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

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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 Seuratoidea (*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

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(2000) also suggested that, in general, kathlaniids develop to the third stage outside the host and then invade various invertebrates, which serve as paratenic hosts.

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′- GCAGGTT CACCTACAGAT-3′) (Brianti *et al.*, 2012). The following PCR conditions were applied to each assay: 50 mM KC1, 10 mM Tris–HCl (pH = 9.0), 1.5 mM $MgCl_2$, 200 µM deoxynucleoside triphosphates (dNTPs), 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 phylogenic 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

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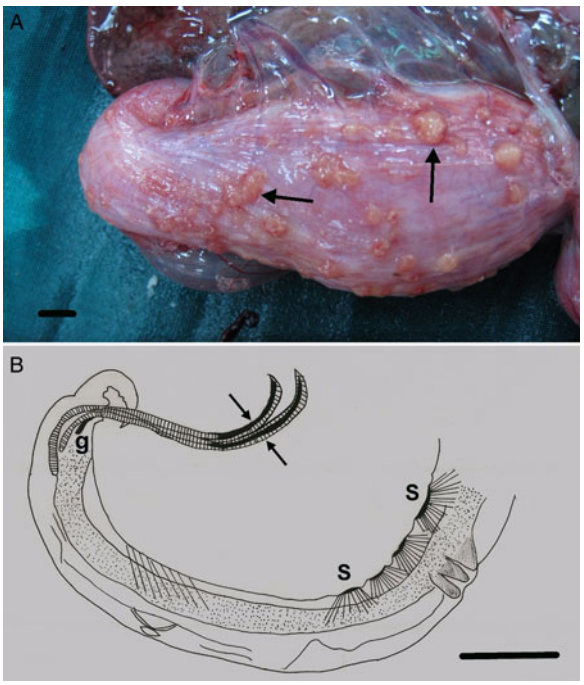

Fig. 1. (A) Nodules (arrowed) on the gastric surface of the European pond turtle (*Emys orbicularis*) caused by *Falcaustra araxiana*, scale bar: 500 µm. (B) Posterior end of male *F. araxiana* with protruding spicules (arrowed), gubernaculum (g) and four pseudosuckers (S), scale bar: 500 µm.

apically. The oesophagus possesses a posterior spherical bulb and anteriorly positioned isthmus. Males possess two elongate spicules with four pseudosuckers and a gubernaculum (fig. 1B). Pairs of caudal papillae showed a 6–6–10 + 1 pattern. There were four pseudosuckers in the posterior end of the body.

Molecular analysis

Using universal amplification as described, all parasites produced a fragment of approximately 1800 bp, which corresponds to the 18S rDNA gene.

Interestingly, sequence analysis showed that all parasite samples originating from different tissue sources were completely identical to each other based on the 18S rDNA sequence. GenBank accession numbers KM200715 and KM200716 were provided for worms separated from the large intestine and stomach, respectively. Comparative sequence analysis of Iranian *F. araxiana* sequences with other related taxa existing in the GenBank database demonstrated the highest homology (>99%) with *Falcaustra catesbeianae* and *Paraquimperia africana*. Unexpectedly, phylogenetic analysis of concatenated data showed that both species of *F. araxiana* and *F. catesbianae* (as the members of Cosmocercoidea) were located within the Ascaridoidea clade, whereas all other Cosmocercoidea members, i.e. *Cosmocercoides* sp., *Raillietnema* sp., *Dacnitoides sp.*, *Cruzia americana* and *Nemhelix bakeri*, form a separated major clade (fig. 2).

According to the phylogenetic tree, the Iranian *F. araxiana* together with *P. africana* and *F. catesbianae* form a sister group for members of Seuratoidea (*Truttaedacnitis truttae*, *Cucullanus baylisi* and *Cucullanus robustus*). In addition, in accordance with the morphological classification, *T. truttae*, *C. robustus* and *C. baylisi* all belong to the superfamily Seuratoidea and are placed in a separate clade based on 18S rDNA analysis.

Histopathology

Small foci of haemorrhage were observed in the thickened mucosal surface of the small intestine, with free worms in the lumen. Also, firm nodules were visible on the gastric serosal and mucosal surfaces, with most of the nodules containing worms.

