BIHAREAN BIOLOGIST 13 (1): 36-38
Article No.: e181308
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http://biozoojournals.ro/bihbiol/index.html

Morphological and molecular taxonomy of helminths of the slow worm, *Anguis fragilis* (Linnaeus) (Squamata: Anguidae) from Turkey

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Received: 21. May 2018 / Accepted: 07. November 2018 / Available online: 12. November 2018 / Printed: June 2019

Abstract. Fifteen specimens of the slow worm, *Anguis fragilis* (two juvenile, five males and eight females), collected in Trabzon and Bursa Provinces, Turkey, were examined for helminths. *Anguis fragilis* was found to harbour four species of helminths: one species of Digenea, *Brachylaemus* sp. and three species of Nematoda, *Entomelas entomelas*, *Oxysomatium brevicaudatum* and *Oswaldocruzia filiformis*. In addition, DNA isolated from the Nematodes was analysed with clustal w and blast computer programs for nucleotide sequences. *Anguis fragilis* from Turkey represents a new host record for *Brachylaemus* sp. Also, 28s rDNA sequencing of *Oxysomatium brevicaudatum* and *Oswaldocruzia filiformis* produced new nucleotide sequences submitted to Genebank (NCBI: National Center for Biotechnology Information). To the knowledge, this is the first DNA analysis of the helminth fauna of *Anguis fragilis*.

Key words: *Anguis fragilis*, Digenea, Nematoda, DNA sequence, taxonomy.

Introduction

The slow worm, *Anguis fragilis* Linnaeus, 1758, inhabits grassy meadows, woodlands and richly vegetated areas of ground cover usually hiding under stones or inside burrows in loose soil. This lizards feeds on soft-bodied invertebrates and insects and is widely distributed in Europe and western Asia, with vertical distribution to 2400 m (Baran & Atatür 1997). In Turkey, *A. fragilis* occurs in North-west Anatolia and on Black Sea coasts. Although some reports of helminths from *A. fragilis* from Turkey are available, there are limited data on this lizard (Schad et al. 1960, Düşen et al. 2010). This is the third helminth investigation for *A. fragilis* from Turkey. There are reports of helminths in *A. fragilis* from other parts of its range (Sharpilo 1968, Sharpilo 1976, Lewin 1990, Shimalov et al. 2000, Borkovcova & Kopriva 2005, Mihalca et al. 2007). However, in these previous studies, helminth species were identified using morphology, anatomy, biogeography or ecology characters. But in this study, molecular techniques were applied to the identified Nematodes.

In some cases important characters which distinguish species are not observed or have disappeared. However, DNA material remains and using DNA sequencing gives important data for identifying species. In recent years the development of PCR (Polymerase Chain Reaction) and DNA sequencing techniques especially ITS (Internal Transcribed Spacer), rDNA (small and large ribozomal subunit) and mitochondrial genome regions have become important tools for taxonomy.

The aims of this study are to identify helminth fauna of the slow worm with classical and molecular techniques, give new locality records for host and helminths (Bursa and Trabzon Provinces, Turkey) and provide new sequence data for genebank.

Materials and Methods

Lizards and study area

Totally fifteen lizard samples were collected between 2006-2008 from two localities in Turkey. Fifteen specimens of *A. fragilis* (two sub adult, five males, eight females, mean snout-vent length = 36.57 ± 11.46 cm; range 25.0-45.0 cm) were collected by hand in Trabzon, Çaykara (40°45′N, 40°15′E, 400 m elevation, n=3) and Bursa (40°10′N, 29° 05′E, 500 m elevation, n=12) and transported to the parasitology laboratory for necropsy.

Collection of helminths

The lizards were euthanized with an ether, sexed and measured for body length. The body cavity was opened and the digestive tract removed. The esophagus, stomach, small and large intestines, and lungs were removed, placed in distilled water in separate petri dishes and examined for helminths under a stereomicroscope.

Morphological taxonomy of helminths

Nematodes were killed in hot saline solution, fixed in 70% ethanol and mounted in glycerol. Digenea were fixed in 70% ethanol, stained with iron-carmine as described by Georgiev et al. (1986), cleared in clove oil and mounted in entellan. Helminth identification was based on keys of Maplestone (1926), Yamaguti (1961, 1963) and Baker (1987). Helminths were identified and the number and location of individuals of each species were recorded. The use of descriptive ecological terms follows Bush et al. (1997). Helminth voucher specimens and lizard specimens were deposited in the helminth collection of Uludag University Museum of Zoology, Bursa, Turkey.

