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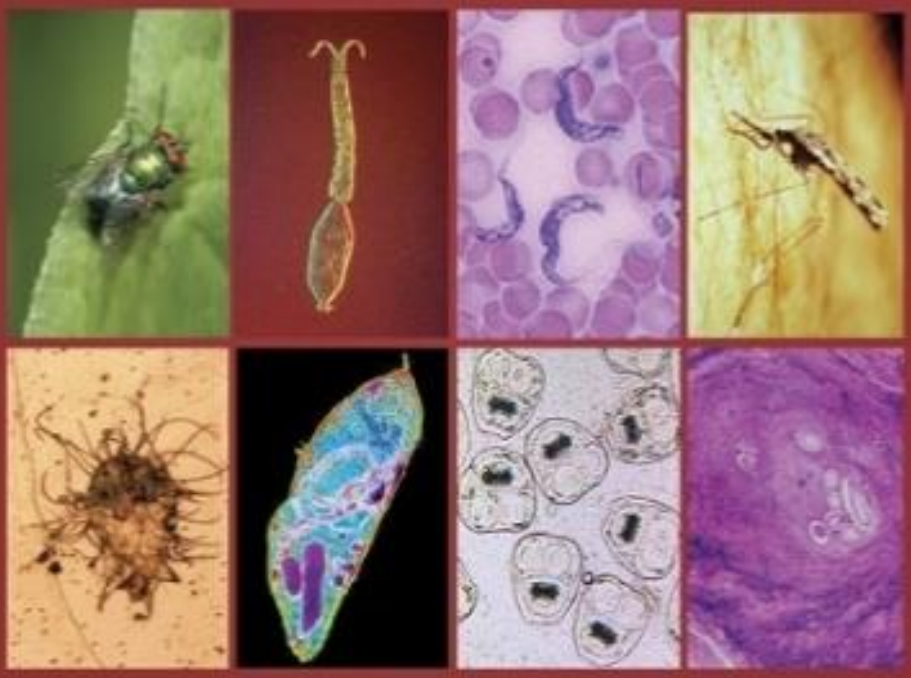

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Morphological and Molecular Characterization Based on ITS-2 of *Moniezia expansa* Rudolphi, 1810 (*Anoplocephalidae*) Isolated from The Intestine of Sheep, *Ovis aries* (Bovidae) from Egypt

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ABSTRACT

One hundred and twenty small intestines of sheep obtained from the Basateen Automated Slaughterhouse in Cairo, Egypt, between February 2021 and May 2021 were examined for cestodes. The cestode observed was *Moniezia expansa* (10.8%). It was measured 175-200 cm in length and varied in width according to the maturation of proglottids. Scolex was measured 0.75-0.88 mm in size and composed of a clear apical region without a rostellum. It had four conspicuous suckers with diameters of 0.25-0.31 mm. Scolex was followed by an unsegmented neck and proglottids. Proglottids were substantially wider than they were long. The immature proglottids measured 0.35-0.60 mm in length and 1.97-3.20 mm in width, without obvious structures. While mature proglottids were measured 0.70-1.40 mm in length and 6.75-8.25 mm in width and possess completely formed reproductive organs. The gravid proglottids were measured 1.38-4.43 mm in length and 14.25-16.75 mm in width, packed with eggs, and came towards the end of the strobila. PCR amplification produced a fragment of approximately 800 bp in size. The analyses showed distinct genotypes with sequence identities that ranged from 98.90% to 100% when compared to the GenBank sequences of *M. expansa*. The variation of evolutionary divergences was extended from 0.000 to 1.356 for *Moniezia* spp. and another genus of cestodes. Based on morphological traits and molecular analyses, the currently isolated worms were confirmed as *M. expansa.*

INTRODUCTION

The majority of cestodes were parasites, and some species may reach as long as several meters. Generally, the adult worms lived in the small intestine of their hosts, where they were held in the intestinal wall by distinctive holdfast organs like suckers and hooks (Mehlhorn, 2016). Due to its direct effects on the host animals, monieziasis has commercial potential where its symptoms include diarrhea, intestinal blockage, anemia, weight loss, weakness, decreased milk output, low-quality meat production, and, in severe cases, death (Prakash *et al*,. 2010).

The most commonly encountered intestinal tapeworms of ruminant animals are the *Moniezia* spp. (Tam *et al.*, 2020). They are unique members of the *Anoplocephalidae* family and have interproglottidial gland groups at the posterior part of each mature proglottid (Gunn and Probert 1983).

