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RESEARCH ARTICLE

Morphological and molecular characterization of *Haemonchus contortus* (Rudolphi, 1803) Cobb, 1898 (Nematoda: Trichostrongyloidea) from sheep, *Ovis aries* in Egypt based on the second internal transcribed spacer of ribosomal DNA

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Abstract

A total of 562 worms (392 females and 170 males) of *H. contortus* were collected from abomasa of 70 sheep, *Ovis aries* slaughtered in Basateen Automated Slaughterhouse, Cairo. Adult male worm, 17.00±0.75 mm long, was smaller than adult female, 28.09±2.86 mm long. Male characterized by having a caudal copulatory bursa at the posterior end, while female characterized by having a vulva that located at the third body length from tail and may or may not be guarded by a cuticular inflation (vulvar flap). According to female vulvar flap characteristic, there were three morphotypes: linguiform (L) (39.5%), knobbed (47.0%) and smooth (13.5%) vulvar flaps. Further classification of the linguiform type of the female vulvar flap revealed the presence of four subtypes: Linguiform A (LA) (60.0%), Linguiform B (LB) (10.3%), Linguiform C (LC) (24.5%) and Linguiform I (LI) (5.2%). Molecular investigation using sequencing of second internal transcribed spacer of ribosomal DNA (ITS-2) revealed that male and each female genotypic sequence was almost identical (99 − 100%). Evolutionary divergence over all sequence pairs was 0.006 among ITS-2 sequences.

INTRODUCTION

Trichostrongylid nematodes are important parasites of domestic ruminants and are responsible for significant economic losses especially in tropical and temperate regions worldwide (Charlier *et al*., 2009). Traits of vulvar flap morphology are considered as morphological markers of ecological adaptation. Variation of vulvar flap morphology indicates manifestation of genetic factors during worm establishment and development (Gharamah *et al*., 2011). Vulvar morphology helps to understand and know more about biology of *Haemonchus* species and determines the type of population that occurs in a certain area (Le Jambre and Whitlock 1968).

Traditional procedures for diagnosis involve cultivation of larvae and microscopic differentiation of third-stage larvae based on morphology (Burger and Stoye, 1968). However, they added that this approach is tedious and not always reliable due to minor morphological characteristics. Genetic characterization is important for accurate identification and also for an effective control due to the anthelmintic resistance problem in this nematode (Gasser *et al*., 2008). Recent advances in DNA technology have resulted in the development of sensitive and rapid hybridization and amplification methods for genetic diversity studies (Masiga *et al*., 2000). In this regard, rDNA

genes and their associated spacer regions provide suitable targets for developing diagnostic probes or makers for species identification (Hoste *et al*., 1995). The ribosomal internal transcribed spacer (ITS) sequences are the most commonly markers to discriminate among nematode species (Cerutti *et al*., 2010) because the ITS regions are one of the most variable nuclear loci and are under concerted evolution (Nadler *et al*., 2000). Thus the ITS-2 region has been widely applied in species identification within the genus *Haemonchus* (Gasser *et al*., 1998 and Heise *et al*., 1999).

The present study included: 1- Incidence and prevalence of *H. contortus* infection among sheep, *Ovis aries* slaughtered in the Basateen Automated Slaughterhouse, Cairo. 2- Carrying out morphological studies of adult worms (male and female) and classifying of females, according to the type of vulvar flap process. 3- Identifying of genetic variations within worms by employing the second internal transcribed spacer region (ITS-2) of nuclear rDNA.

Materials and methods

Sample collection

Regular visits from January to June 2014 to Basateen Automated Slaughterhouse, Cairo, allowed obtaining abomasa of 70 sheep, *Ovis aries* which were randomly originated from different localities in Egypt. Worms recovery was carried out according to the procedures previously described (MAFF, 1986). After slaughtering, abomasa were separated from other parts of stomach, ligated at the both ends and directly transferred to the laboratory in labeled plastic bags. Each abomasum was opened along the greater curvature with a pair of scissors, and contents were poured in a glass beaker. The contents were then processed by repeated washings, sedimentation as well as decantation until the supernatant was clear enough for easy collection of worms. Both the abomasum and contents were carefully examined. Adult worms were collected and washed with a physiological saline solution to remove adherent food residues. Most worms were preserved at 70% ethanol, whereas some were studied as fresh samples.

