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Preliminary Molecular Identification of Two Helminthes (*Moniezia* sp. and *Paramphistomum* sp.) In the Province of Taif, Saudi Arabia

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Abstract: The two ruminant parasites; *Moniezia* sp. and *Paramphistomum* sp. were collected from Taif province and examined molecularly by sequencing 346 nucleotides of cytochrome C subunit 1 gene. The data were used along with those of several other helminthes species from the Genbank to identify these two species genetically and construct their relationship. Neighbor-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) analytical methods were used. The three methods showed sister relationship of *Moniezia* sp. from Taif and *M. benedeni* and clustering of *Paramphistomum* sp. with *Fasciola*. Such relationship was strongly supported for the first and was questionable for the second and did not support the morphological identification of *Moniezia* sp. as *M. expansa* but presumed that it could be *M. benedeni*. We could not be able to identify molecularly to which species *Paramphistomum* belongs. Collecting more samples and DNA data along with conducting electron microscopic description could be necessary to clarify more definitely the exact identification of both species.

Key words: Genetic Identification · Mitochondrial DNA · Digenea · Flatworms · Domesticated Animals

INTRODUCTION

Helminthes are one of the most destructive internal parasites of the vertebrate animals including man [1]. They caused the increase of mortality rate and the decrease in livestock productions [2]. Sheep received the great interest as one of the most important and preferable livestock for human consumption in the Arab countries especially the Gulf ones. *Moniezia expansa* (Family: Anoplocephalidae) and other tapeworms infect sheep, goats, cattle [3] and constituted a big problem in sheep raising countries [4-8]. Little information on the livestock parasites in Saudi Arabia was available [9-11]. Among the different species of genus *Moniezia*, Al-Qureishy [11] recorded only two species (*M. expansa* and *M. denticulata*) infecting the Saudi Arabian livestock with the first being the common.

Recent efforts based on comparative analysis of morphology and on molecules have advanced our understanding of tapeworm systematic considerably [12, 13] and more generally of their position within the phylum Platyhelminthes [14, 15]. The use of molecular data for the study of relationships among tapeworms has been largely limited to those species of medical or economic importance [16, 17], although few recent studies have begun to address the systematics of a wider diversity of tapeworms [18-20].

The aim of the present work is to sequence the cytochrome C oxidase subunit 1 of the mitochondrial genome partially for one taxon of Digenea (*Paramphistomum* sp.) and one taxon of eucestodes (*Moniezia* sp.) in order to identify the genetic relationship of both parasites to their flatworms group.

MARTIALS AND METHODS

Worm samples were freshly obtained from the slaughtered sheep and cow at the city of Taif and were separately washed in normal saline solution and kept in labeled glass containers. Worms were collected and processed for identification. Samples were preserved at - 8°C for further molecular studies. DNA was extracted

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with QIAGEN spin-column kits according to the manufacturer's instructions. Extracted DNA was spectrophotometrically quantified at 260/280nm and was used for polymerase chain reaction (PCR).

PCR was performed in 50 µl total volume of reaction buffer containing 0.2mM dNTPs, 1.5mM $MgCl_2$, 2 µl of DNA solution and 0.25U of DNA *Taq*-polymerase (Invitrogen). 0.2 µM of each of the forward primer (5-TTTTTTGGGCATCCTGAGGTTTAT-3) and the reverse primer (5-TAAAGAAAG AACATAAT GAAAATG-3) as used by Bowles *et al*. [21] were also added. The reaction mixture was put into a 0.2ml thin-walled PCR tube and amplification was performed in PXE 0.5 thermal cycler (Thermo Electron Corporation Co.) with the following profile: 94°C for 5min followed by 30 cycles of 94°C for 1min, 58°C for 1min and 72°C for 1min. A final strand elongation at 72°C was done for an additional 7min.

The resultant solutions were electrophoresed on a 1.5% agarose gel in TAE (40mM Tris, 40mM acetic acid and 1mM ethylenediamine-tetra acetic acid) and gels were stained with ethidium bromide. 100bp DNA Ladder (Biolabs) was used as a marker for the molecular weight size. The PCR products were then purified from gel with the use of spin column according to the Kit Manual.

Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM. BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq-DNA polymerase (FS enzyme) (Applied Biosystems) following the protocols supplied by the manufacturer were used. A single-pass sequencing was performed on each template using the last mentioned PCR-primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. Samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Nucleotide sequences of the mitochondrial CO1 gene (364 bp) were aligned with the same fragment for other related helminthes parasites (from both Digenia and Euocestodes) from the DDBJ database. Two additional outgroup taxa (from Monogenia*; Gyrodactylus turnbulli* and *Gyrodactylus corydori*) were included in the alignment in order to root the tree. The alignment was carried out by using the DNASIS 3.5 (Hitachi) and MacClade 4.03 (Sinauer Associates, Inc.) with manual adjustments. Tree analyses were conducted by neighbor-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) methods. These analyses were done in PAUP* 4.0b10 [22] by heuristic searches with the TBR branch swapping, 10 random taxon additions and 1000 bootstrap replications for each method. Tamura-Nei distance model was used to construct a neighbor-joining tree [23].

