also included in the molecular investigations. The nucleotide sequences resulted in a very low intraspecific variance in both cases, therefore it seems inevitable to carry out revisions in both cases.

Genus *Fridericia* has many, so called species-complexes, in which the observed intraspecific morphological variance hides cryptic species. One of them is *F. ratzeli*, from which two species were recently detached. *F. eiseni* was separated based only on morphological data. The validity of *F. eiseni* was justified by the ITS sequences with maximum support. *F. crassiductata* resembles *F. ratzeli* morphologically, however several

differences can also be noticed. The sequence analysis made it clear that the description of the new species is reasonable. The species in the “*F. bulboides*”-group have spermathecae with identical structure. It can be assumed that *F. bulboides* contains further microtaxa, but the validity of the formerly described species like *F. semisetosa* need confirmation by molecular data as well. *F. semisetosa* proved to be a distinct species by the sequence analyses, moreover we succeeded to separate *F. schmelzi* from *F. bulboides*. A further morphological variant of *F. bulboides* was observed, but the ITS sequences did not indicate divergence, which draws attention to the fact, that not every single morphological difference is a sign of a cryptic species, some species is characterised by significant intraspecific morphological variance. *F. aurita* is considered as a species complex too by the taxonomists. 4 morphological variant were found during our studies, all of them formed monophyletic clades with maximum support, which indicates their relatedness, however strong divergence could be found between the 4 morphological types. It seems highly reasonable that these morphological variants are probably isolated species. The original species description of *F. maculatiformis* mentions 4 praeclitellar nephridia, in contrast variants with 5 pairs were collected from different area. The new variants formed a single clade, while the original type of *F. maculatiformis* was in paraphyletic position to the former group. It is remarkable, that both variants could be sampled from certain collection sites, but they grouped based on the number of nephridia instead of localities. Although the genetic distances between the different *F. maculatiformis* variants seem significant (but lower than distances in the above mentioned species groups), it cannot be declared that the two variants represent diverged species, because there are just a few earlier ITS data from the family Enchytraeidae, and none from the genus *Fridericia*.

5. PUBLICATIONS IN THIS TOPIC

Articles published in referred journals:

- Dózsa-Farkas K., **Cech G.** (2006) Description of a new *Fridericia* species (Oligochaeta: Enchytraeidae) and its molecular comparison with two morphologically similar species by PCR–RFLP. *Zootaxa* 1310: 53-68. **IF= 0,612**
- Pop A. A., **Cech G.**, Wink M., Csuzdi Cs., Pop V. V. (2007) Application of 16S, 18S rDNA and COI sequences in the molecular systematics of the earthworm family Lumbricidae (Annelida, Oligochaeta). *European Journal of Soil Biology* 43:(S) 43-52. **IF= 0,500**

- Boros G., **Cech G.**, Ari E., Dózsa-Farkas K. (2010) Extension of employing ITS region in the investigation of Hungarian *Fridericia* species (Oligochaeta: Enchytraeidae). *Zoology in the Middle East* 49:(2) 23-30. **IF= 0,412**
- Cech G.**, Boros G., Dózsa-Farkas K. (2011) Revision of *Bryodrilus glandulosus* (Dózsa-Farkas, 1990) and *Mesenchytraeus kuehnelti* Dózsa-Farkas, 1991 (Oligochaeta: Enchytraeidae) using morphological and molecular data. *Zoologischer Anzeiger* (in press) doi:10.1016/j.jcz.2011.09.005 **IF = 1,846**

Whole article in conference brochures:

- Cech G.**, Csuzdi Cs., Márialigeti K. (2006) Remarks on the molecular phylogeny of the genus *Dendrobaena* (sensu Pop 1941) based on the investigation of 18S rDNA sequences. In: Pop V V, Pop A A (szerk.) *Advances in earthworm taxonomy II.* Cluj: Univ. Press, Cluj, 2006. pp. 85-98.
- Cech G.**, Dózsa-Farkas K. (2006) Identification of *Fridericia schmelzi* sp.n. combining morphological characters and PCR–RFLP analysis. In: PopVV, Pop AA (szerk.) *Advances in earthworm taxonomy II.* Cluj: Univ. Press, Cluj, 2006. pp. 98-118.

6. FURTHER PUBLICATIONS

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- Tóth Á., Gács M., Márialigeti K., **Cech G.**, Füzi M. (2005) Occurrence and regional distribution of SHV-type extended-spectrum beta-lactamases in Hungary. *European Journal of Clinical Microbiology & Infectious Diseases* 24: 284-287. IF = 2,061
- Mezei M., Balog K., Babic D. Z., Tóth G., **Cech G.**, Vajna B., Tauber T., Seme K., Tomazic J., Vidmar L., Poljak M., Minárovits J. (2006) Genetic variability of gag and env

- regions of HIV type 1 strains circulating in Slovenia. *AIDS Research and Human Retroviruses* 22: 109-113. IF = 2,513
- Molnár K., **Cech G.**, Székely Cs. (2008) Infection of the heart of the common bream, *Abramis brama* (L.), with *Myxobolus s.l. dogieli* (Myxozoa, Myxobolidae). *Journal of Fish Diseases* 31: 613-620. IF = 1,540
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- Frouz J., John R., Rupes V., **Cech G.**, Marialigeti K. (2009) Aggression, cooperation, and relatedness among colonies of the invasive ant, *Monomorium pharaonis*, originating from different areas of the world. *Biologia-Bratislava* 64: 139-142. IF = 0,617
- Molnár K., Eszterbauer E., Marton Sz., **Cech G.**, Székely Cs. (2009) *Myxobolus erythrophthalmi* sp. n. and *Myxobolus shaharomae* sp. n. (Myxozoa: Myxobolidae) from the internal organs of rudd (*Scardinius erythrophthalmus* L) and bleak (*Alburnus alburnus* L). *Journal of Fish Diseases* 32: 219-231. IF = 1,603
- Rangel L. F., Santos M. J., **Cech G.**, Székely Cs. (2009) Morphology, molecular data and development of *Zschokella mugilis* (Myxosporea, Bivalvulida) in a Polychaete alternate host, *Nereis diversicolor*. *Journal of Parasitology* 95: 561-569. IF = 1,208
- Székely Cs., Shaharom-Harrison F., **Cech G.**, Ostoros Gy., Molnár K. (2009) Myxozoan infections in fishes of the Tasik Kenyir Water Reservoir, Terengganu, Malaysia. *Diseases of Aquatic Organisms* 83: 37-48. IF = 1,687
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neapolitana (Polychaeta: Onuphidae) in the Aveiro Estuary (Portugal). Parasitology
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