Journal of Invertebrate Pathology 98 (2008) 299–306

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/yjipa

A molecular revision of the taxonomic status of mermithid parasites of black flies from Quebec (Canada)

Mylène St-Onge [b,*], Bernard LaRue [a], Guy Charpentier [a]

[a] Département de Chimie-biologie, Université du Québec à Trois-Rivières, 3351 Boulevard des Forges, Box 500, Trois-Rivières, Canada G9A5H7
[b] Laboratoire de Lutte Biologique, Département des Sciences Biologiques, Université du Québec à Montréal, Box 8888, Succ. Centre Ville, Montréal, Que., Canada H3C 3P8

ARTICLE INFO

Article history:
Received 16 October 2007
Accepted 1 April 2008
Available online 8 April 2008

Keywords:
Mermithidae
Nematodes
Simuliid hosts
PCR probes
Cryptic species
Multiple parasitism

ABSTRACT

The four currently recognized mermithid (Nematoda) species parasitizing black flies (Diptera: Simuliidae) from Northeast America were distinguished using discriminatory PCR primers aimed at COI and 18S rDNA. *Isomermis wisconsinensis*, *Gastromermis viridis* and *Mesomermis camdenensis* were easily differentiated using either genomic target, even for juvenile mermithids damaged beyond morphological recognition. However, specimens from *Mesomermis flumenalis* being identical in external morphology and producing a unique-sized PCR product were classified by sequence data into four clearly distinguished molecular variants. This quartet was made of two winter and two summer 'physiological variants', including one which also belonged to, but diverged early from the rest of the *Mesomermis* genus. Combining the multiplex PCR and sequencing approaches allowed for the characterization of a multiple parasitism which simultaneously implicated *I. wisconsinensis* and two *M. flumenalis* variants. With another instance where parasites were identified by morphology only, this is the first report of black fly parasitism by multiple mermithid species. A phylogenetic tree built by combining our sequences to previous GenBank entries likely indicates a monophyletic origin for the mermithid family, but also suggests that differentiation between parasite genera sometimes occurred before the evolutionary emergence of the actual host group.

1. Introduction

Among all parasites, mermithids are responsible for the highest known infection rates in larval black flies (Adler et al., 2004). In eastern Nearctic regions, only four species are currently recognized as black fly parasites, namely *Mesomermis flumenalis*, *Gastromermis viridis*, *Isomermis wisconsinensis* (Welch, 1962) and *Mesomermis camdenensis* (Molloy, 1979). Their taxonomy, which has been reviewed by Poinar (1979), but remains poorly developed, relies mostly on adult genitalia, while the more frequently sampled parasitizing juveniles are identified primarily by the shape of their stylus. However, specimens are easily damaged upon extraction from their host, which causes a high level of unidentified or misidentified individuals. More importantly, the paucity of morphological criteria leads to the suspicion that there may be many more species than usually recognized. For instance, two 'physiological variants' of *M. flumenalis*, the so-called winter and summer types, were identified in Newfoundland and each shown to be univoltine and to differ from the other by host range, temperature tolerance and seasonal life cycle (Bailey and Gordon, 1977; Ebsary and Bennet, 1975; Ezenwa, 1974). Given their effective reproductive isolation, these variants stand as likely candidates for the eventual recognition of two distinct species. Contrary to the limited host range of the three other species, *M. flumenalis* also appears to infect a disproportionate number of black fly species (Adler et al., 2004; Colbo and Porter, 1980; the authors, in press). This suggests that one actually sees only the tip of the iceberg and that *M. flumenalis* could in fact correspond to a complex of several species (Gordon, 1984). Others (Colbo, 1990) have also expressed the opinion that the apparent lack of a specific host–parasite relationship may indicate a general taxonomic problem for many mermithid species.