Morphological and morphometric study

Male and female worms were macro- and microscopically studied. Female worms were classified according to their vulvar morphology according to (Jacquiet *et al*., 1995) into three types: linguiform (with a supra vulvar flap), knobbed (with a knob-like vulvar process) and smooth (without any vulvar process). The first type was further classified into four subtypes: linguiform A (LA) had one cuticular inflation, linguiform B (LB) had no cuticular inflations, linguiform C (LC) had two cuticular inflations and linguiform I (LI) had one cuticular inflation that arises from the linguiform process according to Le Jambre and WhitLock (1968). Morphometric study was carried out using ocular micrometer. The study included body length, maximum width, distance of cervical papillae from the anterior end and esophagus length. Distance between vulva to posterior end of the body in female worms and total length of spicule in male worms were also measured.

DNA extraction and ITS-2 amplification

A total genomic DNA was extracted from (5-8) adult worms of male and of each female morphotype, separately (except Linguiform I (LI) subtype because the number of specimens was not enough). Extracted DNA was done using the TIANamp Genomic DNA Kit (Spin Column) according to the manufacturer's protocol and stored at −20◦C.

PCR was performed according the following protocol: The fragment of ITS-2 of rDNA (including flanking sequence) was amplified using the primers set NC1 (forward; 5`- ACGTCTGGTTCAGGGTTGTT-3` and NC2 (reverse; 5`-TTAGTTTCTTTTCCTCCGCT-3`) (Stevenson *et al*., 1995). The standard PCR reaction was carried out on the thermo-cycler (7300 Real Time PCR System, Applied Bio-systems) at the following conditions: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 35 s and extension at 72°C for 45 s, with a final extension of 72°C for 10 min.

The reaction was performed in 50µl of a mixture containing 20µl ddH_2O, 25µl of Thermo Scientific Maxima Hot Start Green PCR Master Mix (2X), 1µl of each primer, and 3µl of genomic DNA (10–20 ng template). Electrophoresis of amplified DNA fragments were conducted in 1.5 % agarose gel in 1xTBE-buffer and stained with ethidium bromide to verify that they represented single bands. These bands were compared with the fragments of GeneRuler 100 bp Plus DNA Ladder, Ready-to-Use 100 to 3000 bp (Thermo Scientific).

DNA purification and sequencing

Amplified DNA with clear bands was purified using Gene JET PCR Purification Kit (Thermo Scientific) according to the protocol provided. The purified products were run on 1.5% agarose gel electrophoresis to check for bands and only clear products were sent for sequencing to a service provider. The sequencing reaction of PCR products was conducted by (GATC Biotech Company, Germany) using ABI 3730xl DNA sequencer. The purified PCR products were sequenced in both directions (forward and reverse) to resolve any potentially ambiguous sites.

Data analyses

The obtained sequences (forward and reverse) were assembled using CAP3 a DNA sequence assembly program online (Huang and Madan 1999). The program used forward–reverse constraints to correct assembly errors and link contigs. The sequences were compared with that of the other studies using BioEdit sequence alignment editor (Hall 1999) for removing unresolved 'noisy' nucleotide sites at both ends and deposited in the GenBank database under the accession numbers of KP090288, KP090289, KP090290, KP090291, KP090292 and KP090293. Sequence alignment was conducted using the program Clustal W within MEGA v.6.0 (Tamura *et al*., 2013) and shown using Jalview v.2.8.1 (Waterhouse *et al*., 2009). Pairwise comparisons and identities (%) were calculated using the servers of NCBI BLAST (Camacho *et al*., 2009). The phylogenetic analysis was conducted using the Neighbour-Joining (NJ) and Maximum Likelihood (ML) methods, respectively, based on the Tamura -Nei model by MEGA v.6.0.

Results

Prevalence of adult *H. contortus* in sheep

Twenty (28.57%) out of 70 sheep were found harbored 562 adult *H. contortus* worms [392 (69.75) females and 170 (30.25) males]. Examination of the cuticular process of female worms revealed the presence of 184 (47.0%) knobbed, 155 (39.5%) linguiform and 53 (13.5%) smooth vulvar morphotypes. The knobbed vulvar flap was encountered as the most predominant morphotype in sheep host. Further classification of the linguiform vulvar flap (L) revealed overall proportions of 93 (60.0%) LA, 16 (10.3%) LB, 38 (24.5%) LC and 8 (5.2%) LI subtypes. Accordingly, Linguiform A was the most dominant linguiform subtype.

