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Molecular Differentiation of *Haemonchus longistipes* Railliet & Henry, 1909 (Nematoda: Trichostrongyloidea) of Female Morphotypes and Male Worms Infecting the Dromedary *Camelus dromedarius* Using RAPD Technique

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ABSTRACT

Molecular variability among female morphotypes (smooth, knobbed and linguiform B) and male of *Haemonchus longistipes* from *Camelus dromedarius* were analyzed by means of random amplified polymorphic DNA (RAPD) using 5 selected decamer primers. Results showed that primers OPA-04 and OPC-1 showed monomorphic bands for female morphotypes and male worms. In contrast, primers OPB-03, OPB-18, and OPD-05 yielded polymorphic bands among female morphotypes and male worms. A number of bands amplified by each primer varied from two to ten bands and ranged from 200 to 1750 bp. Band frequency per *H. longistipes* worm and per primer was estimated. The neighbor-joining clustering data based on pairwise distances among female morphotypes and male indicated that each sample had its own genetic peculiarity.

INTRODUCTION

Cameline haemonchosis caused by *H. longistipes* has an extremely wide geographical range particularly in tropical and subtropical areas on the occurrence and incidence (El Bihari & Kawasmeh, 1980; Hussein & Hussein, 1985). The parasite inhabits abomasum of camel, sucking blood from the mucosal vessels, leading to hemorrhagic anemia. Larvae and their piercing lancet develop just before the final molt, enabling them to obtain blood from blood vessels (Bahirathan *et al.,* 1996 a & b; Urquhart *et al.*, 1996 & 2006). Infection takes place through ingestion of these infective larvae from pasture. Browsing animals such as camels are highly susceptible to *Haemonchus* infection, particularly when they derive their entire food intake from pasture (Soulsby, 1982 & 1986).

Morphology and morphometrics of spicules of male (Jacquiet *et al*., 1997) and vulvar flap morphology of female worms that vary in both shape and size (Roberts *et al.*, 1954; Rose, 1966; Le Jambre & Whitlock, 1968) are reliable tools for species identification of *Haemonchus* spp. Tod (1965) stated that the vulvar morphology of *Haemonchus* worms is manifestation of some genetic factors necessary to establish and develop inside hosts. Therefore, it is important to develop genotyping methods that could be used for accurate

identification and also for an effective control because of the anthelmintic resistance problem of this nematode (Gasser *et al*., 2008). On one hand, techniques such as macro-restriction analysis of genomic DNA, followed by pulsed-field gel electrophoresis (REA-PFGE) (Cormican *et al.*, 1996), Amplified Fragment Length Polymorphism (AFLP) (Brost *et al.*, 2003), PCR melting profile (MP-PCR) (Krawczyk *et al.*, 2009) and other techniques based on sequencing e.g. multi-locus sequence typing (MLST) (Robles *et al.*, 2004) guarantee a high discriminatory power, but nonetheless are expensive, time-consuming and require specialized equipment and well-trained staff (Szweda *et al*., 2013). On the other hand, the RAPD technique has been successfully used for taxonomic delineation of numerous parasites such as sandfly species identification (Adamson *et al.*, 1993); identification of strains and species of schistosomes (Dias *et al.*, 1993); lungworms and *Toxocara canis* (Epe *et al*., 1995 & 1999); trichostrongylid nematodes (Humbert & Cabaret 1995); *Eimeria* spp. (Procunier *et al.*, 1993); *Echinococcus granulosus* (Siles-Lucas *et al.*, 1994) as well as *Trypanosoma cruzi* strains (Steindel *et al.*, 1993). The aim of current investigation was to evaluate the possibility of detecting genetic differences among female morphotypes and males of *H. longistipes* using a simple molecular method, RAPD technique.

MATERIALS AND METHODS

Parasites and DNA Extraction:

H. longistipes worms were previously collected (Elsayed *et al*., 2019) from Arabian camels *Camelus dromedarius* slaughtered at Elbasateen automated abattoir (Cairo, Egypt). A total genomic DNA was extracted from twenty worms (5 males and 15 females; 5 knobbed, 5 smooth, 5 linguiform B) using QIAamp DNA Mini Kit according to the manufacturer's protocol and stored at −20°C until used.

