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Evolutionary Loss of Parasitism by Nematodes? Discovery of a Free-Living Filaroid Nematode

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ABSTRACT: A cattle-drinking pool in nature reserve "Zwin" on the Belgian coast contained free-living third-stage infective filaroid juveniles. These juveniles clearly differ morphologically from all known nematodes. Morphological and molecular analyses indicate a position within the Filaroidea. The aberrant biology of this nematode, namely, a free-living stage in an aquatic environment, is unknown within this superfamily, and the evolution of the parasitic phenotype to a free-living state is generally thought to be unlikely. However, the obtained placement in the small subunit molecular phylogenetic tree suggests that this free-living stage is most likely a secondary adaptation. It is reasonable to assert that nematodes with complex life cycles still have the genetic potential for a reversion from parasitism to a (partial) free-living stage.

Adult filaroids are parasites of tissues and tissue spaces of all classes of vertebrates other than fishes (Anderson and Bain, 1976). They are all transmitted by hematophagous arthropods, which include most of the major groups known to suck the blood of higher vertebrates, i.e., biting midges, black flies, fleas, horse and deer flies, mosquitoes, lice, louse flies, mites, and ticks (Anderson, 2000). In these intermediate hosts, microfilariae develop to infective third-stage juveniles that are inoculated in the final host. The life cycle is completed without a free-living stage, and, unlike other Spirurida, e.g., *Dracunculus medinensis* with copepods as intermediate hosts, members of Filaroidea do not have strict aquatic intermediate hosts in their life cycle. Counter to what is normally recognized within Filaroidea, we have detected free-living third-stage infective filaroid juveniles in the bottom sediment of a cattle-drinking pool.

During the multidisciplinary project "MANSCAPE" (integrated management tools for water bodies in agricultural landscapes), bottom samples were taken in several cattle-drinking pools in Belgium. In this survey, the top 10 cm of the sediment was sampled at 8 arbitrarily chosen points within each pool by using a Perspex core (inner diameter 5.7 cm); nematodes were extracted by the centrifugal-floatation technique. Two replicas of a cattle-drinking pool in nature reserve "Zwin", close to the Belgian coast (51°21,418′N, 3°20,536′E), contained the unknown juvenile filaroids. To find other possible life cycle stages, tadpoles and arthropods (chironomid larvae) that inhabit and surround the pool were screened, but without success.

The unknown filaroids were morphologically characterized using an Olympus BX 51 differential interference contrast microscope; measurements and illustrations were prepared using a camera lucida; the drawings (Fig. 1) were prepared using Illustrator 10.0 software (Adobe Systems, Mountain View, California). The morphology also was recorded as video clips that mimic multifocal observation through a light microscope following the video capture and editing procedures developed by De Ley and Bert (2002). The resulting virtual specimens are available on the Web at http://www.nematology.ugent.be/VCE/filarioid.htm.

The juvenile body was large (2.1–2.3 mm) and almost straight after fixation. The cuticle had a lateral field with fine annulations. The mouth opening was surrounded by conspicuous protruding liplike structures; the anterior part of the stoma was squarish, and the posterior part was indistinct and surrounded by 2 conspicuous cells. The stoma-pharynx transition was indistinct; the pharynx was 0.72–0.76 mm long. It was divided into an anterior muscular region and a posterior pharyngeal part, both about equal in length; cardia were well developed. The excretory pore was 0.17 mm posterior from the head. The intestine possessed a wide lumen; the long rectum was surrounded by distinct cells. The tail had 2 distinct ventrolateral subterminal projections, each with 3 tubercles. The terminal portion of the tail had 2 terminal handlike structures, which are 6- or 7-fingered; the handlike structures are opposite each other. With this remarkable tail structure, the juveniles clearly differ from all other known nematodes; the other morphological features are, however, in accordance with third-stage infective juveniles of the Onchocercidae (Bain and Chabaud, 1986).