Morphological characteristics of *H. contortus*

Macroscopically, adult worms were easily seen, mostly in the fundus region of the abomasum. There was a significant difference in length between adult male and female of *H. contortus*. Male worms were ($P<0.05$) smaller than females, measuring 17.00±0.75 mm in average length, while females averaged 28.09±2.86 mm in length. Whereas, there were no significant differences in length among all female morphotypes (except between the knobbed and linguiform B vulvar flaps). The knobbed was 29.30±2.49 mm in average length that was significantly ($P<0.05$) longer than linguiform B that was averaging 24.66±3.78 mm in length.

Microscopically, adult male worm was characterized by a caudal copulatory bursa at the posterior end (Fig. 1). The latter was well developed and consisted of two lateral lobes and asymmetrically placed dorsal one. Dorsal lobe situated against the left lateral lobe and was supported by a characteristic Y-shape dorsal ray. The two spicules were relatively long, equal to each other, brownish in color, 0.51±0.02 mm in average length, and ended with a barbed tip. Gubernaculum located anteriorly between the spicules.

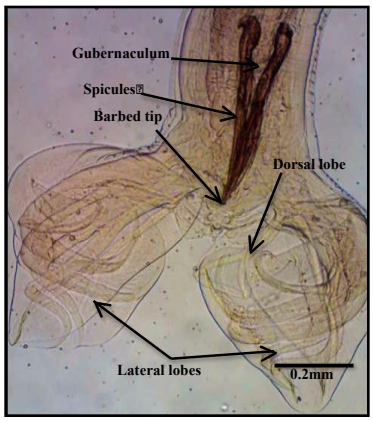

Fig. (1) Male *H. contortus* showing a caudal copulatory bursa

Vulva was located at the anterior limit of the last third of the female's body length. Its position averaged 4.70±0.49 mm from the end limit of a worm. There was no significant difference in distance between vulva and end limits of worms in all morphotypes. Vulva was either smooth (not covered with a flap), so called ″Smooth vulvar morph″(Fig. 2A) or covered with a small knob-like flap (Knobbed vulvar morph) (Fig. 2B) or covered with a relatively large and prominent flap (Linguiform vulvar morph (L)). Further classification of the latter revealed the presence of four types: LA subtype (Fig. 2C) with vulva covered with a large linguiform flap and had only one cuticular inflation; LB subtype (Fig. 2D) without cuticular inflation; LC subtype (Fig. 2E) with two cuticular

inflations in addition to the linguiform flap and LI subtype (Fig. 2F) with one cuticular inflation raised from the linguiform process. Table (1) summarized the morphometric study of adult male and all female morphotype worms.