RESULTS AND DISCUSSION

A fragment of 364 unambiguous sites from CO1 gene for two digenean species belonging to the genera *Moniezia* and *Paramphistomum* were sequenced in this study. These data were deposited in DDBJ/EMBL GenBank database with their accession numbers (AB683980 and AB688990). The sequences were aligned and were used for genetic analysis where 158 sites were constant and 206 were variables. Fifty nine of the variable sites were parsimony-uninformative and 147 were informative under parsimony criterion. The estimated uncorrected pairwise distance (Table 1) showed low distances between *Moniezia* sp. from Taif and the other *Moniezia* species (D= 0.08 and 0.09) and that the former exhibited the closest distance to *M. benedeni* (D = 0.01). *Paramphistomum* sp. showed closer distance (Table 2) to Schistosomatidae (*Schistosoma*, 0.20 and *Trichobilharzia*, 0.22) than to *Fasciola* (D= 0.26).

Figure 1 depicts an NJ tree that has been constructed using the aligned sequences and neighbor-joining algorithm [23]. The same tree topology was obtained by maximum-parsimony under similar conditions. An optimal ML tree was also found with similar topology and a negative log likelihood of 1369.479. The three analytical methods (NJ, MP and ML) showed 100% and 95% sister relationship of *Moniezia* sp. from Taif and *M. benedeni* while the position of *M. expansa* was questionable within the genus.

Discrepancy between the calculated pair wise distance and the tree topology was found regarding the situation of *Paramphistomum* sp. The species was closer to *Fasciola* in the tree topology but acquired very close distance to Schistosomatidae as the genetic distance indicated. This topology is not trustable because there was no strong statistical support at the node of the group including *Paramphistomum* and there was no DNA data for its congeneric taxa in the Genbank to use. Unless we collect more molecular data and more samples, we could not resolve this discrepancy. This relationship was also untrustable because of the different topological positions of *Paramphistomum* when different analytical methods were used.

The sequenced fragment in the present study has been translated into its corresponding amino acids and the obtained data were aligned with that of the different

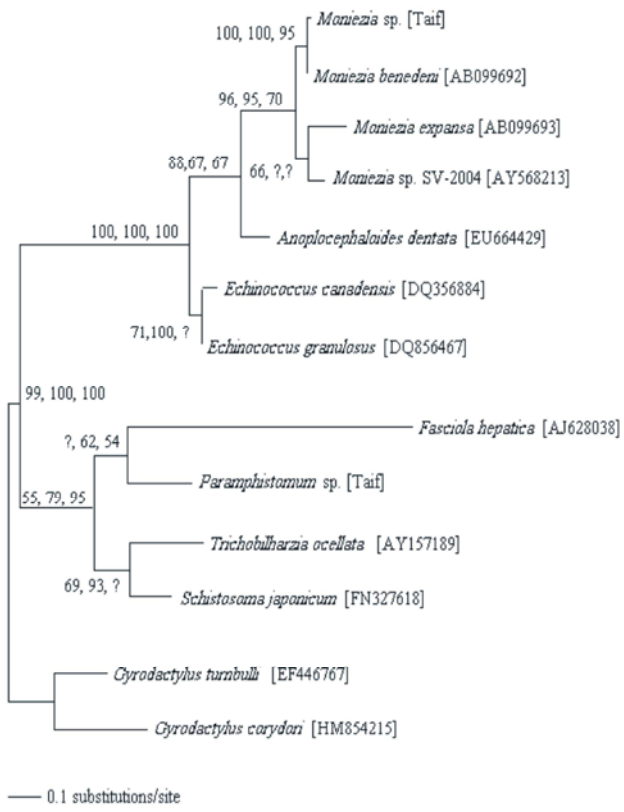

Fig. 1: Neighbor-joining tree constructed from 364 bp of CO1 gene among the different studied digenean species. The bootstrap values are showed at nodes for maximum-parsimony, Neighbor-joining and maximum-likelihood methods, respectively, when they are more than 50%. The sign "?" means that the bootstrap value was not recorded for the corresponding analytical method.