RAPD-PCR Reaction:

The reaction was carried out in 50.0 µl of a mixture containing 22.0 µl ddH_2O, 25.0 µl of iNtRON's Maxime PCR Premixe (2X PCR Master mix Solution i-Taq™ South Korea), 1.0 µl of the decamer primer (10 pmol) (Thermo Fisher Scientific, USA), and 2.0 µl of genomic DNA (10–20 ng template). The standard PCR reaction was carried out on a thermal cycler (STEC PC-815 Program Temp. Control System, Japan). Thermocycling conditions were 95°C for 2 min (initial denaturation), followed by 35 cycles of 94°C for 30 sec (denaturation), 40°C for one min (annealing), 72°C for 30 sec (extension) and one cycle of 72°C for 5 min (final extension). RAPD reactions were conducted using 5 random decamer primers (Table 1).

Table 1. The used primers in RAPD technique for differentiation of *H. longistipes* samples

	Primer code	Sequence
1	OPA-04	5`-AATCGGGCTG-3`
2	OPB-03	5`-CATCCCCCTG-3`
3	OPB-18	5`-CCACAGCAGT-3`
4	OPC-01	5`-TTCGAGCCAG-3`
5	OPD-05	5`-TGAGCGGACA-3`

Genetic Analysis of RAPD Data:

After PCR amplification, the banding pattern of randomly amplified DNA was visualized. Resulted bands were analyzed using gel-pro analyzer 3.1, and the size of bands was determined against 100-2000 bp range plus DNA ladder (Fisher scientific, Thermo fisher scientific, USA). For each primer in RAPD assay, a total number of scored bands and polymorphic/monomorphic bands were recorded. DNA fingerprint was scored for the presence (1) or absence (0) of similar-sized DNA bands in order to generate a binary data matrix of female

morphotypes and male worms. RAPD data binary matrix based on 29 characters/bands were subjected to the neighbor-joining clustering (Saitou & Nei, 1987), under distance criterion, using PAUP version 4.0a150 (Swofford, 2001), with a distance measure based on the mean character differences. The reliability of NJ tree was validated by the bootstrap analysis with 1000 replicates as all characters were included.

RESULTS

RAPD Banding Patterns:

Polymorphic profile of RAPD amplification of female morphotypes and male of *H. longistipes* using decamer primers was tested and yielded differences in banding patterns. A number of amplified bands by each primer varied from two to ten and ranged from 200 to 1750 bp in size (Figs. 1-5). Two of the five tested primers showed identical bands (Figs. 1 & 4). Primer OPA-04 (Fig. 1) showed two bands 200 and 1000 bp for all. Primer OPC-01 (Fig. 4) showed no different frequency among samples of six bands 250, 300, 400, 550, 600 and 750 bp. Whereas the other three tested primers showed an obvious polymorphism, each sample had distinct fragments and some other fragments common to one or more samples, allowing easy distinction among them (Figs. 2, 3 & 5). Primer OPB-03 amplified common bands 430, 500 and 550 bp, which were observed in all examined samples. The band at 300 bp was intraspecific for all samples, except knobbed morphotype, while bands at 700 bp and 1000 bp were intraspecific for smooth and LB morphotypes. Bands 250 bp and 350 bp were unique for male worm. Primer OPB-18 amplified common nine bands 200, 250, 300, 350, 450, 550, 700, 750 and 800 bp which were observed in all examined samples and the band 1750 bp was unique for male. Primer OPD-05 amplified the common band 370 bp and this was noticed at all examined samples. The band 480 bp was intraspecific for all samples, except LB, while band 550 bp was intraspecific for all examined samples, except male ones.