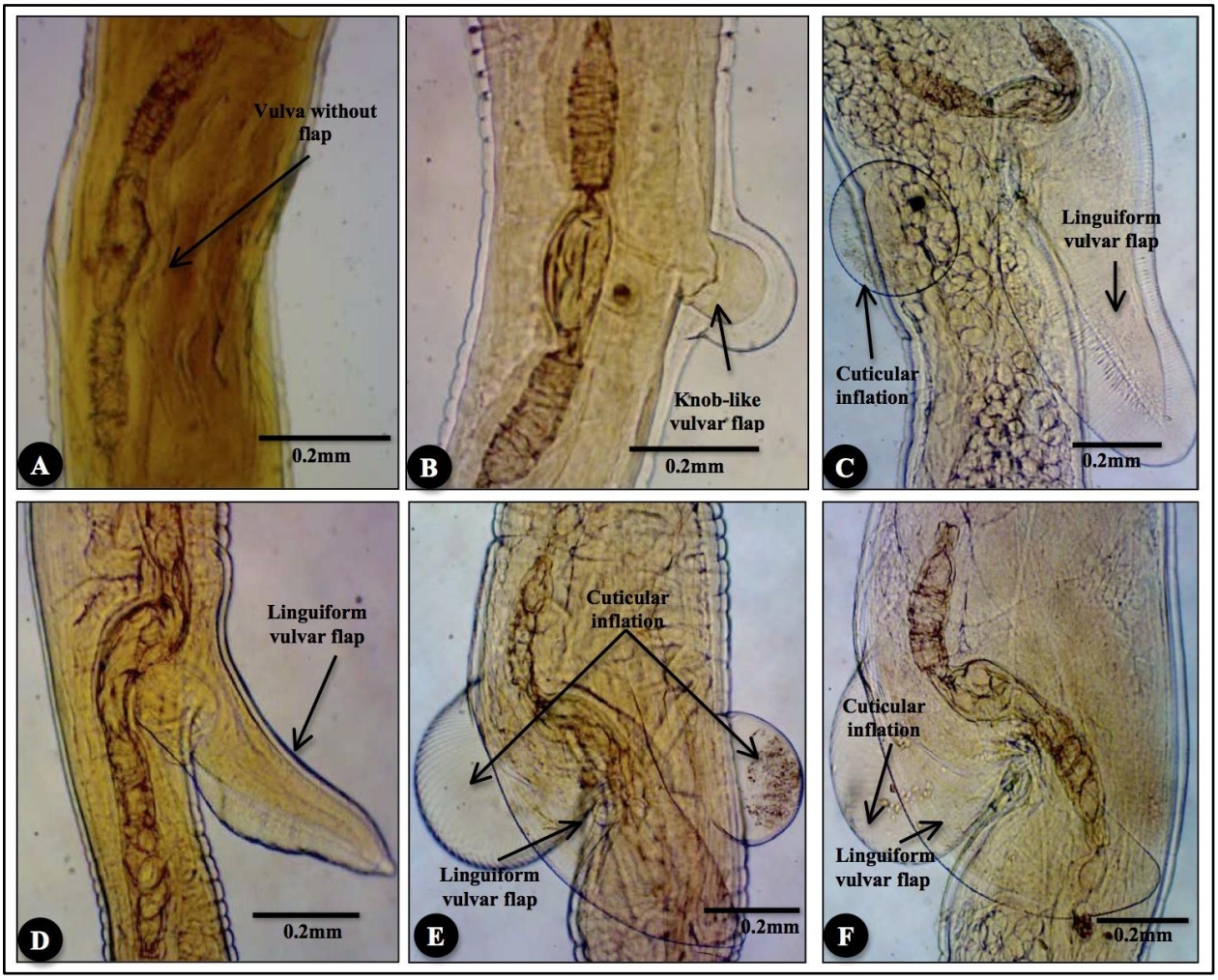

Fig. (2): Different vulvar morphotypes of female *H. contortus*: **A**- Smooth, **B**- Knobbed, **C**- Linguiform A, **D**- Linguiform B, **E**- Linguiform C & **F**- Linguiform I.

Table (1): Morphometric study of adult male and all female morphotypes of *H. contortus*

Worms	Male	Female					
		Smooth	Knobbed	Linguiform A	Linguiform B	Linguiform C	Linguiform I
Body length	17.00±0.75	27.80±2.15	29.30±2.49	28.40±2.59	24.67±3.78	28.25±2.86	27.00±5.19
Max. width	0.34±0.04	0.37±0.05	0.44±0.05	0.46±0.07	0.36±0.16	0.45±0.11	0.37±0.06
Cervical papillae from ant. end	0.44±0.02	0.43±0.05	0.51±0.07	0.46±0.04	0.42±0.01	0.42±0.04	0.45±0.04
Esophagus length	1.72±0.08	1.79±0.09	1.94±0.12	1.82±0.08	1.85±0.24	1.82±0.09	1.76±0.03
Vulva from post. end	----	4.71±0.34	4.77±0.31	4.74±0.27	4.27±1.58	4.81±0.48	4.53±0.50
Spicules length	0.51±0.02	----	----	----	----	----	----

- All measurements were in mm (mean ± standard deviation). max.= maximum, ant.= anterior, post.= posterior.

Isolation and purification of ITS-2 region

PCR amplification yielded a fragment of 321 bp in size for each adult female morphotype and male worms that included 231 bp of the ITS-2 plus 90 bp of flanking regions (20 bases from the 3**'** end of the 5.8**S** gene and 70 bases from the 5**'** end of the 28**S** gene) (Fig. 3).