Histopathological examinations of the tissue sections of gastric nodules revealed granulomatous reactions on larvae, which were surrounded by necrotic debris, eosinophils, mononuclear cells and a few giant cells within a connective tissue capsule. No lesions were observed on the liver or spleen.

Discussion

Up to now, 95 species of *Falcaustra* have been identified from different hosts. Reptiles and amphibians are final hosts for these species (Anderson, 2000; Bursey & Rivera, 2009); among them, freshwater turtles and lizards are common hosts for these parasites (Anderson, 2000). Despite the identification of many species of this parasite, the life cycle of the family Kathalaniidae (the Oxyascaridinae, with *Oxyascaris* and *Pteroxyascaris*) have not been completely identified (Anderson, 2000). Moravec *et al.* (1995) reported that freshwater fishes can act as paratenic hosts for *Falcaustra* spp. Also, third-stage larvae of *Falcaustra wardi* have been found in the tissue of the freshwater snail *Lymnea stagnalis* (Bartlett & Anderson, 1985).

In this study we found that both larvae and adults of *F. araxiana* were located in the gastric nodules, and phylogenetic analysis using 18S rDNA gene sequencing confirmed that the two types were similar, suggesting that nodule formation is part of the life cycle or possibly a survival strategy. In addition, larval stages of *F. araxiana* develop in the gastric mucosa before migrating to the large intestine.

Some nematodes, such as *Oesophagostomum*, have nodule formation in their life cycles (Soulsby, 1982; Zajac & Conboy, 2012). Nodular formation caused by hypobiotic or arrested larvae is seen in strongylid and ascarid nematodes (Zajac & Conboy, 2012), such as *Toxocara*, *Amplicaecum* and *Ophidascaris* (Wharton, 1986; Anderson, 2000). Consistently, our study concludes that the nodule formation is a part of the life cycle of *F. araxiana*. Thus, we can argue that nodule formation is part of a strategy for survival of the parasite, which can be caused by arrested or normal larvae, but further studies are needed to confirm this issue.

Previous studies have focused on the morphology of the genus *Falcaustra* but few studies exist on gene sequencing and phylogeny (Hasegawa *et al.*, 2013). In the present study, BLAST searches and phylogenetic analysis showed a close relationship between *F. araxiana* and *P. africana*, a member of the Ascaridoidea (fig. 2). Because of the difficulties in studying turtle nematodes, associated with

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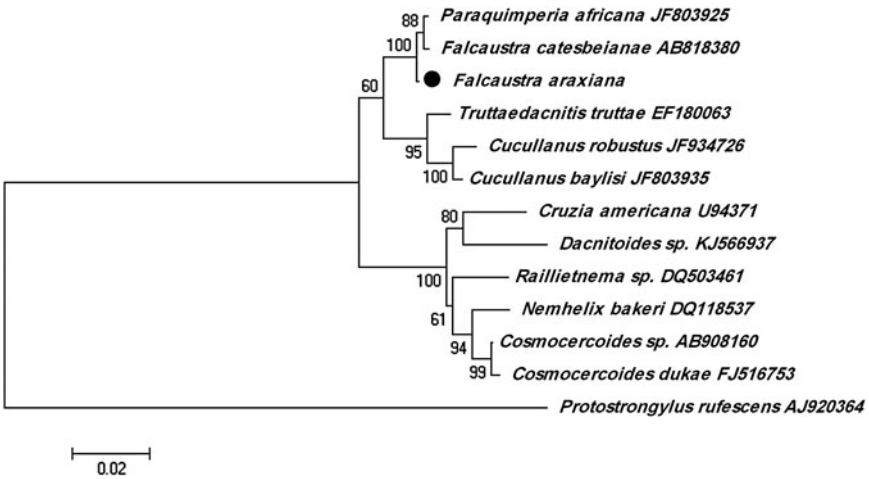

Fig. 2. Phylogenetic analysis of the partial 18S rDNA gene sequences of Iranian *F. araxiana* (KM200715 and KM200716) and related genera and species, with the tree constructed using neighbour-joining methods and bootstrap percentage values given at the nodes.

their morphological and biological peculiarities, most species of these parasites are poorly characterized (Anderson, 2000). It seems that their present classification system does not perfectly reflect phylogenetic relationships and a taxonomic revision of this nematode group, based on detailed morphology (including scanning electron microscopy and transmission electron microscopy), life cycle and molecular studies of individual species, is necessary.

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Conflict of interest

None.

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