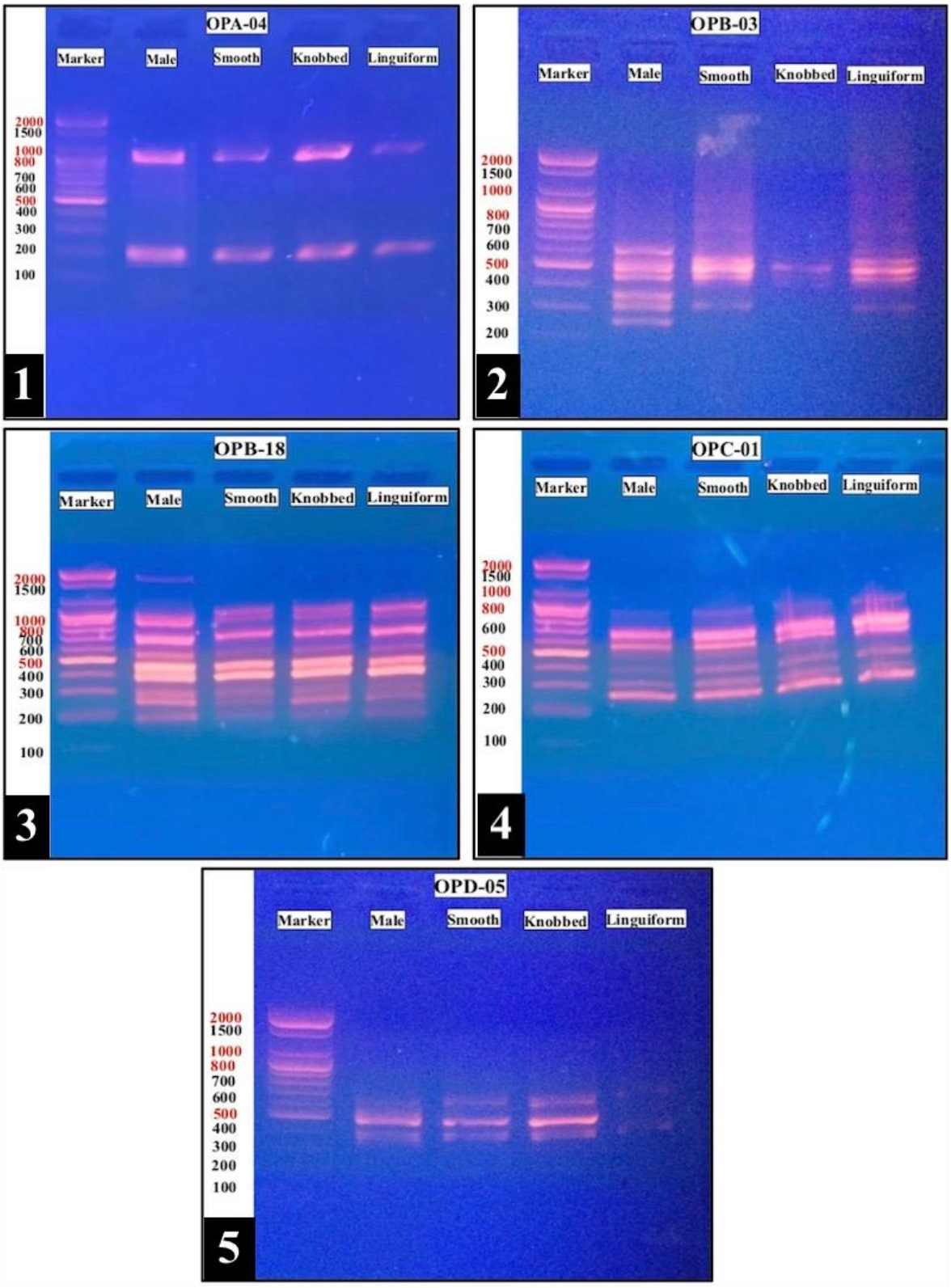
Characteristics of Fragments:

All characteristics of RAPD primers used for generating reliable fingerprints pattern and assessing polymorphism of female morphotypes and male *H. longistipes* were shown in Table (2). Band frequencies per sample were 0.8966, 0.8966, 0.7931, 0.8621 for male, smooth, knobbed and LB respectively, whereas, band frequencies per primers ranged from 0.0690 to 0.3448 (Figs. 6&7).