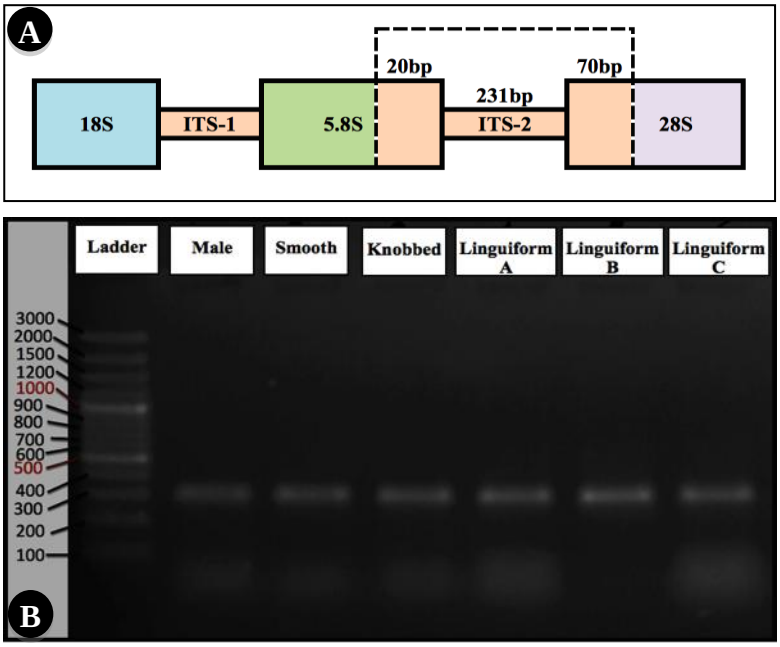

Fig. (3): PCR amplification: (**A**) diagram indicating an amplified region of the rDNA, with base pair (bp) numbers as described by Stevenson *et al*. (1995). ITS = internal transcribed spacer. 18S, 5.8S and 28S are rDNA genes (Fig. without scale). (**B**) amplification product of ITS-2 rDNA region of *H. contortus.*

Sequencing and phylogenetic analyses

ITS-2 sequences for male and female morphotypes (except LI subtype) were shown in Fig. (4A) revealing that distinct genotypes of *H. contortus* sequence identities ranged from 99−100%, when compared with each other. Alignment of ITS-2 sequences revealed that there were three single nucleotide polymorphisms (SNPs) resulted from substitutions at the nucleotide positions (187, 196 and 208). These substitutions represented one transversion at the nucleotide position 196 (A <−> T) and two transitions at the nucleotide positions 187 and 208 (A<−>G).

Phylogenetic tree of ITS-2 sequence alignment was performed using Neighbour-Joining method (Fig. 4B). The dendrogram represented the similarity level among the ITS-2 sequences of morphotypes. It was marked only two clusters at the phylogenetic trees, LA and LB subtypes opposite other morphotypes: LC, smooth and knobbed in addition to male sequence. Further analyses did not identify significant grouping between the different morphotypes.

Estimates of average evolutionary divergence overall sequence pairs among the six ITS-2 sequences were 0.006. Numbers of base substitutions per site form between different sequences were shown in Table (2). Analyses were conducted using the Maximum Composite Likelihood model.

Table (2): Estimates of evolutionary divergence between the ITS-2 sequences

Sample code	1	2	3	4	5	6
Hc_KP090288_Male	---	---	---	---	---	---
Hc_KP090289_Smooth	0.004	---	---	---	---	---
Hc_KP090290_Knobbed	0.004	0.000	---	---	---	---
Hc_KP090291_Linguiform_A	0.009	0.013	0.013	---	---	---
Hc_KP090292_Linguiform_B	0.004	0.009	0.009	0.004	---	---
Hc_KP090293_Linguiform_C	0.004	0.000	0.000	0.013	0.009	---

