Parasitology
Research Article

ACTA ZOOLOGICA BULGARICA
Acta zool. bulg., 71 (2), 2019: 273-278

Intestinal Helminth Parasites of Two Bat Species of the Genus *Myotis* Kaup, 1829 (Chiroptera: Vespertilionidae) from Turkey, with DNA Sequencing of Helminth Nuclear LSR rDNA

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Abstract: Three individuals of *Myotis alcathoe* von Helversen & Heller, 2001 (males) and twenty three individuals of *Myotis daubentonii* (Kuhl, 1817) (three females and twenty males) from Bursa Province, Turkey, were examined for helminth parasites. *Myotis alcathoe* was found to harbour four species of digeneans: *Prosthodendrium ascidia, Lecithodendrium linstowi, Plagiorchis koreanus* and *Plagiorchis elegans*. *Myotis daubentonii* was found to harbour eight species of digeneans: *Prosthodendrium chilostomum, Prosthodendrium longiforme, Prosthodendrium hurkovae, Lecithodendrium linstowi, Plagiorchis koreanus, Plagiorchis muelleri, Plagiorchis elegans* and *Plagiorchis vespertilionis*, as well as two species of nematodes: *Capillaria moraveci* and *Molinostrongylus skrjabini*. All these species were identified using both morphological and molecular methods, obtaining consistent results. To our knowledge, this is the first helminthological examination of *Myotis alcathoe* and four new host and geographical records are reported. New host and geographical records for five species of helminths are reported for *Myotis daubentonii*. The 28s rDNA partial sequences of two helminth species (*Prosthodendrium ascidia* and *Molinostrongylus skrjabini*) are new records for GenBank.

Key words: Bats, *Myotis*, Digenea, Nematoda, DNA sequencing

Introduction

The mammalian order Chiroptera (bats) is one of the most diverse, abundant and widely distributed orders of mammals and the only one with the capacity of powered flight (Omatsu et al. 2007, Li et al. 2010). Nearly 1000 species are found worldwide, except in the northern and southern polar areas, representing approximately 20% of all mammalian species (Teeling et al. 2005, Omatsu et al. 2007). Suborder Microchiroptera (includes species of the genus *Myotis* Kaup, 1829) has spread throughout the World with the exception of mountain peaks, Arctic and Antarctic regions and some isolated islands.

Bats are hosts for a large number of endo- and ectoparasites. They may serve as either intermediate hosts or final host for endoparasites, both platyhelminths (trematodes and cestodes) and nematodes.

There are 37 species of bats in Turkey (Karataş et al. 2006); of these, 29 species are assigned to the family Vespertilionidae. To our knowledge, there is one record of nematode parasites of Turkısh bats (Schad et al. 1960). Of the two species of *Myotis* examined in this study, to our knowledge, there is no record of helminth fauna for *Myotis alcathoe* von Helversen & Heller, 2001; however, there are two reports of helminths for *Myotis daubentonii* (Kuhl,

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1817) from Hungary (Matskasi 1967) and Belarus (Shimalov et al. 2002).

Ribosomal DNA genes encoding the rRNA subunits have proven particularly useful in taxonomic studies since they embody many of the features desirable in a gene study. They include regions with varying rates of evolution from highly conserved to highly variable (Hasegava et al. 1985). In this context, several studies have been done with DNA sequencing of helminth parasites from various vertebrate hosts (Tkach et al. 2000, 2001, 2003, Bentz et al. 2001, Foronda et al. 2004, Choudhury et al. 2007, Kuznetsov 2007, Farjallah et al. 2009, Lord et al. 2012, Yildirimhan et al. 2012, Sümer & Yildirimhan 2017) but a few of them relate to bat parasites (Tkach et al. 2000, Lord et al. 2012, Sümer & Yildirimhan 2017).

The aims of this study are to determine the helminth fauna of two species of *Myotis* with traditional taxonomical methods utilizing morphological, anatomical and ecological characteristics; to provide new host-parasite records and locality records; to present infection rate of parasite species; and to provide DNA sequencing of partial 28S ribosomal gene region of each helminth species as well as presentation of any new nucleotide sequence of helminth gene regions.

