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NURHAN SÜMER

HİKMET SAMİ YILDIRIMHAN

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DNA sequencing of Digenea nuclear lsrDNA of the whiskered brown bat, *Myotis aurescens* (Vespertilionidae: Chiroptera), from Turkey

Nurhan SÜMER*, Hikmet Sami YILDIRIMHAN
Department of Biology, Faculty of Science and Literature, Uludağ University, Bursa, Turkey

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Abstract: Six species of Trematoda (*Prosthodendrium ascidia, Prosthodendrium longiforme, Lecithodendrium linstowi, Plagiorchis muelleri, Plagiorchis vespertilionis,* and *Plagiorchis koreanus*) of *Myotis aurescens* from Bursa Province, Turkey, were examined for DNA sequencing of the 28S ribosomal gene region. A partial sequence of the 28S ribosomal gene from digenean species was studied using BLAST and found to be a useful region for helminth species diagnosis.

Key words: Turkey, bat, Digenea, DNA sequence

1. Introduction

Until recently, diagnosis of Trematoda helminth parasites of bats has been done according to morphological, anatomical, and ecological features. Related to this subject, some studies have been performed to date in other countries (Matskasi, 1967, 1968, 1973, 1980; Sawada, 1983; Botella et al., 1993; Shimalov et al., 2002), but in Turkey there is only one study about helminth parasites of bats (Schad et al., 1960). In that article the bat species *Plecotus auritus*, *Miniopterus schreibersii*, *Myotis myotis*, and *Rhinolophus ferrumequinum* were studied in terms of Nematoda helminth parasites. Therefore, neither in Turkey nor in the rest of the world has any research been done on Digenea of *Myotis aurescens*. With developing technology, molecular methods began being used for helminths' genome. DNA sequencing analysis of species is important and gives useful data for determining gene structure and base composition, detecting several types of mutations, calculating identification rates between species, diagnosing species, understanding phylogeny in the one taxonomic category, population studies, and generating a gene database. There were some studies about ribosomal DNA sequences of digenean helminth parasites of bats (Tkach et al., 2000, 2001, 2003; Lord et al., 2012).

To the best of our knowledge, there is no report on *Prosthodendrium ascidia* based on this gene region nucleotides sequence in GenBank. The purpose of this study was to present the first records of the DNA sequence related to the Digenea genome from *M. aurescens* found in this study and test the classical taxonomy of helminth species.

2. Materials and methods

Fifteen bats were collected by mist net from 4 localities in a forested area, in Bursa Province, Turkey (2 samples from Uludağ 40°7′N, 29°7′E, 6 samples from Keles 39°55′N, 29°4′E, 2 samples from Misi 40°10′N, 28°58′E, and 5 samples from Doğancı Dam 40°6′N, 28°57′E), and taken to the parasitology laboratory for necropsy. They were identified as *Myotis aurescens* based upon the keys published by Dietz and Helverson (2004). The bats were euthanized with an overdose of 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 dissected, placed in distilled water in separate petri dishes, and examined for helminths under a stereomicroscope. Digeneans were fixed in 70% ethanol, stained with iron-carmine as described by Georgiev et al. (1986), cleared in clove oil, and mounted in Entellan. Helminths were identified with a light microscope and identification was based on the morphological and anatomical descriptions given by Yamaguti (1961, 1963), Matskasi (1973), and Tkach et al. (2000). 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 follows: HADYEK (Uludağ University, Animal Experiments Local Ethics Committee) number: B.30.2.ULU.0.8Z.00.00/53, decision number: 2011-05/06 and Forest and Water Affairs Ministry number: B.23.0.DMP.0.15.01.-510-29610 from Turkey.

* Correspondence: n.sumer@hotmail.com

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 the PCR mixture was 25 µL. Forward 300F (5′-CAAGTACCGTGAGGGAAAGTTG- 3′) and reverse ECD2 (5′-CTTGGTCCGTGTTTCAAGACGGG-3′) primer sequences (Tkach et al., 2003) were used with the following conditions: first denaturation for 10 min at 94 °C, then 38 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 60 °C, and extension for 30 s at 72 °C, ending with a 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 version 3.1 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 s, 50 °C for 5 s, 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 was used for determining differences between helminth species.

DNA sequences of digeneans were deposited in GenBank (National Center for Biotechnology Information; NCBI) as follows: KT369203 (*Prosthodendrium ascidia*), KT369204 (*Prosthodendrium longiforme*), KT369205 (*Lecithodendrium linstowi*), KT369206 (*Plagiorchis muelleri*), KT369207 (*Plagiorchis vespertilionis*), and KT369208 (*Plagiorchis koreanus*) for lsu rDNA (28S rDNA).

3. Results

Partial nuclear ribosomal gene (28S rDNA, LSU) sequence data of helminth species were determined. Moreover, this gene region pointed out 6 Digenea species from *Myotis aurescens*, similar to classical taxonomy. *Prosthodendrium ascidia* 574 bp, *Prosthodendrium longiforme* 646 bp, *Lecithodendrium linstowi* 563 bp, *Plagiorchis muelleri* 618 bp, *Plagiorchis vespertilionis* 613 bp, and *Plagiorchis koreanus* 610 bp DNA nucleotide sequence was obtained from these species. According to these sequence data, some nucleotide sequence regions are constant in all 6 Digenea species and some regions are variable between species. 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. Because of having different nucleotide sequence length and location in the GenBank sequence, in this study *Prosthodendrium ascidia* (100%) was compared with another species with similar sequence length and the following identification rates were obtained: *Prosthodendrium ascidia* 100%, *Prosthodendrium longiforme* 91%, *Lecithodendrium linstowi* 87%, *Plagiorchis muelleri* 74%, *Plagiorchis vespertilionis* 74%, and *Plagiorchis koreanus* 75%.

4. Discussion

Digenean helminth parasites of *Myotis aurescens* were studied using classical taxonomy rules and the results obtained pointed out 6 Digenea species. With this study we compared the 28S ribosomal gene of the digenean species using BLAST to understand the percentage similarity rate: *Prosthodendrium longiforme* 9%, *Lecithodendrium linstowi* 13%, *Plagiorchis muelleri* 26%, *Plagiorchis vespertilionis* 26%, and *Plagiorchis koreanus* 25% different from *Prosthodendrium ascidia* (100%, query). Thus the classical taxonomy of these species agreed with molecular methods. The 28S ribosomal gene is a useful region for helminth species diagnosis. There are 28S rDNA nucleotide sequences data about Digenea helminth species in GenBank. Some of them have the same gene regions while others have different gene regions. 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.

This study provides a new partial 28S DNA sequence for *Prosthodendrium ascidia* worldwide. It also provides partial 28S DNA sequences of *Prosthodendrium longiforme*, *Lecithodendrium linstowi*, *Plagiorchis vespertilionis*, *Plagiorchis koreanus*, and *Plagiorchis muelleri* from Turkey.

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