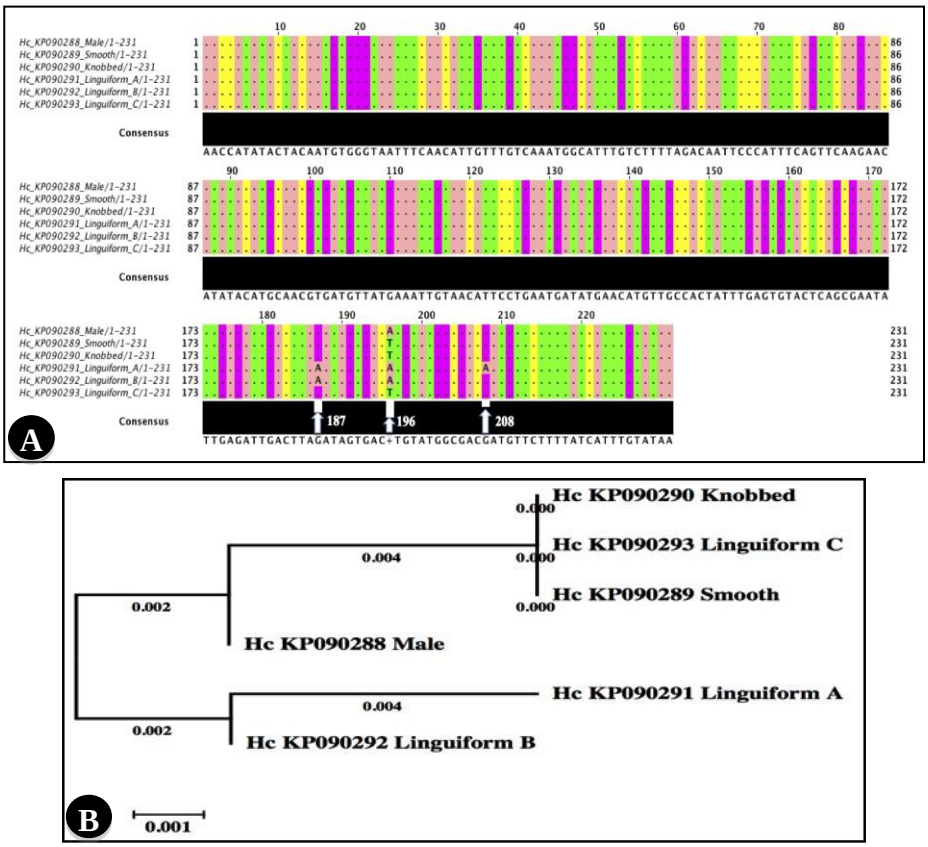

Fig. (4): **A-** Alignment of nucleotide sequences for the ITS-2 region (5'to3') male and all female morphotypes (except LI subtype) of *H. contortus* (Hc), identical bases were represented by dots. **B-** Phylogenetic tree of ITS-2 sequence of *H. contortus* (Hc) using the Neighbour-Joining method analysis based on the variability of their sequence alignment.

Discussion

Vulvar flap polymorphism of *H. contortus* has a great taxonomic importance and consider as a phenotypic marker for physical adaptation and diversity. The current results revealed the presence of knobbed (47%), linguiform (39.5%) and smooth (13.5%) vulvar morphotypes. The knobbed vulvar flap was encountered as the most predominant type. These results were in accordance in part with that of Rahman and Abd Hamid (2007) in Penang, Malaysia, Gharamah *et al*. (2011) in Al-Huaydah, Yemen, Abdel-Hafez *et al*. (2013) in Sharkia, Egypt and Vadlejch *et al*. (2014) in the Czech Republic. However, the current results were not in accordance with that of Jacquiet *et al*. (1995) in Mauritania, Thomas *et al*. (2007) in Ethiopia, Kumsa *et al*. (2008) in Eastern Ethiopia, Gharamah *et al*. 2010 in the Kelantan Stat, Peninsular Malaysia and Akkari *et al*. (2013) in North Tunisia, who recognized that linguiform vulvar was the predominant type. Gharamah *et al*. (2011) in Sana'a, Yemen showed that linguiform and knobbed vulvar types were equal, each was (42%).

In the present investigation, further classification of linguiform vulvar flap revealed presence of four subtypes: LA (60.0%), LB (10.3%), LC (24.5%) and LI (5.2%). These results coincided with that of Akkari *et al*. (2013) and Abdel-Hafez *et al*. (2013), where LA was the predominant linguiform vulvar morphotype, followed by LC, LB and LI. While, when comparing the present results with the findings obtained by Kumsa *et al*. (2008), the proportions were nearly similar to only LA and LI morphotypes, but differed in case of LB and LC. However, the current results differed from those obtained by Thomas *et al*. (2007) who found LC the predominant linguiform vulvar morphotype, followed by LI, LB and LA. Variations in prevalence rates of different morphotypes of female *H. contortus* obtained from different countries might be due to differences of ecological parameters (Jacquiet *et al*., 1995). Whereas, Vadlejch *et al*. (2014) stated that the vulvar morphology of female *Haemonchus* could be affected by the worm's ability to establish and develop within a host.

Morphometrical study of adult male and female worms of the current *H. contortus* revealed presence of a significant difference in length between them, but there were no significant differences in length among all female morphotypes, except knobbed vulvar type that was significantly longer than LB. Whereas, Hunt *et al*. (2008) found that both LB and knobbed vulvar types of *H. contortus* were significantly shorter than LA and LC. However, Abdel-Hafez *et al*. (2013) stated that no significant differences in length among all female morphotypes of *H. contortus* were noticed. There are certain factors influencing morphological features of nematode worms, the host immune system can regulate female length (Strain and Stear 2001). They observed a significant association between reduced length of adult female worm and increasing of immunoglobulin (Ig) A against third-stage larvae. Host breed (Aumont *et al*., 2003), host gender (Gruner *et al*., 2004), diet (Hoste *et al*., 2008) and concurrent parasitic infection (Terefe *et al*., 2005) can also affect the nematode worm length.