Materials and Methods

Morphological taxonomy of helminths

Between 2012-2013, from different localities and time periods, three *Myotis alcathoe* von Helversen and Heller, 2001 and twenty three *Myotis daubentonii* (Kuhl, 1817) were collected by mist net from a forested area, Bursa Province, Turkey, and returned to the parasitology laboratory for necropsy. They were identified based upon the keys of Dietz & Helverson (2004), euthanized with an overdose of ether, sexed and body length was measured. The body cavity was opened and the digestive tract removed. The oesophagus, stomach, small and large intestines and lungs were opened, placed in distilled water in separate petri dishes and examined for helminths under a stereomicroscope. Digeneans were fixed in 70% ethanol, stained with iron acetocarmine as described by Georgiev et al. (1986), cleared in clove oil and mounted in entellan. Nematodes were killed in hot saline solution, fixed in 70% ethanol and mounted in glycerol. Helminths were examined with a light microscope and identification was based on the morphological and anatomical descriptions given by Maplestone (1926), Yamaguti (1961, 1963), Matskasi (1973, 1980), Baker (1987), Genov et al. (1992) and Tkach et al. (2000). 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 bat specimens were deposited in the collection of Uludağ University Museum of Zoology, Bursa, Turkey. Collection and animal use permits are as follow: HADYEK (Uludağ University, Animal Experiments Local Ethics Committee), no. B.30.2.ULU.0.8Z.00.00/53, decision no. 2011-05/06, and Forest and Water Affairs Ministry, no. B.23.0.DMP.0.15.01.-510-29610.

Molecular taxonomy of helminths

One specimen was selected from each helminth species and stored at 4°C for spin column DNA isolation. Obtained DNA was stored at –20°C for subsequent studies. For polymerase chain reaction (PCR) studies, a Bioron Kit was used. Total volume of PCR mixture was 25 µl. Forward 300F (5′-CAAGTACCGTGAGGGAAAGTTG-3′) and reverse ECD2 (5′-CTTGGTCCGTGTTTCAAGACGGG-3′) 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 extension for 30 sec at 72°C, ending with final extension for 10 min at 72°C. A 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 v3.1 Cycle Sequencing Kit which was modified to optimize our results: 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. 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 analyzed 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 the studied species of Digenea and Nematoda were deposited in GenBank (NCBI) as follows: KT369203 (*Prosthodendrium ascidia*), KU711924 (*Prosthodendrium chilostomum*), KT369204 (*Prosthodendrium longiforme*), KU711925 (*Prosthodendrium hurkovae*), KT369205 (*Lecithodendrium linstowi*), KT369208 (*Plagiorchis koreanus*), KU711926 (*Plagiorchis elegans*), KT369206

Table 1. Helminth species, site of infection, prevalence (P%), mean intensity (MI), mean abundance (MA), number of infected host (N_{inf}), total number of helminth individuals and minimum – maximum numbers of helminth individuals in studied specimens of *Myotis alcathoe.*

Helminth species	Site	P%	MI	MA	N_{inf}	Total no. of helminths	Min – max no. of helminths
Prosthodendrium ascidia Beneden, 1873	Intestine	66.66	3.5	2.33	2	7	2-5
Lecithodendrium linstowi Dollfus, 1931	Intestine	33.33	12.0	4.00	1	12	0-12
Plagiorchis koreanus Ogata, 1938	Intestine	33.33	1.0	0.33	1	1	0-1
Plagiorchis elegans Rudolphi, 1802	Small intestine	33.33	1.0	0.33	1	1	0-1

Table 2. Helminth species, site of infection, prevalence (P%), mean intensity (MI), mean abundance (MA), number of infected host (N_{inf}), total number of helminth individuals and minimum – maximum numbers of helminth individuals in studied specimens of *Myotis daubentonii.*