Molecular taxonomy of helminths

One specimen of each Nematode species was selected and stored at 4 °C for spin column DNA isolation. The specimen of *Brachylaemus* was retained as museum voucher material. A RTA genomic DNA isolation kit from tissue was used for DNA isolation. Isolated DNA was stored at -20 °C for subsequent studies. For PCR analyses, a Bioron kit was used. Total volume of PCR mixture was 25 µl (10 µl water, 2 µl buffer, 1 µl $MgCl_2$, 0,3 µl dNTPs, 2 µl (2 pmol) forward and 2 µl reverse primer, 0,3 µl super hot taq DNA polymerase 5u/ µl, 7 µl DNA). Forward 300F (5′-CAAGTACCGTGAGGGAAAGTTG-3′) and reverse ECD2 (5′-CTTGGTCCGTGTTTCAAGACGGG-3′) (Tkach et al. 2003) primer sequences were used with the following conditions: first denaturation for 10 min at 94 °C, then 38 cycles of denaturation for 30 sec at 94 °C, annealing for 30 sec at 60 °C and extention for 30 sec at 72 °C, ending with final extention for 10 min at 72 °C. Gene Amp PCR System 9700 (in 9600 emulation mode) was used for first PCR and sequencing PCR.

Following PCR, the samples were checked by 2% agarose gel electrophoresis in which the bands were stained with ethidium bromide. Then cycle sequencing protocols were used for the Big Dye Terminator version 3.1 Cycle Sequencing Kit which was modified to optimize our results (4 µl water, 3 µl sequencing buffer, 1 µl big dye ready reaction mix, 1,5 µl forward or reverse primer, same primer

with first PCR and 1,5 µl first PCR product so final volume 11 µl): initial denaturation at 96 °C for 1 min, then 25 cycles (96 °C for 10 sec, 50 °C for 5 sec, 60 °C for 4 min) and hold until ready to purify. DNA sequencing was done in two directions (forward and reverse). A Zymo Research DNA Sequencing Clean-up Kit was used for purification. Pure DNA was loaded directly into the sequencer (10 µl, 3130 ABI genetic analyzer) and sequences were analysed by Sequencing Analysis V.3.1 computer program. DNA sequence alignments of helminth species were created with Clustal W (Multiple Sequence Alignment) program. BLAST (Basic Local Alignment Search Tool) was used for determining differences between helminth species.

DNA sequences of Nematodes were deposited in Genbank (NCBI: National Center for Biotechnology Information) as: KT124550 (*Entomelas entomelas*), KT124551 (*Oxysomatim brevicaudatum*) and KT124552 (*Oswaldocruzia filiformis*) for lsu rDNA (28s rDNA). Sequence data to be released after publication.

Results

A total of 241 helminths representing four species were collected from the fifteen slow worms: *Brachylaemus* sp. Dujardin, 1843 (Digenea); *Entomelas entomelas* (Dujardin 1845), *Oxysomatium brevicaudatum* (Zeder 1800), *Oswaldocruzia filiformis* (Goeze 1782) (Nematoda). Each *A. fragilis* (100%) was infected with one or more helminth species. Helminth species, location on host, prevalence, mean intensity, abundance, number of infected host, total helminths number and minimum - maximum helminth number of the *A. fragilis* were given in Table 1.

Nuclear ribozomal rDNA (LSU rDNA, 28s) sequence data obtained from the Nematode species confirmed the species. *Entomelas entomelas* 548 bp, *Oxysomatim brevicaudatum* 530 bp and *Oswaldocruzia filiformis* 532 bp DNA nucleotides sequence were obtained from these species. All three species have deletion in this gene region. Some nucleotides sequence region constant in all species and some region variable between species. When we compared *Entomelas entomelas* (query) another species that was sequenced using blast program, these identification ratios were found; *Entomelas entomelas* %100, *Oswaldocruzia filiformis* %96 and *Oxysomatim brevicaudatum* %90. These are the percentage identity for the best match (top hit) from blasting the sequences. Because of the different percentage ratios, we can say these helminth species are different from each other. Ribosomal target gene region of helminths determined in this study have high similarity with recorded sequence data in Genbank.

Discussion

Fifteen slow worms lizard (two juveniles, eight females, five males) from Trabzon and Bursa in October 2006-June 2008 have been studied helminthologically. Two lizards harboured one species of helminth, twelve lizards harboured two species of helminths and one lizard harboured three species of helminths. *Entomelas entomelas* and *Oxysomatium brevicaudatum* have the highest prevalence, 93.3% (Table 1). Although *E. entomelas* is considered a specific parasite of the slow worm, prevalance was equal to and abundance was less than *O. brevicaudatum.*

There are two reports of helminths in *A. fragilis* in Turkey and there are several studies about helminth fauna of slow worms in other parts of its range (Schad et al. 1960, Sharpilo 1976, Lewin 1990, Shimalov et al. 2000, Borkovcova & Kopriva 2005, Mihalca et al. 2007, Düşen et al. 2010). Diversity of helminths in *A. fragilis* found by various authors are given in Table 2.