Consequently, the development of DNA-based typing methods seems desirable not only for the purpose of mermithid identification, including damaged or fragmentary specimens, but also as a tool for ecological studies. The latter includes a clearer understanding of the specificity, or lack of it, of the host–parasite relationship, especially in the case of multiple parasitism. Also, comparative DNA sequence analysis could open the way for a more detailed understanding of mermithid phylogeny. The present report describes such a molecular tool and shows that '*M. flumenalis*' consists of at least four molecular variants which are clearly distinguished based on partial sequences from the nuclear 18S rDNA (18S) and from the mitochondrial gene of subunit I of the cytochrome oxidase complex (COI).

* Corresponding author. Fax: +1 514 987 4647.
E-mail address: st-onge.mylene@courrier.uqam.ca (M. St-Onge).

0022-2011/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2008.04.001

2. Methods

2.1. Sample collection

Mermithids were collected in streams from various locations in Quebec (Canada) over a 2-year period. Specimens, most of them parasitizing juveniles extracted from their host, were identified under the microscope using criteria from Molloy (1979), Phelps and DeFoliart (1964) and Poinar (1979) and stored afterwards at 4 °C in 95% ethanol prior to DNA extraction. Black flies were identified under a binocular microscope using the keys of Wood et al. (1963) and Adler et al. (2004).

2.2. DNA purification

Following a brief hydration in TE buffer (Sambrook and Russell, 2001), the mermithid was transferred to a microcentrifuge tube containing 120 µl of a lytic buffer originally developed for *Drosophila* (Zyskind and Bernstein, 1989) and DNA was extracted as previously described for insect material (Léry et al., 2003). The final purification step involved cleaning on a commercial resin (Wizard DNA clean-up resin, Promega) and recovery of DNA in 50 µl of sterile water.

2.3. PCR assays, characterization of amplification products and DNA sequencing

DNA amplification was performed in a 25 µl volume containing 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 200 µM of each dNTP, 0.4 µM of each PCR primer, 1 U of *Taq* DNA polymerase (Roche) and 1 µl of purified mermithid DNA. The thermocycling profile was 180 s at 95 °C, followed by 38 cycles of 30 s at 95 °C, 50 s at annealing temperature and 60 s at 72 °C and ended by a final extension of 180 s at 72 °C. Sequences, targets and annealing temperatures of PCR primers are described in detail by Table 1. For size determination purposes, amplified DNA was analyzed by electrophoresis on a 5% polyacrylamide + 2.7 M urea gel running in TBE buffer (Sambrook and Russell, 2001) at 12 V/cm for 1 h and DNA bands were stained with ethidium bromide prior to photography. If sequencing was required, the PCR products were instead separated shortly on a 1% agarose gel, after which the band of interest was cut and DNA extracted (QIAquick PCR purification kit from QIAGEN). Products from cycle sequencing performed through the BigDye Terminator v3.1 Cycle Sequencing Kit were characterized on the AB13130XL Genetic Analyser and sequence data were processed using the AB DNA Sequencing Analysis Software v5.2 (reagents, equipment and software all from Applied Biosystems; analyses performed at the Centre de Séquençage de l'Université Laval, Quebec, Canada).

Table 1
Sequences, targets and annealing temperatures of PCR primers

Name[a]	Sequence[a] (5′ → 3′)	Target[b]; annealing temperature
JB3	TTTTTTGGGCATCCTGAGGTTTAT	COI/codon 235; 47 °C
JB4.5	TAAAGAAAGAACATAATGAAAATG	COI/codon 382; 47 °C
cCOI-R	TGNCCAACYACAAARTAGGTRTCATG	COI/codon 375; 61 °C
COI-F.Mf	TTCTTATTTTACCTGCTTTTGGGATAG	COI/codon 245; 61 °C
COI-F.Mc	AGGGGTAAAAAATATGTATTTGGTACTA	COI/codon 262; 61 °C
COI-F.Gv	TTTACTGTGGGGTTGGATATTGATTC	COI/codon 292; 61 °C
COI-F.Iw	GCAATAATTAGAATTAGAATTCTTGGTA	COI/codon 276; 61 °C
c18S-F	CAGGTNTGTGATGCCCTTAGATG	18S/−350; 52 °C, 61 °C[c]
c18S-R	ATGATCCAGCTGCAGGTTCACCTAC	18S/−27; 52 °C
18S-R.Mf	TTACGACTTTTACTTCCTCTAGAGG	18S/−35; 61 °C
18S-R.Mc	CCAGTTTAAAAAACTTCTCCAAAACAT	18S/−64; 61 °C
18S-R.Gv	CGTACTTGCCACAGTCCAAGCG	18S/−101; 61 °C
18S-R.Iw	TCAACTCGAGCTTATGACTCGCAT	18S/−187; 61 °C