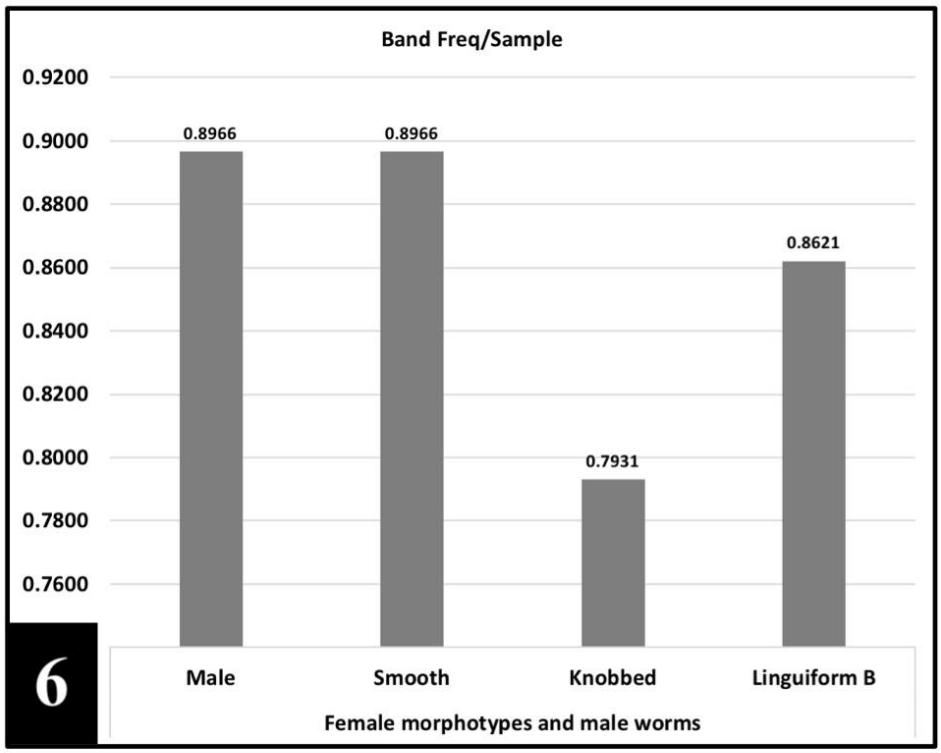
Table 2. Characteristics of fragments generated by the used primers

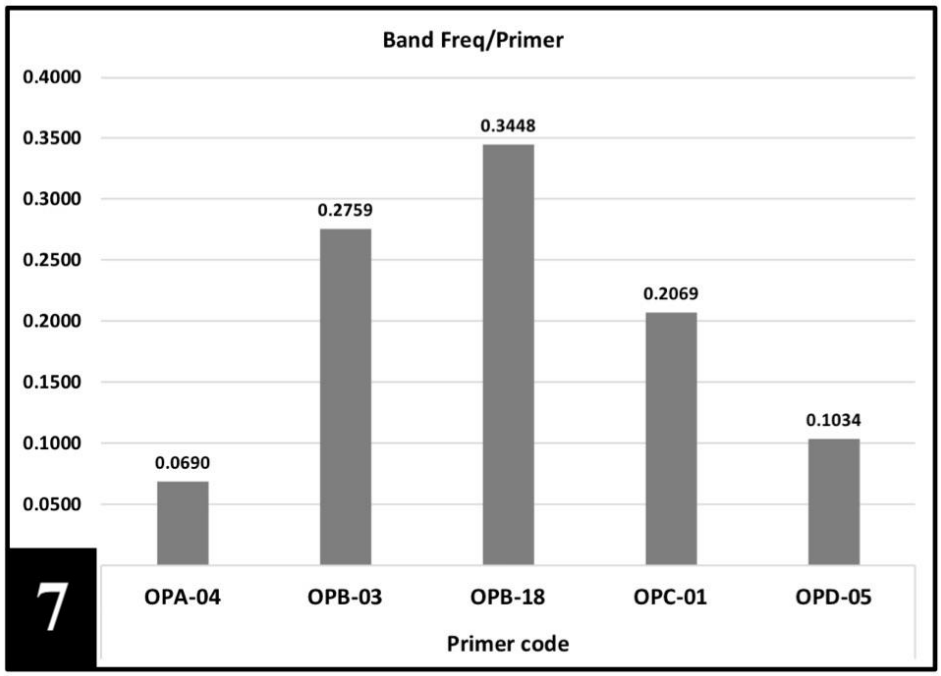
Primer code	NA bands/ Sample				TNA bands	Band Freq/ Primer	NU bands	NP bands	NM bands	% POL	RAF (bp)
	Male	Smooth	Knobbed	LB							
OPA-04	2	2	2	2	2	0.0690	0	0	2	0	200-1000
OPB-03	6	6	3	6	8	0.2759	2	3	3	28	250-1000
OPB-18	10	9	9	9	10	0.3448	1	0	9	10	200-1750
OPC-01	6	6	6	6	6	0.2069	0	0	6	0	250-750
OPD-05	2	3	3	2	3	0.1034	0	2	1	2	370-550
Total	26	26	23	25	29						
Band Freq/ Sample	0.8966	0.8966	0.7931	0.8621							