M. expansa has a typical cestode in length and begins with an anterior scolex, followed by a long-unsegmented neck and strobila (Mehlhorn, 2016). The interproglottidial glands can be utilized as a conventional approach to distinguish between *M. expansa* and the other *Moniezia* spp. (Haukisalmi *et al.,* 2018).

However, in certain individuals, interproglottidial glands are lacking, making morphological species identification tricky (Taylor, 1928; Diop *et al.,* 2015). Because there are few or no morphological characteristics that may be used to distinguish between individuals of various *Moniezia* spp., genetic markers must be developed to accurately identify *Moniezia* spp. and to serve as the foundation for taxonomic investigations (Chilton *et al.,* 2007). The nuclear ribosomal DNA's second internal transcribed spacer (ITS-2) is a highly helpful marker for the interspecies relationship of helminths (Ando *et al.,* 2006).

The current investigations attempt to determine the prevalence of cestode worms infecting the small intestine of domestic sheep, *Ovis aries*, in the Basateen Automated Slaughterhouse in Cairo, Egypt. The isolated worms will be identified based on their morphological traits and molecular characterization using ITS-2 sequences.

MATERIALS AND METHODS

Samples Collection:

A total of 120 small intestines of freshly slaughtered domestic sheep from Basateen Automated Slaughterhouse, Cairo, Egypt, were obtained during the period from February 2021 to May 2021. These intestines were transported as soon as possible in plastic bags to the Parasitology Laboratory, Zoology Department, Faculty of Science, Al-Azhar University. The contents of each small intestine were opened separately, emptied into a glass beaker, and repeatedly washed with water. Then, the contents were thoroughly checked for cestode parasites. The worms were recovered and relaxed in the fridge for an body that can reach from six to ten meters hour. Then, each worm was divided into small pieces (1-2 cm). Certain pieces of worms were prepared for morphological and molecular studies, while other components were preserved in 70% alcohol.

The Morphological Study:

Various pieces of worms were sandwiched between two slides, overnight fixed using 4% formalin, then stained with acetocarmine, differentiated in 70% acid alcohol, dehydrated in an ascending ethanol series, cleared in xylene, and mounted in Canada balsam (Georgiev *et al.,* 1986). Worms were carefully studied and morphologically identified according to Yamaguti (1959) and Soulsby (1986). Light microscopes called NOVEL (NTB-2B) and XSZ-107T were used to take ocular micrometer measurements and photomicrographs.

The Molecular Study:

Using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), genomic DNA was extracted from two *M. expansa* worms following the manufacturer's instructions and stored at -20 °C until used. PCR was performed to amplify the majority of 5.8S and the complete ITS-2 using the primer pairs 3S (5′–GGT ACC GGT GGA TCA CTC GGC TCG TG–3′) (forward) and BD2 (5′–TAT GCT TAA ATT CAG CGG GT–3′) (reverse) (Bowles *et al.,* 1993). The reaction was performed in 50 µl of a mixture containing 20 µl ddH2O, 25 µl of Thermo Scientific Maxima Hot Start Green PCR Master Mix (2X), 1 µl of each primer, and 3 µl of genomic DNA (10–20 ng template). The thermo-cycler (7300 Real-Time PCR System, Applied Bio-Systems) was used to perform the standard PCR reaction under the following conditions: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 35 sec and extension at 72°C for 45 sec, with a final extension of 72°C for 10 min. DNA fragments that had been amplified were electrophoresed on 1.5 % agarose gel in 1xTBE-buffer and

stained with ethidium bromide to confirm that they were single bands. These bands were compared with the fragments of Gene Ruler 100 bp (Thermo-Scientific).

DNA amplification with clear bands was purified using the Gene JET PCR Purification Kit (Thermo-Scientific) according to the provided instructions. Purified PCR products were sequenced using an ABI 3730xl DNA sequencer (GATC Biotech Company, Germany).

Sequence alignment was carried out in MEGA v.7.0's Clustal W software (Kumar *et al.,* 2016)**,** and it was displayed in Jalview v.2.11.0 (Waterhouse *et al.,* 2009)**.** Using NCBI BLAST servers, pairwise comparisons and identities (%) were determined (Camacho *et al.,* 2009). The Maximum Composite Likelihood method was used to compute the evolutionary distances (Saitou, 1988). ITS-2 sequences of *M. expansa* worms deposited in the GenBank.