One live specimen was fixed in acetone to allow molecular analysis. DNA extraction and sequencing were as described in Tandingan De Ley et al. (2002). The 18S rDNA sequence of the unknown filaroid (GenBank DQ103704) was aligned with the published sequences of 12 other taxa and 1 unpublished sequence by using the program Clustal_X, version 1.64 under default settings (Thompson et al., 1997). Phylogenies were estimated using maximum parsimony (MP) and maximum likelihood (ML) criteria as implemented in PAUP, version 4b10 (Swofford, 1998), and Bayesian inference as implemented in MrBayes, version 3.0b4 (Huelsenbeck and Ronquist, 2001). Modeltest, version 3.06 (Posada and Crandall, 1998), was used to determine the best-fit maximum likelihood models. This model was the general time reversible model (GTR+I+G). The parameters for base frequencies, substitution rate matrix, and shape and proportion of invariant sites were allowed to vary throughout the Bayesian analysis. The total number of generations in this analysis was set to 1 million, 200 times greater than the burn-in value. Four parallel chains (1 cold and 3 heated) were used. Trees were sampled every 1,000 generations. The burn-in value was set to 10,000

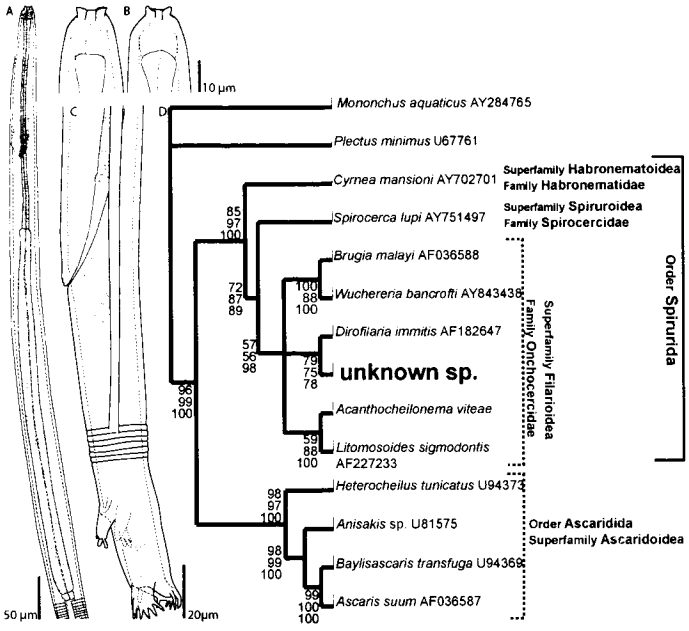

FIGURE 1. Morphological and molecular analyses of unknown free-living juvenile filarioid. Illustrations of neck region (**A**), head regions (**B**), and tail (**C**). Phylogenetic position of unknown filarioid inferred from 18S rDNA (**D**). Summary cladogram obtained from MP, ML, and Bayesian analysis. Branches support indicated by bootstrap values >50% (MP-ML), calculated by 1,000 replicates, and Bayesian probability values, all expressed as percentages. MP bootstrap values, up; ML bootstrap values, middle; and Bayesian probabilities, down. Outgroup: *Mononchus aquaticus* and *Plectus mimimus*. The species used are associated with GenBank accession numbers, except *Acanthocheilonema viteae*, which is an unpublished sequence.

generations, which equated to the next 7,000 generations above the level at which the log likelihood reaches a stable value in a preliminary run. Majority-rule consensus trees were reconstructed from the fundamental trees. To estimate nodal support in MP and ML, 1,000 bootstrap replicates were calculated using heuristic search criteria.