* NA bands/ Sample = Number of amplified bands per Sample
* TNA bands = Total number of amplified bands
* Band Freq/Primer = Band frequency per primer
* NU bands = Number of unique bands
* NP bands = Number of polymorphic bands
* NM bands = Number of monomorphic bands
* %POL = Polymorphic percentage = NP bands/TNA bands of the primerX100
* RAF bp = Range of amplified fragment in base pair
* Band Freq/Sample = TNA bands of one sample per TNA bands of all samples



Figs. 1-5. RAPD amplification products generated by primers 1- OPA-04, 2- OPB-03, 3- OPB-018, 4- OPC-01 and 5- OPD-05 resolved by electrophoresis on 2% agarose gel from marker or ladder 100 bp and *H. longistipes* worms (male, smooth, knobbed and Linguiform B).





Figs. 6&7. Band frequencies recorded: (6) for *H. longistipes* (female morphotypes and male) and (7) per different primers.

Phylogenetic Analysis of RAPD Data:

The neighbor-joining clustering of RAPD data based on pairwise distances among female morphotypes and male of *H. longistipes* worms (Table 3) produced a tree (Fig. 8) displaying the degree of relatedness among *H. longistipes* worms. As shown in Table (3), the mean values of *H. longistipes* worms ranged from 0.01818 to 0.12727, reflecting such relationships. Additionally, the bootstrap analysis with 1000 replicates produced a tree with branches reaching bootstrap support of 90%.

Table 3. Pairwise distance among *H. longistipes* samples. Below diagonal: total band differences. Above diagonal: mean band differences

	Male	Smooth	Knobbed	Linguiform B
Male	-	**0.10909**	**0.09091**	**0.12727**
Smooth	**6**	-	**0.05455**	**0.01818**
Knobbed	**5**	**3**	-	**0.07273**
Linguiform B	**7**	**1**	**4**	-