Sequence analyses of the nuclear multi-copy ribosomal DNA (rDNA) genes encoding for structural RNAs of ribosomes have been widely used in the phylogenetic studies and identification of nematodes (Cerutti *et al*., 2010). rDNA was arranged in tandem repeats in one or few chromosomal loci with thousands of repeats. The ITS-2 region has been widely applied in species identification within genus *Haemonchus* (Gasser *et al*., 1998 and Heise *et al*., 1999). The only investigation similar to the present one, in dealing with ITS-2 sequences of different female morphotypes and male of *H. contortus* in detail, was done in the Czech Republic by Vadlejch *et al*. (2014). The results of both studies revealed very low levels of variation within the worms (Fig. 5).

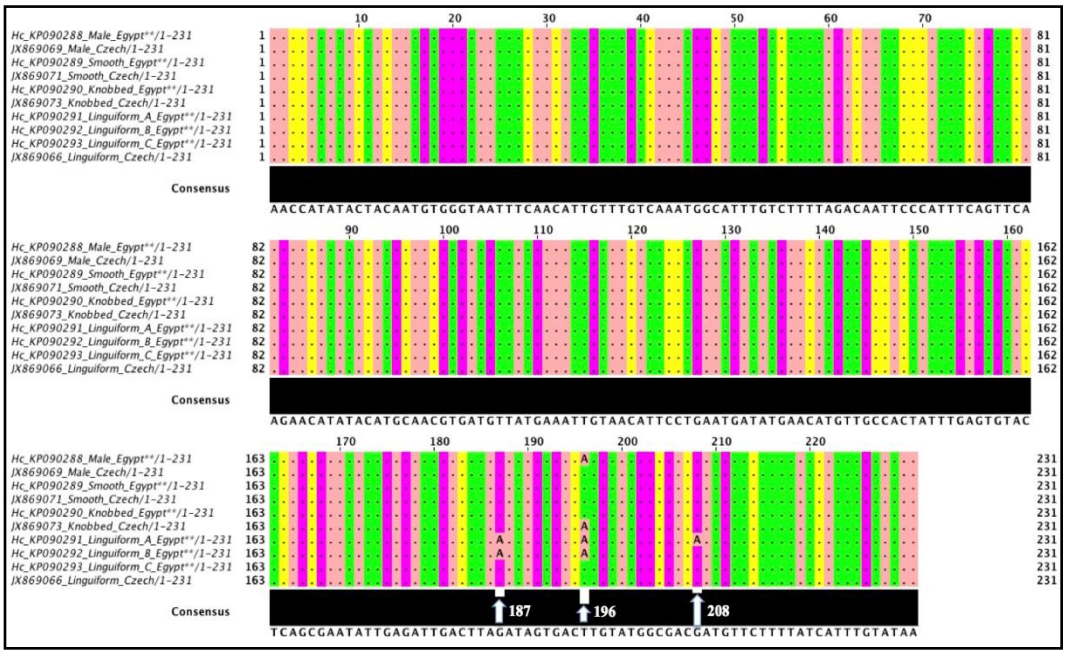

Fig. (5): Alignment of nucleotide sequences for the ITS-2 region (5'to3') of the present *H. contortus*** (male, smooth, knobbed, linguiform (LA, LB & LC), and of Vadlejch *et al*. (2014) in the Czech Republic (male, smooth, knobbed & linguiform). Identical bases were represented by dots.

A comparison of ITS-2 sequences for different morphotypes of the present female worms with those of the other *H. contortus* and also of *H. placei* and *H. longistipes*, was recorded in Table (3) and Fig. (6).

A standard PCR was performed in this study to isolate an ITS-2 region as previously described. The resulted unified bands of the present samples were within the expected range with an expected size which almost 321 bp. The evolutionary history between ITS-2 of 100 sequences of different *Haemonchus* spp. was inferred using the Maximum Likelihood method. The tree with the highest log likelihood (-483.6904) was shown in Fig. (6). The initial tree(s) for the heuristic search were automatically obtained by the applying Neighbor-Joining method and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value was carried out. The analysis involved all the 100-nucleotide sequences from different genotypes isolated from different countries. All positions containing gaps and missing data were eliminated. There were a total of 220 positions in the final data set.