RESULTS

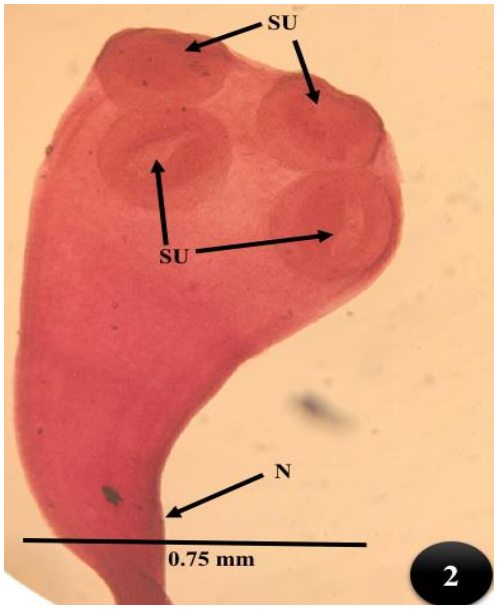
The Morphological Study:

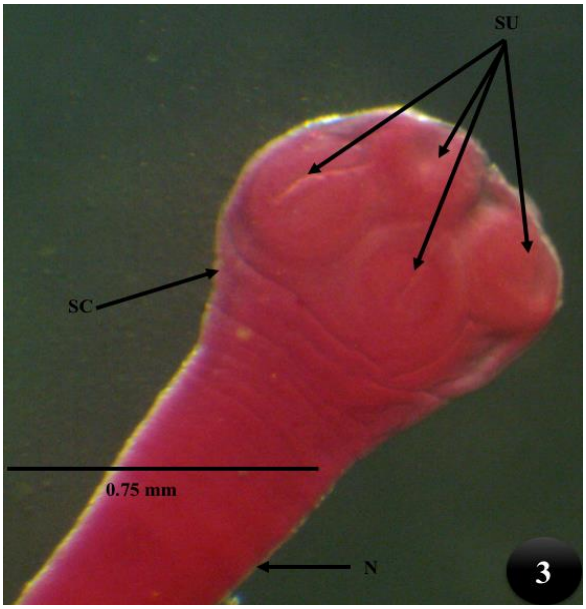
One hundred and twenty small intestines of sheep obtained from Basateen Automated Slaughterhouse were examined for cestodes, which revealed that 13 (10.8%) of the sheep only had one species of cestode. The isolated worms were identified as *M. expansa* based on their morphological traits. The adult worm was white in color and had a long, dorso-flattened body segmented. The whole worms measured 175 - 200 cm in length and 0.19 - 1.67 cm in width and consisted of an anterior scolex followed by an unsegmented neck and a long strobila. The proglottids of the strobila were much wider than they were long, and they got wider as they went down the strobila, from the immature following the neck to the mature to gravid proglottids at the end (Fig. 1).

Scolex was measured 0.75 - 0.88 mm in size, without rostellum, and comprised a distinct apical region with four conspicuous suckers that were each measured 0.25 - 0.31 mm in diameter (Figs. 2&3).

Immature proglottids were measured 0.35 - 0.60 mm in length and 1.97 - 3.20 mm in width, which was newly generated and small with unrecognized internal structures **(**Fig. 4).

Fig. 1 Photograph of the *M. expansa* worm isolated from domestic sheep, showing the three main body parts of the whole worm: the scolex (SC), the neck (N), and the strobila (ST).





Figs. 2-3 Photomicrographs showing the structure of *M. expansa* scolex (SC), it had four prominent suckers (SU), without a rostellum, followed by a neck (N).