[a] F, -R, forward, reverse primer. The prefix 'c' designates a common primer while .Mf, .Mc, .Gv and .Iw identify primers being specific for *M. flumenalis*, *M. camdenensis*, *G. viridis* and *I. wisconsinensis*, respectively. JB3 (forward) and JB4.5 (reverse) COI primers are from Hu et al. (2005). *N* = mixture of all four bases, Y = C/T, R = A/G.

[b] Target: hybridization site of the primer 3′ terminal base, with reference to COI (GenBank Accession No. X54252) and 18S rDNA (upstream position from ITS1 start; Ellis et al., 1986) sequences from *C. elegans*.

[c] Annealing at 52 °C with c18S-R as second primer, 61 °C otherwise.

2.4. Sequence alignment and phylogenetic tree

18S sequences were aligned with Clustal W and a neighbor-joining (PHYLIP v3.67 software) phylogenetic tree was built using the Kimura 2 parameters distance.

3. Results

3.1. Parasite sample

The mermithid sample was composed of 47 individuals, 85% of them being juveniles. This included 22 ambiguous or damaged specimens. The remaining 25 comprised 13 individuals from *M. flumenalis* (Mf), five from *M. camdenensis* (Mc), five from *I. wisconsinensis* (Iw) and two from *G. viridis* (Gv). For each species, a single adult female which could be clearly identified based on genitalia morphology was set aside as a reference specimen in order to extract the sequence information needed for specific primer design as described below.

3.2. Designing the PCR primers

The COI-specific primers JB3 and JB4.5, originally used by Hu et al. (2005) for the molecular characterization of cestode species, gave large amounts of PCR product with Mf and Mc DNA, but worked so poorly with Gv and Iw that reamplification proved essential to obtain enough material for sequencing purposes. The information from the resulting 440 bp sequences was used to create five new internal primers (Fig. 1), including a partly degenerate reverse primer cCOI-R hybridizing to a common target being shared by all four species and four specific primers, COI-F.Mf, COI-F.Mc, COI-F.Gv and COI-F.Iw. The 3′ terminal base of each of these four primers was chosen such that it would hybridize to the DNA of only one species and also targeted a first or second codon position to avoid amplification failure due to possible intraspecific third position polymorphism. Predicted and observed (Figs. 1 and 3) PCR product sizes were 394, 344, 302 and 250 bp for Mf, Mc, Iw and Gv, respectively. To create 18S primers, we first compared sequences from *Drosophila melanogaster* (Tautz et al., 1988) and *Caenorhabditis elegans* (Ellis et al., 1986), which revealed two long nucleotide stretches, with approximate locations at 15 and 360 bases upstream from the 18S 3′ end, that were highly homologous between the two species. Assuming that these sequences would be very similar in the mermithid genome, we used parts of these conserved elements to create the c18S-F and c18S-R primers. The sequence information (Fig. 2) available after a successful amplification using these external primers led to the design of four internal specific reverse primers which, in combination with the leftward common primer c18S-F, enabled a distinction between all four species.

3.3. Specificity of the discriminatory PCR assays

As shown by Fig. 3A, a COI or 18S assay using a combination of the four specific primers plus the common primer led to a full species discrimination following either PCR. The band profile of both COI and 18S matched perfectly the morphological identification of all specimens for which this information was already available and each of the remaining dubious or damaged individuals could