Helminth species	Site	P%	MI	MA	N_{inf}	Total no. of helminths	Min – max no. of helminths
Prosthodendrium chilostomum Mehlis, 1831	Intestine	47.82	7.4	3.5	11	81	0-28
Prosthodendrium longiforme Bhalerao, 1926	Intestine	69.56	14.2	9.9	16	227	0-102
Prosthodendrium hurkovae Dubois, 1960	Intestine	13.04	2.0	0.3	3	6	0-4
Lecithodendrium linstowi Dollfus, 1931	Intestine	47.82	5.1	2.4	11	56	0-17
Plagiorchis koreanus Ogata, 1938	Intestine	30.43	2.8	0.9	7	20	0-7
Plagiorchis muelleri Tkach ve Sharpilo, 1990	Intestine	39.13	2.0	0.8	9	18	0-5
Plagiorchis elegans Rudolphi, 1802	Intestine	8.69	2.0	0.2	2	4	0-2
Plagiorchis vespertilionis Müller, 1784	Intestine	13.04	1.7	0.2	3	5	0-3
Capillaria moraveci Esteban, Mas-Coma, Oltra-Ferrero ve Botella, 1991	Intestine	39.13	2.4	1.0	9	22	0-7
Molinostrongylus skrjabini Skarbilovitch, 1934	Intestine	86.95	5.2	4.5	20	103	0-13

(*Plagiorchis muelleri*), KT369207 (*Plagiorchis vespertilionis*), and KU711928 (*Molinostrongylus skrjabini*) for LSU rDNA (28s ribosomal DNA); no accession number (*Capillaria moraveci*).

Results

A total of twenty one helminths representing four species of digeneans (*Lecithodendrium linstowi, Prosthodendrium ascidia, Plagiorchis elegans* and *P. koreanus*) were collected from three males of *M. alcathoe*. The September sample had a greater number of helminths than the August sample. One *Myotis alcathoe* was infected with two species (*Lecithodendrium linstowi* and *Prosthodendrium ascidia*). The second *Myotis alcathoe* harbored three helminth species (*Prosthodendrium ascidia, Plagiorchis koreanus* and *P. elegans*) and the third *Myotis alcathoe* was not infected.

A total of 543 helminths representing eight species of digeneans (*Prosthodendrium chilostomum, P. longiforme, Lecithodendrium linstowi, Plagiorchis koreanus, P. muelleri, P. elegans, P. vespertilionis* and *Prosthodendrium hurkovae*) and two species of nematodes (*Capillaria moraveci* and *Molinostrongylus skrjabini*) were collected from twenty three *Myotis daubentonii* (three females and twenty males). Generally, September samples had greater helminth numbers than those from June, July or August. There were 144 helminths from female hosts and 399 from male hosts. All twenty three *Myotis daubentonii* (100%) were infected with one or more helminth species: two were infected by one helminth species, six were infected by three helminth species, eight were infected by four helminth species, five were infected by five helminth species, one was infected by six helminth species and one was infected by eight helminth species.

Helminth species, site of infection, prevalence (% of infected bats), mean intensity (average number of parasites per bat), abundance (total helminth number per total of bat individuals), number of infected host, total helminth number and range related to each bat species are given in Tables 1 and 2.

First, identification of helminth species was done according to morphological and anatomical aspects. For the species of Digenea, we measured length of body, width of body, oral sucker size, pharynx size,

ventral sucker size, right testes size, left testes size, ovary size and cirrus sac size. For the male nematodes, we studied length of body, width of body, length of oesophagus, lengths of tail, lengths of right and left spicules. For female nematodes, we measured lengths of body, width of body, lengths of oesophagus, lengths of tail and position of vulva. All these characters are important diagnostic characters of helminth species.