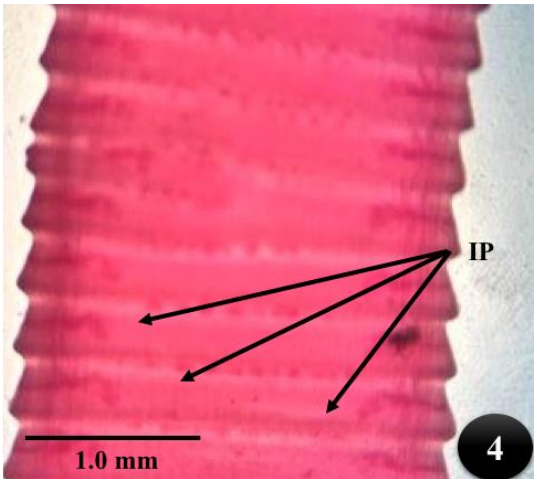
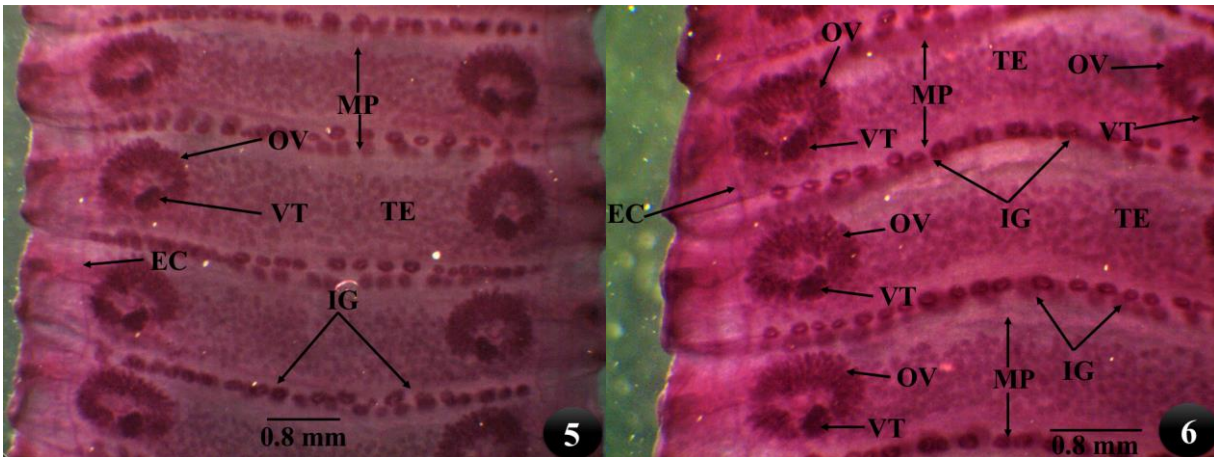


Fig. 4: Photomicrograph showing several immature proglottids (IP) of the *M. expansa* worm with unrecognized internal structures.

Mature proglottids were measured 0.70 - 1.40 mm in length and 6.75 - 8.25 mm in width and contained completely formed reproductive organs, vitelline glands, two bilateral excretory canals, and interproglottidial glands (Figs. 5&6).

The ovary and the vitelline glands are located on both sides of the mature proglottid, which made an ovoid shape. Each ovary was measured 0.17 - 0.21 mm and appeared a horseshoe shape (Fig. 7). The testes disseminated in the central part of the mature proglottid between the two longitudinal excretory canals and surrounding the ovaries. Interproglottidial glands are located at the posterior border of the mature proglottid and arranged in a row of rosette shapes. The number of these glands was variable, ranging from 20 - 27 glands.

Gravid proglottids were measured 1.38 - 4.43 mm in length and 14.25 - 16.75 mm in width, filled with eggs and came at the end of the strobila of the adult worm (Fig. 8).



Figs. 5-6: Photomicrographs showing the internal structures of mature proglottids (MP) of two *M. expansa* worms: bilateral ovaries (OV), vitelline gland (VT), Testes (TE), excretory canal (EC), and interproglottidial glands (IG).

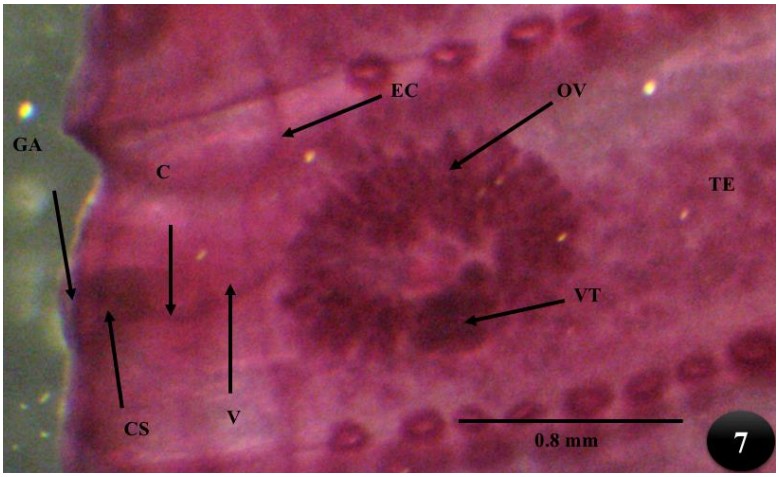


Fig. 7 Photomicrograph showing the reproductive organs of the *M. expansa* worm, which contain the following: testes (TE), ovary (OV), vitelline gland (VT), genital atrium (GA), cirrus (C), cirrus sac (CS), and vagina (V).