Table 1. Helminth species, location, prevalence, mean intensity, abundance, number of infected host, total helminths number and minimum - maximum helminth number of the *A. fragilis.*

Helminth species	Site of infection	Prevalence (%)	Mean intensity	Abundance	Number of infected host	Total helminth number	Min.–max. helminth number
Brachylaemus sp.	İntestine	6.7	1	0.07	1	1	0-1
E. entomelas	Esophagus, Lung	93.3	5.4	5	14	75	0-15
O. brevicaudatum	İntestine	93.3	11.6	10.9	14	163	0-60
Os. filiformis	Small intestine	6.7	2	0.13	1	2	0-2

Table 2. Diversity of helminths found by various authors in different countries from *A. fragilis*.

Countries	Authors	Diversity of helminths
Turkey	Schad et al. (1960)	*Entomelas entomelas, E. dujardini, Oswaldocruzia skrjabini, Oxysomatium brevicaudatum*
Russia	Sharpilo (1976)	*Pleurogenoides medians, Aplectana acuminata, Cosmocerca ornata, Neoxysomatium caucasicum, E. entomelas, Paraentomelas dujardini, O. brevicaudatum, Os. filiformis, Agamospirura minuta*
Poland	Lewin (1990)	*E. entomelas, P. dujardini, O. brevicaudatum, Os. filiformis, Brachylaime* sp., *Mesocestoides* sp., *Protostrongylidae* sp. larvae
South Belarus	Shimalov et al. (2000)	*Acanthocephalus ranae, Agamospirura minuta, C. ornata, E. entomelas, N. brevicaudatum, Os. filiformis, Pa. dujardini, Physocephalus sexalatus*
Czech Republic	Borkovcova&Kopriva (2005)	*N. brevicaudatum, Rhabdias fuscovenosus, Abbreviata* sp., *Os. filiformis*
Romania	Mihalca et al. (2007)	*E. entomelas*
Turkey	Düşen et al. (2010)	*R.bufonis, E. entomelas, Os. filiformis, C. ornate, O. brevicaudatum*

Of the fifteen individuals studied (eight females, five males, two juveniles) more individual helminths were found in female lizards (three samples in juveniles, 141 samples in females and 97 samples in males host). These results show concurrency with Lewin's research (1990). Only one species and one or two helminths were found in the juvenile lizards. Juvenile lizard samples have fewer (only *Entomelas entomelas* or only *Oxysomatium brevicaudatum*) helminth species than adults.

Brachylaemus sp. was found as a larva. This species is considered to be an accidental parasite for reptiles so it is less common in reptiles. This species is a new record for the slow worm in Turkey. *Oxysomatium brevicaudatum* is a parasite species especially of anura but is found in reptiles. This helminth species has been reported in several studies of helminth fauna in anurans of Turkey (Yıldırımhan 1999, Yıldırımhan et al. 2005b, Yıldırımhan et al. 2006). *Oswaldocruzia filiformis* is commonly reported form anurans, turtles and other reptiles. This species was reported in anurans from Turkey (Yıldırımhan 1999, Yıldırımhan et al. 2005b, Yıldırımhan et al. 2006).

This study is correlates well with other studies of *A. fragilis* in that the number of helminth species infecting *A. fragilis* is few. Most likely, when more slow worm individuals are studied from different localities, the number of helminth species will increase.

To our knowledge, there are no records of DNA sequences of helminths from *A. fragilis*. There is one record of DNA sequences for *Entomelas entomelas* in Genbank (Tkach et al. 2014). According to Tkach et al. (2014), partial sequence of internal transcribed spacer 1, 5.8S ribosomal RNA gene, complete sequence of internal transcribed spacer 2 and partial sequence of 28S ribosomal RNA gene regions were used to understand molecular phylogeny of the Nematode family Rhabdiasidae. According to Tkach et al. (2014) and this study ıt can be said that 28s rDNA is informative as a helminth gene marker.As a result of this study, the slow worm *A. fragilis* was examined for helminth parasites from Turkey. We studied DNA sequences of helminths from *A. fragilis* hosts first time in the world. Additionally new helminths, localities and sequences records and also diversity of helminths found by various authors in different countries from *A. fragilis* are presented.

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