Table (3): 100 ITS-2 sequences of *Haemonchus* spp. from 13 countries, including the current one from the GenBank database

GenBank accession no.	No. of sequences	Parasite	Host	Country
KP090288 – KP090293	6	*Hc*	Sheep (The present study)	Egypt
AB682686 – AB682687	2	*Hc*	Sheep	
AB682683 – AB682684	2	*Hl*	Camels	
KJ724250 – KJ724272	23	*Hc*	Randomly from sheep, goat, cattle and buffalo	Pakistan
KJ724326 – KJ724328	3	*Hp*		
KJ724324 – KJ724325	2	*Hl*		
HQ683710 – HQ683713	4	*Hc*	Sheep and goat	Malaysia
HQ683714 – HQ683715	2	*Hc*	Sheep and goat	Yemen
KF364628 – KF364632	5	*Hc*	Cattle	Australia
X78803	1	*Hc*	Sheep	
X78812	1	*Hp*	Cattle	
KC415117 – KC415134	18	*Hc*	Sheep and goat	China
JX869066 – JX869075	10	*Hc*	Sheep	Czech
KJ188203 – KJ188206	4	*Hc*	Sheep	Turkey
FN432335 – FN432336	2	*Hc*	Goat and chamois	Italy
JX901146 – JX901153	8	*Hc*	Sheep; goats and cattle	Tunisia
JQ342246 – JQ342247	2	*Hc*	Sheep, goat, cattle and buffalo	Brazil
JN128897 – JN128898	2	*Hc*		
KC503915	1	*Hc*	Sheep	Uzbekistan
KC503916	1	*Hp*	Cattle	
AJ577461	1	*Hl*	Undefined	Mauritania

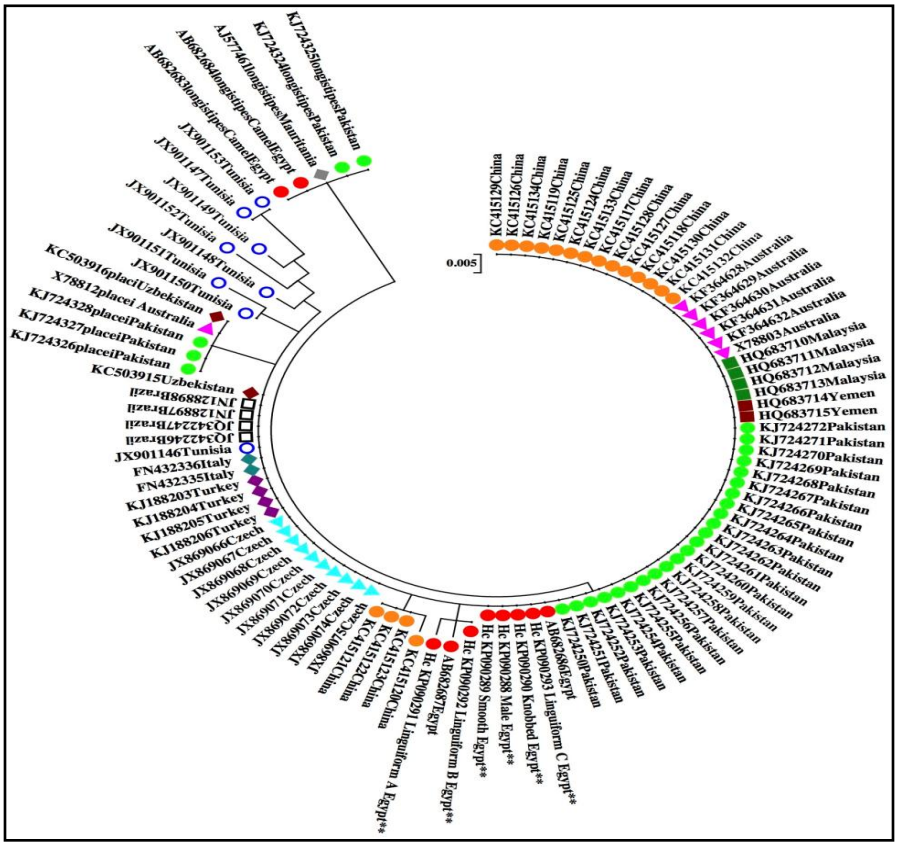

Fig. (6): Phylogenetic tree of *Haemonchus* spp. including the present one** using the Maximum Likelihood method.

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