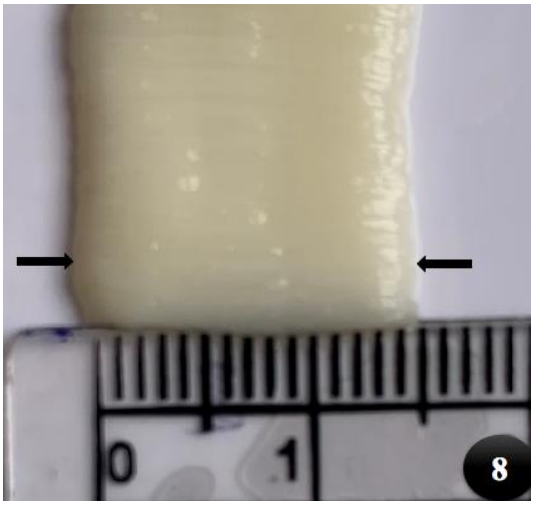


Fig. 8 Photograph showing the width (between arrows) of gravid proglottids of the *M. expansa* worm.

The Molecular Study:

A total genomic DNA was successfully extracted from two worms of *M. expansa*, PCR amplification with the described primers (3S and BD2) yielded a fragment of 800 bp in size for ITS-2 (Fig. 9). The sequencing of PCR products using an ABI 3730xl DNA sequencer was determined. Only 635 bp of the ITS-2 sequences were used for phylogenetic studies. Representative sequences were deposited in the GenBank under accession numbers ON112102 and ON112103. The analysis of ITS-2 sequences revealed that distinct genotypes with sequence identities ranged from 98.90 to 100% (Table 1) when compared to each other and GenBank sequences.

The alignment of these sequences revealed that there were nine SNPs (single nucleotide polymorphisms), resulting from substitution, and addition, or deletion at the nucleotide positions (126, 152, 153, 154, 207, 208, 267, 347, and 617). The substitutions represented four transversions at the nucleotide position 126, where the nucleotide C (pyrimidine) in all sample sequences was replaced by the nucleotide G (purine) in KX425620 at the nucleotide position 267, where the nucleotide G (purine) in all sample sequences replaced to the nucleotide C (pyrimidine) in ON112103 at the nucleotide position 347, where the nucleotide A (purine) in all sample sequences replaced to the nucleotide C (pyrimidine) in ON112103 and 617, where the nucleotide A (purine) in all sample sequences replaced to the nucleotide T (pyrimidine) in KX425620, and addition/deletion at the nucleotide positions 152, 153,154, 207 and 208 (Fig. 10).

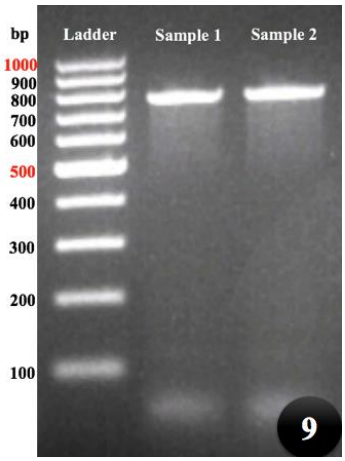


Fig. 9 Photograph showing agarose gel electrophoresis of PCR products of partial 5.8S and complete ITS-2 genes of *M. expansa* worms. The first lane represented DNA Ladder, and the second and third lanes represented samples (1&2).

Table 1: Data showing pairwise identities (%) among six ITS-2 genotypes of *M. expansa* worms, representing two sequences (from the present study) from Egypt and four sequences from the GenBank database.