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Moniezia benedeni      FFGHPEVYVLIIPGFGMISHICFSLSMVSDVFGFYGLLFAMFAIVCLGSS
Moniezia sp. [Taif]    FFWGPEVYVLIIPGFGMISHMCFSLSMVSDVFGFYGLLFAMFAIVCLGSS
Moniezia expansa       FFGHPEVYVLILPAFGMVSHICFSLSMVSDVFGFYGLLFAMFSIVCLGSS
Moniezia sp.SV-2004    FFGHPEVYVLIIPGFGMVSHICFSLSMVSDVFGFYGLLFAMFSIVCLGSS

                                                                   100
Moniezia benedeni      VWGHHMFTVGLDVKTAVFFSSVTMIIGVPTGIKVFTWLYMLLNSNVSKWD
Moniezia sp. [Taif]    VWGHHMFTVGLDVKTAVFFSSVTMIIGVPTGMKVFTWLYMLLKSKVSKWD
Moniezia expansa       VWGHHMFTVGLDVKTAVFFSSVTMIIGVPTGIKVFTWLYMLLNSNVSKWD
Moniezia sp.SV-2004    VWGHHMFTVGLDVKTAVFFSSVTMIIGVPTGIKVFTWLYMLLNSNVSKWD

                                                  121
Moniezia benedeni      PVLWWVISFIVLFTFGGVTGI
Moniezia sp. [Taif]    PVLWWVISFMVLFTFGGVTGI
Moniezia expansa       PILWWVVSFIVLFTFGGVTGI
Moniezia sp.SV-2004    PVLWWVISFIVLFTFGGVTGI
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Fig. 2: The aligned translated amino acids of the sequenced fragment of CO1 gene for the different digenean species. The underlined letters are the different amino acids in *Moniezia* from Taif compared to other *Moniezia*.

Table 1: Uncorrected pairwise distances among different species of *Moniezia* and the related cestodes

species	*Moniezia* sp. [Taif]	*Moniezia benedeni*	*Moniezia* sp. [AY568213]	*Anoplocephaloides dentata*	*Echinococcus canadensis*
Moniezia sp. [Taif]	-				
Moniezia benedeni	0.01	-			
Moniezia sp. [AY568213]	0.09	0.08	-		
Anoplocephaloides dentata	0.16	0.14	0.15	-	
Echinococcus canadensis	0.18	0.17	0.29	0.14	-
Echinococcus granulosus	0.20	0.19	0.19	0.15	0.08

Table 2: Uncorrected pairwise distances among different trematode species related to *Paramphistomum*.

species	*Paramphistomum* sp. [Taif]	*Fasciola hepatica*	*Schistosoma japonicum*
Paramphistomum sp. [Taif]	-		
Fasciola hepatica	0.26	-	
Schistosoma japonicum	0.20	0.29	-
Trichobilharzia ocellata	0.22	0.26	0.16

taxa of *Moniezia* in order to record the non synonymous differences among them. The translated amino acids for the complete sequence of CO1 gene of *Schistosoma mansoni* was obtained from the Genbank (accession number) and was aligned with those of *Moniezia* in order to know the location of the amino acids of *Moniezia* within the CO1 gene. It was found that the obtained sequence for *Moniezia* is located between Phenylalanine at 268 and isoleucine at 389 and our sample was shown to be different from other *Moniezia* taxa in 7 amino acids most of which was methionnine (Figure 2).

The most common cestode in domesticated animals in the Kingdom of Saudi Arabia is *M. expansa* while *M. benedeni* and *M. deuticulata* were recorded very rarely and for the first time in Riyadh [11, 24]. Since there is no mitochondrial DNA data available for *M. deuticulata* in the GenBank, we could not be able to assume that our sample could be *M. deuticulata*. Meanwhile, the Taif sample showed clear genetic difference to the other available *Moniezia* species. It is therefore most likely to identify our sample as *Moniezia* sp.-Taif right now until more samples and more molecular data are collected.

Paramphistomes require an aquatic snail as an intermediate host and the pre-parasitic stages of the lifecycle (miracidia and stages in the snail) are very similar to those of liver flukes (*Fasciola hepatica*) [25]. The life cycle of *Paramphistomum* is also similar to that of *Fasciola hepatica* in that they require two hosts to complete their life cycle; a mammalian definitive host and a snail intermediate host [26]. Moreover, Abrous *et al.* [27] revealed that some species of both parasites infect the same intermediate host (*Lymnaea glabra* and *Lymnaea truncatula*). The abovementioned similarities could be a reasonable interpretation of the clustering of both digenean parasites in the genetic tree. This clustering could be not accurate because of the unavailability of the data for the closely related taxa to *Paramphistomum*. *Paramphistomum cervi* was recorded commonly in indigenous cattle for the first time in livestock in Saudi Arabia [10]. The author recoded this parasite in cattle, goats, sheep and imported sheep. As we could not identify our sample morphologically and there is no available mitogenome data for any of *Paramphistomum* in the GenBank, we could not be able to exactly identify our sample. We therefore suggested to identify it as *Paramphistomum* sp.-Taif.

The Kingdom of Saudi Arabia is a very large country which is annually visited from all over the world for Pilgrimage. It needs therefore a huge number of domesticated animals to be slaughtered. As Mohammed and Koshak [28] recommended examining the immigrant employees and workers from many nations annually for the different parasites, we belief that examining the resident and introduced domesticated animals is more necessary.

Recommendations: This work needs to conduct morphological study by light and electron microscopes to exactly identify the studied samples. We also recommend to conduct further molecular studies on more samples in order to clearly identify these parasites as one of Saudi Arabian biodiversity strategy.

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