Second, molecular data of helminths were also determined; partial DNA sequence of 28S ribosomal gene region was done. The length of sequence region was 550 – 650 bp. This target gene region had some difference identification rates between species. When comparing *Prosthodendrium longiforme* (100%, query sequence) with other helminth species (subject sequence), the sequence target gene region gave the following results of identification rates: *Prosthodendrium chilostomum* 94%, *P. ascidia* 91.5%, *P. hurkovae* 90.5%, *Lecithodendrium linstowi* 86%, *Plagiorchis vespertilionis* 89%, *P. muelleri* 90%, *P. elegans* 91%, *P. koreanus* 92%, *Capillaria moraveci* 88% and *Molinostrongylus skrjabini* 87%. Moreover, this gene region pointed out eleven helminth species from *Myotis alcathoe* and *M. daubentonii* similar to classical taxonomy. According to these sequence data, some nucleotide sequence regions are constant (most of DNA sequence) in all Digenea and Nematoda helminth species and some regions are variable (some of DNA sequence) between species. *Lecithodendrium linstowi* species had nine points of heterozygous base variation (T > C), (C > T), (G > A), (T > C), (T > G), (A > G), (G > C), (A > G) and (C > T). Sequence data obtained from helminth species were compared with the 28S rDNA region from GenBank using BLAST. This gene region exhibited a high similarity rate. Classical taxonomy and molecular records of helminth species from bats from Turkey are given in Table 3.

Discussion

Previously, *Plecotus auritus, Miniopterus schreibersii, Myotis myotis* and *Rhinolophus ferrumequinum* from Turkey were studied by Schad et al. (1960) and found the harbour the nematode species *Molinostrongylus ornatus, Strongylacantha glycirrhiza* and *Litomosa chiropterorum*.

In this study, *Myotis alcathoe* was found to harbour four digenean species: *Prosthodendrium ascidia, Lecithodendrium linstowi, Plagiorchis koreanus* and *Plagiorchis elegans.* This is the first report of parasites in this bat species throughout it geographical range.

Myotis daubentonii was found to harbour ten helminth species. These were eight Digenean species *Prosthodendrium chilostomum, Prosthodendrium longiforme, Prosthodendrium hurkovae, Lecithodendrium linstowi, Plagiorchis koreanus, Plagiorchis muelleri, Plagiorchis elegans, Plagiorchis vespertilionis*, as well as two nematode species, *Capillaria moraveci* and *Molinostrongylus skrjabini.* Previous studies have found *Myotis daubentonii* to harbour nine digenean species in Hungary: *Lecithodendrium linstowi, L. granulosum, Prosthodendrium chilostomum, P. ascidia, P. hurkovae, P. carolinum, Plagiorchis vespertilionis, Parabascus duboisi* and *Moedlingeria amphoraeformis* (Matskasi 1967), and four digenean species (*Lecithodendrium linstowi, Prosthodendrium chilostomum, P. longiforme* and *Plagiorchis vespertilionis*) and one nematode species, *Capillaria romana* in Belarus (Shimalov et al. 2002).

In this study, *Prosthodendrium ascidia* was found only in *Myotis alcathoe* and had the highest value of prevalence (66.66%) (Table 1). *Myotis alcathoe* represents a new host record for *Prosthodendrium ascidia* and Turkey is a new geographical record.

Prosthodendrium longiforme was found only in *Myotis daubentonii* and had the highest mean intensity (14.18) for this host (Table 2). This report is the second record of *Myotis daubentonii* as a host for *Prosthodendrium longiforme*. Turkey is a new geographical record for this parasite species.

Lecithodendrium linstowi was found in both *Myotis alcathoe* and *Myotis daubentonii.* Mean intensity, abundance and total helminths number were greater in *Myotis alcathoe* than in *Myotis daubentonii* (Tables 1 and 2). *Myotis alcathoe* represents a new host record for *Lecithodendrium linstowi*. Turkey is a new geographical record for this species.

Plagiorchis koreanus was found in both *Myotis alcathoe* and *M. daubentonii. Myotis alcathoe* represents a new host record for *Plagiorchis koreanus*. Turkey is a new geographical record.

Plagiorchis elegans was found in both *Myotis alcathoe* and *Myotis daubentonii.* It has a lower prevalence value, abundance, number of infected host and total helminths number in *Myotis daubentonii* than in *Myotis alcathoe* (Table 1 and 2). *Plagiorchis elegans* represesnts new host records for *Myotis alcathoe* and *Myotis daubentonii*. Turkey is a new geographical record for this species.