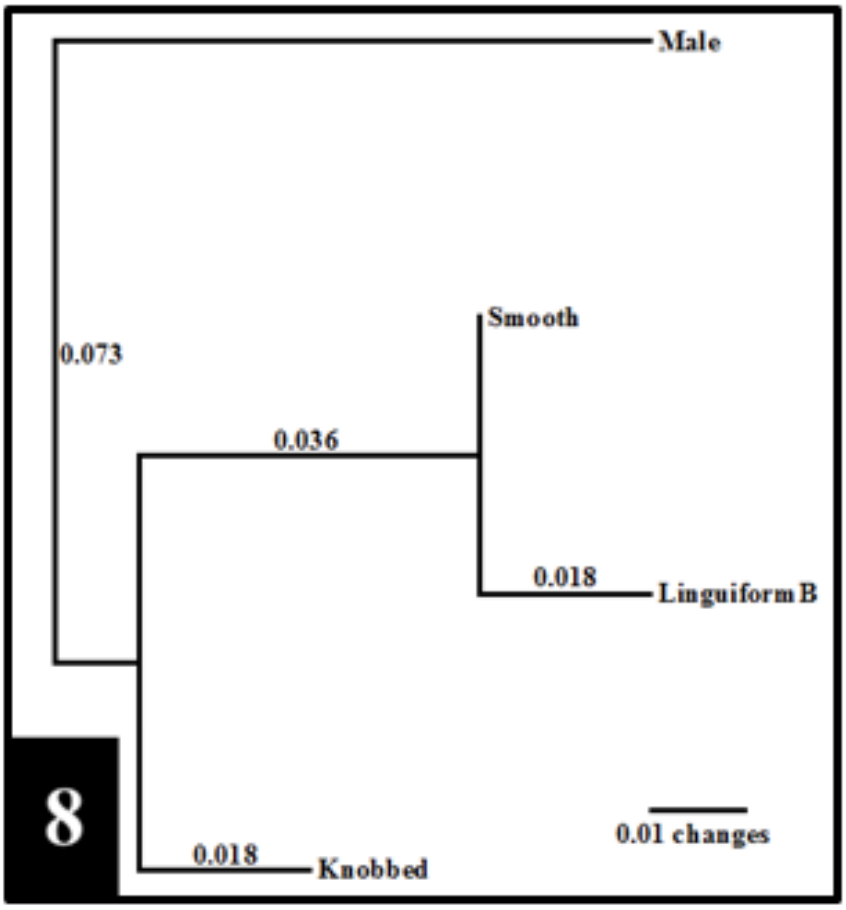


Fig. 8. The neighbor-joining (NJ) tree computed by PAUP for RAPD markers generated from distance measures based on the mean band differences

DISCUSSION

Parasites management and control of camel's husbandry represent a huge problem worldwide, where camels become susceptible to parasitic or viral infections during feed intake (Abdallah & Faye, 2013). *Haemonchus* spp. are major species that cause economic losses in ruminants' livestock worldwide (Lichtenfels *et al.*, 1994; Moudgil *et al.*, 2017).

In the present study, RAPD technique showed that the efficiency to differentiate among female morphotypes (smooth, knobbed and LB) and male of *H. longistipes* worms through producing a variable number of polymorphic fragments. RAPD fragments seemed to descend from the amplification of different sequences of DNA. Thus, it is likely that RAPD could prove to be valuable for the genetics differentiation of *Haemonchus* worms. RAPD technique was successfully applied to monitor genetic variations in other parasitic models, such as schistosomes (Barral *et al.*, 1993; Dias Neto

et al., 1993), plant-parasitic nematodes (Caswell-Chen *et al.*, 1992; Cenis 1993), and coccidia (McPherson & Gajadhar, 1993; Procunier *et al*., 1993). Furthermore, RAPD technique was also used to check the genetic variations in vertebrate animals, such as parrotfishes (EL-Mahdi, 2018). At the current study, the neighbor-joining clustering of RAPD data based on pairwise distances among *H. longistipes* (female morphotypes and male) produced a tree displaying the degree of relatedness. A similar finding in other *Haemonchus*, *H. contortus*, was previously reported (Humbert & Cabaret, 1995), where genetic variations among individuals of three morphotypes (smooth, knobbed and linguiform vulvar flap) were detected. Moreover, morphological polymorphism was genetically determined (Le Jambre, 1977; Le Jambre & Royal, 1977), and each morphotype thus had its own genetic peculiarity (Humbert & Cabaret, 1995).

The major role of molecular techniques in nematode taxonomy is the identification of sibling species, subspecies as well as other intraspecific groupings (Curran, 1990). Interestingly, Humbert & Cabaret (1995) stated that the advantages of RAPD technique versus other molecular techniques are very wide because of seemed fairly well-calibrated, as distances were arranged in descending order; among genera, species, morphological morphotypes of the same species for polymorphic ones, no nucleotide sequence information is necessary, and the required quantity of DNA is very small (1-10 ng). Furthermore, the last authors (Humbert & Cabaret, 1995) added that this method is very simple, rapid, reproducible and inexpensive compared to the other molecular techniques.

Since there were inadequate studies regarding the molecular characterization of *H. longistipes* worms infecting camels worldwide, the present study provided some molecular data of this species which may facilitate its further discrimination from other *Haemonchus* spp. However, extreme care must be taken during using RAPD technique for comparative studies; sterilization of buffers, tubes, and tips and use of the same standard conditions for DNA extraction, preparation and amplification are highly recommended (Liu & Berry, 1995).

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