These molecular analyses (Fig. 1) place the unknown filarioid within the superfamily Filaroidea, order Spirurida. Monophyly of the Spirurida is robustly supported, and the filaroid clade is moderately supported (67% MP bootstrap, 69% ML bootstrap, 75% Baysian probability); however, the phylogenetic relations within the Filaroidea could not been determined unequivocally. The position of the arthropod-parasitic *Brumptaemilius justini* (Rhigonematida), which comprises free-living juveniles in its life cycle, is uncertain in relation to the Ascaridida and Spirurida, and according to Wijová et al. (2005), this species actually forms a paraphyletic assemblage with Ascaridida and Spirurida. Conversely, in our analyses, which are based on a slightly larger data set, this arthropod-parasitic rhigonematid is consistently retrieved outside the Spirurida clade.

Our combined morphological and molecular results indicate that this filaroid is an undescribed genus within the Onchocercidae. Formal description of this new filaroid genus is not possible at present because adults could not be found.

The aberrant biology of this nematode, namely, a free-living stage in an aquatic environment, is unknown within the Filaroidea. The placement in the small subunit molecular phylogenetic tree suggests that a free-living stage is most likely a secondary adaptation. However, the evolution of the parasitic phenotype requires coordinate acquisition of many novel traits and thus reversion to a free-living state is thought to be unlikely. Hence, irreversibility of adaptation to a parasitic mode of existence is widely postulated (for an overview, see Siddall et al., 1993). This view also has been supported by genomic analyses of intracellular eubacterial and eukaryote parasites, where parasitism is associated with genome reduction and loss of many enzymatic pathways (Katinka et al., 2001). The lost metabolism is replaced by nutrient scavenging from the host. In nematode parasites, however, such reductive loss compared with free-living taxa is unlikely. The parasites have added environments to their life cycles, and they also must survive the immune responses and other protective mechanisms they now encounter (Blaxter et al., 2004). For example, *Ascaris suum* retains normal aerobic metabolism for survival as a first- to third-stage larva before it relies on a reduced, relatively inefficient, anaerobic energy metabolism (Duran et al., 1998). Similarly, although tissue-dwelling filarial nematodes secure sugars and nucleosides from the vertebrate host, they also must exploit the very different environment provided by arthropod vectors. It is significant to note that the filarial nematode *Brugia malayi* expresses many genes that are apparently missing from the genome of the free-living *Caenorhabditis elegans* but that are present in other eukaryote phyla (Blaxter et al., 2002). This relation indicates that gene loss, and genomic simplification, is not necessarily associated with parasitism in the Nematoda. Based on extended phylogenetic analyses, Siddall et al. (1993) already demonstrated reversibility of parasitism in unicellular organisms, and these authors pointed out that parasitism may prove to be less rigid if more phylogenies were accurately reconstructed. In this respect, the Nematoda were especially mentioned, because they contain species with a variety of life history modes, including wholly autonomous, free-living taxa, those with alternating free-living and parasitic cycles, and others with the most intimate intracellular associates. Blaxter et al. (2004) suggest that for parasites with multiple hosts or with complex life cycles, the rule that parasitism leads to reduction is unlikely to be true at a genomic level, no matter that the simplifications in morphology are demonstrable. Thus, from this point of view, reversibility of parasitism is genetically not impossible in nematodes.

Nevertheless, isolation from the host caused by some irregularity, e.g., postsampling isolation, cannot be excluded in this case. There are several arguments in favor of an actual free-living stage: (1) live free-living individuals were found almost immediately after sampling, but they were equally well be retrieved live after 7 mo in the same sample (stored by 6 C); (2) the juveniles were found in 2 independently acquired replicates, which reduces the possibility of an erroneous finding; and (3) the infective juveniles could not be retrieved from arthropods from the samples (chironomid larvae, copepods, and ostracods) at the same site. To complete the knowledge of this organism, multiple benthic samples, vertebrates, and potential transmitting insects must be further assessed. Hopefully, this approach will lead to a resolution of the life cycle of this aberrant organism and provide insight regarding the mode of parasitic evolution and host relations within the Filaroidea.