Plagiorchis muelleri was found in only *Myotis daubentonii. Plagiorchis muelleri* represents a new host record for *Myotis daubentonii*, and Turkey is a new geographical record.

Plagiorchis vespertilionis was found in only *Myotis daubentonii*, with low mean intensity (Table 2).

Capillaria moraveci is a common species par-

Table 3. Classical and molecular taxonomy records of helminth parasite fauna in bats from Turkey. 1. Schad et al. (1960). 2. Sümer & Yildirimhan (2017). 3. Sümer & Yildirimhan (2018). 4. Present study.

	Classical taxonomy				**Classical and molecular taxonomy**		
Bat Species	***Myotis myotis***	***Plecotus auritus***	***Miniopterus schreibersii***	***Rhinolophus ferrumequinum***	***Myotis aurescens***	***Myotis alcathoe***	***Myotis daubentonii***
Helminth Species							
Digenea							
Lecithodendrium linstowi					2, 3	4	4
Prosthodendrium longiformae					2, 3		4
Prosthodendrium chilostomum							4
Prosthodendrium ascidia					2, 3	4	
Prosthodendrium hurkovae							4
Plagiorchis vespertilionis					2, 3		4
Plagiorchis müelleri					2, 3		4
Plagiorchis koreanus					2, 3	4	4
Plagiorchis elegans						4	4
Nematoda							
Litomosa chiropterorum		1	1				
Molinostrongylus alatus	1						
Molinostrongylus ornatus			1				
Molinostrongylus skrjabini							4
Strongylacantha glycirrhiza				1			
Capillaria moraveci							4
Rictularia lucifugus					3		

asitic in birds and mammalian hosts. This helminth was found in only *Myotis daubentonii. Capillaria moraveci* represents a new host record for *Myotis daubentonii.* Turkey is a new geographical record.

Molinostrongylus skrjabini was found only in *Myotis daubentonii*. This helminth had the highest level of prevalence (86.95) and number of infected host (twenty) values in our samples (Table 2). *Molinostrongylus skrjabini* represents a new host record for *Myotis daubentonii*. Turkey new geographical record.

In conclusion, the digeneans are the dominant helminth group in the gastrointestinal tract of the studied Turkısh vespertilionid bats. A possible explanation is that most studies focused on insectivorous species, with the composition of the helminth fauna determined by life style, habitat use, diet and type of prey. The life cycle of digeneans, aside from the bats as definitive host, includes two intermediate hosts, mollusks and insects. Metacercariae of certain digeneans develop inside aquatic insects, which, upon their metamorphosis into the adult stage, can be preyed upon by bats that favour wetland and aquatic habitats (Hall 1960, Macy 1960).

Subject sequences of *Prosthodendrium chilostomum* (6%), *P. ascidia* (8.5%), *P. hurkovae* (9.5%), *Lecithodendrium linstowi* (14%), *Plagiorchis vespertilionis* (11%), *P. muelleri* (10%), *P. elegans* (9%), *P. koreanus* (8%), *Capillaria moraveci* (12%) and *Molinostrongylus skrjabini* (13%) are different from *Prosthodendrium longiforme* (query sequence). When comparing the 28S rDNA sequence of helminth species diagnosed in this study with GenBank records, it is seen that they have very similar rates. Two of the eleven species, *Prosthodendrium ascidia* (Digenea) and *Molinostrongylus skrjabini* (Nematoda), are new gene records for GenBank; the remaining nine species have partial sequences of ribosomal large unit gene published in GenBank as a result of studies by other researchers (Tkach et al. 2000, 2003). Generally, DNA sequence data used purpose of phylogeny between species by that mentioned studies.

All of sequence data showed eleven helminth species new for Turkey gene records. Species of in the same genus have closer identification rate to each other. There were 1% differences of sequences among the species of the genus *Plagiorchis*.

Acknowledgements: We thank the Uludağ University Scientific Research Council of Turkey for their financial support with UAP (F) – 2011 / 77 project number.

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Received: 13.05.2018
Accepted: 10.07.2018