	1	2	3	4	5	6	Country
ON112102	--						**Egypt**
ON112103	99.69	--					**Egypt**
LC422625	99.69	99.37	--				**Vietnam**
LC422627	100	99.69	99.69	--			**Vietnam**
AB367793	100	99.69	99.69	100	--		**Japan**
KX425620	99.22	98.90	98.91	99.22	99.22	--	**India**

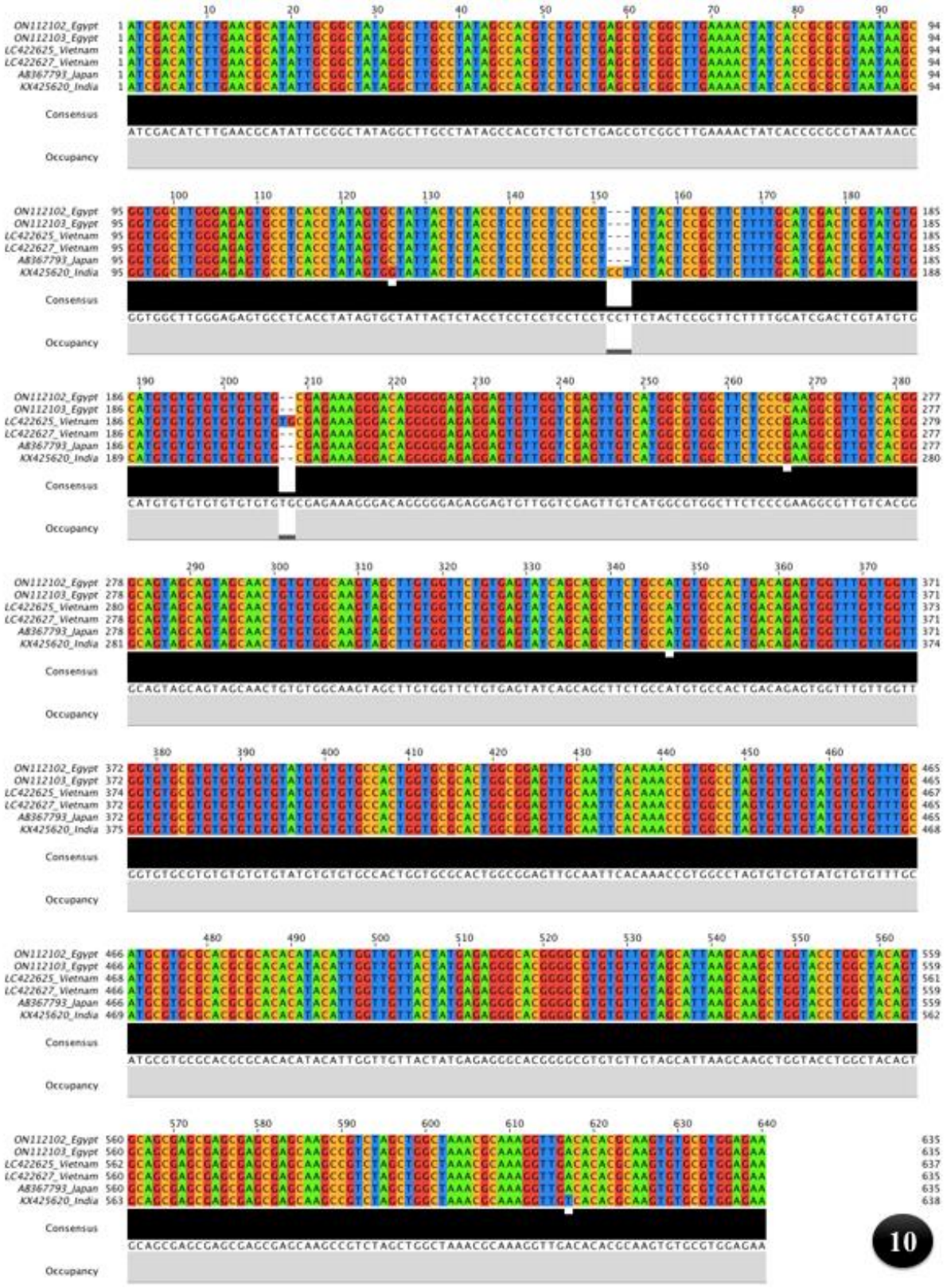


Fig. 10 Alignment of nucleotide sequences for the ITS-2 region (5′ to 3′) of *M. expansa* worms.