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LITERATURE CITED

ANDERSON, R. C. 2000. Nematode parasites of vertebrates. Their development and transmission, 2nd ed. CABI Publishing, Wallingford, Oxon, U.K., 650 p.

———, AND O. BAIN. 1976. Keys to the genera of the order Spirurida. Diplotriaenoidea, Aproctoidea and Filarioidea. *In* CIH keys to the nematode parasites of vertebrates 3. Part 3, R. C. Anderson, A. G. Chabaud, and A. G. Willmort (eds.). CABI Publishing, Wallingford, Oxon, U.K., p. 87–100.

BAIN, O., AND A. G. CHABAUD. 1986. Atlas des larves infestantes de filaires. Tropical Medicine and Parasitology **37:** 301–340.

BLAXTER, M., J. DAUB, D. GUILIANO, J. PARKINSON, AND C. WHITTON. 2002. The *Brugia malayi* genome project: Expressed sequence tags and gene discovery. Transactions of the Royal Society of Tropical Medicine and Hygiene **96:** 7–17.

———, R. FLOYD, M. DORRIS, E. ABEBE, AND P. DE LEY. 2004. Utilizing new nematode phylogeny for studies of parasitism and diversity. *In* Proceedings of the Fourth International Congress of Nematology, 8–13 June 2002, Tenerife, Spain. Nematology Monographs and Perspectives 2, R. Cook, and D. J. Hunt (eds.). Brill, Leiden, The Netherlands, p. 615–632.

DE LEY, P., AND W. BERT. 2002. Video capture and editing as a tool for

the storage, distribution and illustration of morphological characters of nematodes. Journal of Nematology **34:** 296–302.

DURAN, E., D. J. WALKER, K. R. JOHNSON, P. R. KOMUNIECKI, AND R. W. KOMUNIECKI. 1998. Developmental and tissue-specific expression of 2-methyl branched-chain enoyl CoA reductase isoforms in the parasitic nematode, *Ascaris suum*. Molecular and Biochemical Parasitology **91:** 307–18.

HUELSENBECK, J. P., AND F. RONQUIST. 2001. MrBayes: Bayesian inference of phylogeny. Bioinformatics **17:** 754–755.

KATINKA, M. D., S. DUPRAT, E. CORNILLOT, G. METENIER, F. THOMARAT, G. PRENSIER, V. BARBE, E. PEYRETAILLADE, P. BROTTIER, P. WINCKER, F. DELBAC, H. EL ALAOUI, P. PEYRET, W. SAURIN, M. GOUY, J. WEISSENBACH, AND C. P. VIVARES. 2001. Genome sequence and gene compaction of the eukaryote parasite *Encephalitozoon cuniculi*. Nature **414:** 450–453.

POSADA, D., AND K. A. CRANDALL. 1998. MODELTEST: Testing the model of DNA substitution. Bioinformatics **14:** 817–818.

SIDDALL, M. E., D. R. BROOKS, AND S. S. DESSER. 1993. Phylogeny and the reversibility of parasitism. Evolution **47:** 308–313.

SWOFFORD, D. L. 1998. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer, Sunderland, Massachusetts.

TANDINGAN DE LEY, I., P. DE LEY, A. VIERSTRAETE, G. KARSSEN, M. MOENS, AND J. VANFLETEREN. 2002. Phylogenetic analysis of *Meloidogyne* SSU rDNA. Journal of Nematology **34:** 319–327.

THOMPSON, J. D., T. J. GIBSON, F. PLEWNIAK, F. JEANMOUGIN, AND D. G. HIGGINS. 1997. The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research **25:** 4876–4882.

WIJOVÁ, M., F. MORAVEC, A. HORÁK, D. MODRÝ, AND J. LUKEŠ. 2005. Phylogenetic position of *Dracunculus medinensis* and some related nematodes inferred from 18S rRNA. Parasitology Research **96:** 133–135.