Morphological and molecular analyses of the spiruroid nematode, *Falcaustra araxiana* Massino, 1924 (= *Spironoura araxiana*) from the European pond turtle (*Emys orbicularis*)

M. Rajabloo¹, H. Sharifiyazdi²*, F. Namazi¹, H. Shayegh³, E. Rakhshandehroo¹ and G. Farjanikish⁴

¹Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, PO Box 1731, Shiraz 71345, Iran; ²Department of Clinical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, PO Box 1731, Shiraz 71345, Iran; ³School of Veterinary Medicine, Shiraz University, Shiraz, Iran; ⁴Department of Pathobiology, School of Veterinary Medicine, Lorestan University, Khorram Abad, Iran

(Received 2 November 2015; Accepted 7 March 2016)

Abstract

There is little information on the phylogenetic position and life cycle of family Kathlaniidae. *Falcaustra araxiana* is a member of this family which infects the large intestine of the European pond turtle (*Emys orbicularis*). In the present study, morphological data and molecular analyses based on the 18S rDNA were performed on different types of *F. araxiana* originating from the large intestine and gastric nodules in the turtle. Morphological data revealed both larvae and adult stages in the gastric nodules. In addition, all nematodes recovered in the large intestine were adult worms. GenBank accession numbers KM200715 and KM200716 were provided for adult *F. araxiana* located in the intestine and stomach, respectively, of *E. orbicularis*. The results of sequencing proved that these two types are completely similar. Accordingly, it can be hypothesized that nodule formation is a part of the life cycle of the parasite or a survival strategy. Furthermore, *F. araxiana* develops to the adult stage in the gastric mucosa prior to migrating to the large intestine. Phylogenetic analysis revealed that *F. araxiana* unexpectedly branched away from other members of the superfamily Seuratoidae (Truttaedacnitis truttae, Cucullanus robustus and C. baylisi) and showed a closer relationship with *Paraquimperia africana*, a member of the Ascaridoidea. It seems that phylogenetic reconstruction for the present parasite needs more detailed morphology, life cycle and molecular studies.

Introduction

Species of *Falcaustra* Lane, 1915 (= *Spironoura* Leidy, 1856) (Cosmocercoidea, Kathlaniidae) have been reported in the digestive tract of fish, amphibians and reptiles (Bursey et al., 2012). Generally, findings on the life cycle of different species of *Falcaustra* are not adequate. Specifically, Bartlett & Anderson (1985) described third-stage larvae of *Falcaustra* in tissues of a freshwater snail, whereas Moravec et al. (1995) recovered larvae of *Falcaustra* spp. from freshwater fish collected in Texas. Anderson

*E-mail: Hassansharifi@gmail.com*
and DNA was stored at −20°C until further processing. To date, there is little information about the phylogenetic position of the genus and only one gene sequence has been published (Hasegawa et al., 2013). There are two reports of Falcaustra araxiana in the large intestine of the European pond turtle, Emys orbicularis, from Armenia and Iran, albeit without molecular analysis (Yamaguti, 1962; Bursey & Rivera, 2009; Shayegh et al., 2016). In our previous study, F. araxiana was found in the large intestine of the European pond turtle (Shayegh et al., 2016). However, other helminths similar to F. araxiana in appearance, but different in size, were also discovered in gastric nodules. In the present study, helminths from the nodules and the large intestine of the European pond turtle have been described, as well as the phylogenetic position of F. araxiana using molecular analysis of the 18S rDNA gene, together with histopathological changes to host tissue.

Materials and methods

Sample preparation

Worms were recovered from the large intestine and gastric nodules of European pond turtles (E. orbicularis) as described previously by Shayegh et al. (2016). The worms were then examined for morphological features according to the previous reports (Yamaguti, 1962). In order to determine probable similarities between helminths originating from gastric nodules and those from the large intestine, we examined primarily the dimensional differences in the recovered worms. In the next step, 18S rDNA gene analysis was conducted. For molecular examination two males and two females of F. araxiana recovered from the large intestine and two of each from the nodules were selected randomly. Formalin-fixed nodules were also used for histopathological studies.