DISCUSSION

In the current study, the prevalence of *M. expansa* worm was 10.8% (13/120) in the small intestines of sheep obtained from the slaughterhouse in Cairo, Egypt, during the period from February 2021 to May 2021. This result was in line with that recorded by Al-Otaibi *et al.* (2021) in Al Taif Governorate, Saudi Arabia (10%), but it was lower than those recorded by Lone *et al.* (2012) in Ganderbal, Kashmir (29.10%); Nguyen *et al.* (2012) in Vietnam (16.4%); Ibrahim *et al.* (2014) in Jimma Town, Western Ethiopia (13.1%); Ndom *et al.* (2016) in Dakar, Senegal (15.4%); and Fol *et al.* (2020) in Cairo, Egypt (50%). This fluctuation in the prevalence of this worm might be due to the sampling time, host behaviors, vectors, and a variety of local geo-climatic conditions.

The current investigation showed that the adult *M. expansa* worm was white in color and had a long, dorso-flattened, segmented body that was 175 to 200 cm in length. The latter (length) was lesser than the lengths of the same worms from sheep recorded by Bashtar *et al.* (2011) in Cairo, Egypt (470 cm); Kuchai *et al.* (2013) from sheep in Ladakh, Kashmir (453 cm); and Fol *et al.* (2020) from sheep in Cairo, Egypt (421 cm). On the other hand, the length of the present worm was longer than that obtained by Tam *et al.* (2020) from goats and cattle in Bac Giang Province, northern Vietnam (114 cm), and Gómez-Puerta *et al.* (2008) from a domestic pig in Tumbes, Peru (50 cm). The current investigation revealed that the morphological features and morphometrics of scolex, immature proglottids and mature proglottids almost agreed with that of the same worms obtained by Kaufmann (1996) from sheep in Basel, Switzerland, Gómez-Puerta *et al.* (2008) from a domestic pig in Tumbes, Peru, Bashtar *et al.* (2011) from sheep in Cairo, Egypt, Kuchai *et al.* (2013) from sheep in Ladakh, Kashmir, Fol *et al.* (2020) from sheep in Cairo, Egypt, and Tam *et al.* (2020) from goats in Bac Giang Province, northern Vietnam. In the worm studied herein, the interproglottidial glands were located at the posterior border of the mature proglottid and appeared as a linear row of rosette-shaped. Their numbers were variable (20 - 27). The last result was nearly similar to the same cestode recorded by Bashtar *et al.* (2011) from sheep in Cairo, Egypt (26 ± 4) and Gómez-Puerta *et al.* (2008) from a domestic pig in Tumbes, Peru (20 – 31). However, the number of these present glands was less than the number of glands recorded by Taylor (1928) from sheep in the USA (0 - 60).

The gravid proglottid of the worm studied herein measured 1.38 - 4.43 mm in length and 14.25 - 16.75 mm in width, this result was in agreement with that obtained from the same host (sheep) by Kaufmann (1996) in Switzerland (16.00 mm in width), but it was less than that of Mehlhorn (2016) (25.00 mm in width) and more than that of Taylor (1928) in the USA (2.00 – 8.00 mm in width), Kuchai *et al.* (2013) in Ladakh, Kashmir (3.05 - 4.87 mm in width), Fol *et al.* (2020) in Cairo, Egypt (2.10 – 5.02 mm in width), and from goats and cattle by Tam *et al.* (2020) in Bac Giang Province, northern Vietnam (6.00 – 6.80 mm in width).

The factor responsible for variation in measurements of the current worm and those of previous scientific studies may be attributed to the host's food, where animals that feed on a highly nutritive diet harbored lengthier parasites compared to those that fed on a low diet (Kuchai *et al.,* 2013). These authors also noted that the morphology and body measurements of the worm were directly influenced by the parasite species' severity and the host animal's immunity.

A conventional approach used to distinguish between *Moniezia* spp. was performed by comparing the shape and number of interproglottidial glands (Haukisalmi *et al.,* 2018). However, Taylor (1928) noted that this method was not always accurate because the interproglottidial glands could be absent in a particular area of the strobila or even the

entire strobila. These linear glands could be difficult to detect in specimens with poor staining, which could prevent morphological identification at the species level (Spasskii, 1951). Additional studies had reported the absence of interproglottidial glands from *M. expansa* recovered from goats, or sheep in Ethiopia and Senegal (Diop *et al.,* 2015), as well as in central Vietnam from sheep, goats, or cattle (Nguyen *et al.,* 2012). Therefore, the molecular technique (ITS-2 sequences) was used to confirm that the present worm was *M. expansa*. ITS-2 sequences work great as interspecies identification markers (Ando *et al.,* 2006).