Molecular examination

Genomic DNA from all collected samples was extracted and purified individually using the DNeasy Tissue Kit (Qiagen, Valencia, California, USA), according to the manufacturer’s recommended protocol, and used as template DNA for the polymerase chain reaction (PCR). Briefly, to achieve the desired results, we used a 2–3 h incubation time for samples at 56°C with 180 μl tissue lysis buffer (ATL, Qiagen) and 20 μl (50 μg/ml) of proteinase K, vortexing every 30 min. After adding 200 μl of lysis buffer (AL, Qiagen) containing guanidine hydrochloride and 200 μl ethanol, the mixture was vortexed for 15 s and then added to a DNA-binding column and spun down for 1 min. The column was then washed several times using 500 μl of AW1 and AW2 buffers (Qiagen). The genomic DNA extract was diluted to a working concentration of 20 ng/μl, and 4 μl of it was used as a template in PCR. All extracted DNA was stored at −20°C until further processing.

Primers and PCR amplification

The 18S rDNA sequences (~1800 bp) were amplified by universal primers NC18SF1 (5′-AAAGATTAAGCCATGCA-3′) and NC5BR (5′-GCAGGTTCACCTACAGAT-3′) (Brianti et al., 2012). The following PCR conditions were applied to each assay: 50 mM KCl, 10 mM Tris–HCl (pH = 9.0), 1.5 mM MgCl₂, 200 μM deoxynucleoside triphosphates (dTTPs), 20 pmol of each primer and 2 U Taq DNA polymerase (Fermentas, Burlington, California, USA) per 50-μl reaction, using 4 μl of the DNA extracted as the template. For amplification, the samples were cycled in a Bio-Rad thermocycler (Bio-Rad Laboratories, Hercules, California, USA). Cycling conditions included an initial DNA denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 1 min and an extension at 72°C for 1 min. Sterile water was used as a negative control. The presence of amplicons and their size was assessed by electrophoresis of 5 μl of each reaction product in 1.5% (w/v) Tris–acetate/EDTA agarose gel and visualized by staining with ethidium bromide (final concentration of 0.5-μg/ml) under UV light. Images were captured on a computer and printed.

Amplified products were purified with a PCR product purification kit (BioNeer, Korea) and sequenced directly using a capillary DNA analyser (ABI 3730; Applied Biosystems, Foster City, California, USA) after sequencing reactions with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). Forward and reverse nucleic acid sequence data were used to construct a continuous sequence of each inserted DNA. The 16S rDNA sequence obtained was compared to GenBank entries using the BLAST tool provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

To determine the phylogenetic position of F. araxiana, the sequence obtained for this parasite was compared with homologous sequences previously reported for other nematodes in Ascaridida. Creating multiple-sequence alignment was established using the Clustal W program in the MEGA 4.0 software for each queried DNA sequence (Tamura et al., 2007). Data sequences were also used for construction of the phylogenetic trees using maximum parsimony and neighbour-joining methods.

Histopathology

Tissues removed from the liver, stomach, intestines and spleen of the pond turtle were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm and stained with haematoxylin and eosin (H&E) for light microscopic examination.

Results

Morphological study

Different stages of nematodes recovered from the gastric nodules (fig. 1A) were previously identified as F. araxiana using morphological characteristics (Shayegh et al., 2016). Two stages of nematodes were identified, including larvae or pre-adults and adult worms with genitalia, some of which were in the nodules but the majority of adults occurred in the large intestine. All worms were cylindrical with truncated anterior and tapered posterior ends. The mouth was surrounded by three large vesiculated cephalic lips, each with a medial V-shaped indentation, giving a hexagonal appearance to the oral opening when viewed...