According to the GenBank DNA database, the ITS-2 sequences of *M. expansa* were only found in a limited number of specimens from Vietnam, India, and Japan. The nucleotide sequences of *M. expansa* were nearly the same and distinct from those of *M. benedeni*. The phylogenetic tree of the current *M. expansa* sequences revealed that they share a degree of similarity with *M. expansa* of the previous sequences on GenBank, which clustered together in a tree, and other species of *M. benedeni* clustered in a separate branch, while ITS-2 of another genus, *Anoplocephala perfoliata* (MZ648229) in the same family, clustered as an outgroup (Fig. 11). Estimates of average evolutionary divergence over all sequence pairs among the seven ITS-2 sequences were 0.346 and extended from 0.000 to 1.356. Table (2) displays the number of base substitutions per site formed between various sequences.

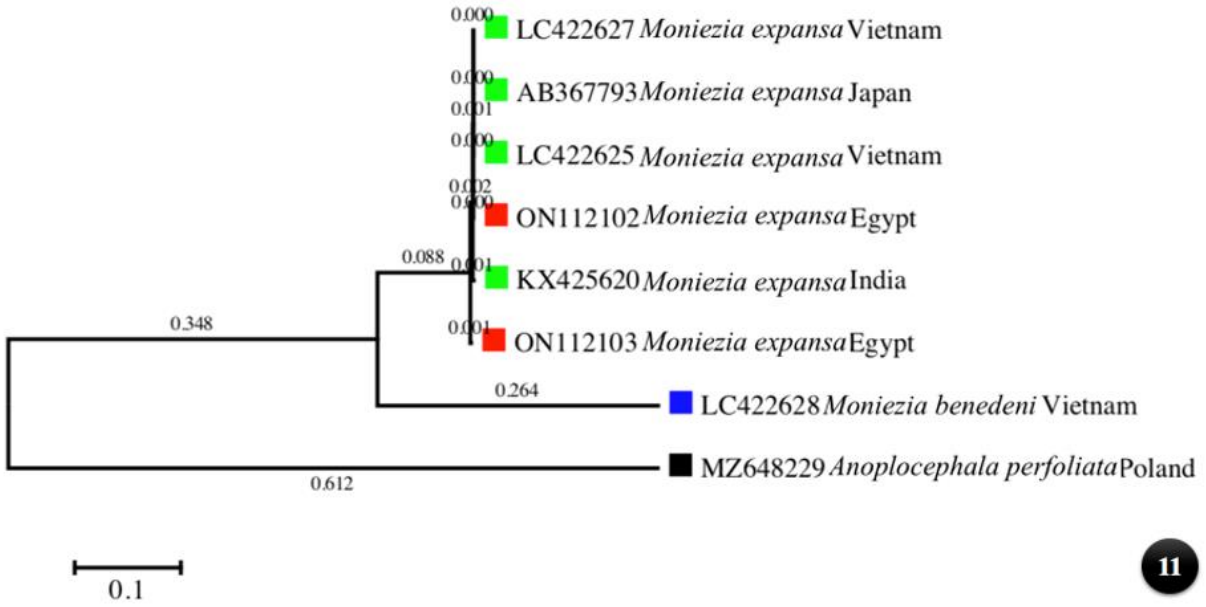


Fig. 11 Diagrammatic representation of a phylogenetic tree of ITS-2 sequences of the *M. expansa* worm using the Maximum Likelihood method analysis based on the variability of their sequence alignment.

Table 2: Data showing estimation of evolutionary divergence among six ITS-2 genotypes of *M. expansa* worms, representing two sequences (from the present study) from Egypt and sequences from the GenBank database

	1	**2**	**3**	**4**	**5**	**6**	**7**
ON112102 *M. expansa* Egypt							
ON112103 *M. expansa* Egypt	0.003						
LC422625 *M. expansa* Vietnam	0.000	0.003					
LC422627 *M. expansa* Vietnam	0.000	0.003	0.000				
AB367793 *M. expansa* Japan	0.000	0.003	0.000	0.000			
KX425620 *M. expansa* India	0.002	0.005	0.002	0.002	0.002		
LC422628 *M. benedeni* Vietnam	0.353	0.359	0.353	0.353	0.353	0.356	
MZ648229 *Anoplocephala perfoliata* Poland	1.052	1.042	1.052	1.052	1.052	1.048	1.224

Declarations:

Conflict of interest: The authors declare that they have no conflicts of interest.

Ethical approval: No approval of research ethics committees was required.

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