# COMPARATIVE BIONOMICS OF *Haemonchus contortus* IN SHEEP AND GOATS BETWEEN MALAYSIA AND YEMEN AND ITS RESPECTIVE MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION

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**By**

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## LIST OF ABBREVIATION AND SYMBOLS

| | | |
|---|---|---|
| MAFF | = | Ministry of Agriculture, Fisheries and Food |
| BL | = | Body length |
| CP | = | Cervical papillae |
| RSL | = | Right spicule length |
| LSL | = | Left spicule length |
| THr | = | Tip-to-hook distance of the right spicule |
| THl | = | Tip-to-hook distance of the left spicule |
| GL | = | Gubernaculum length |
| CR | = | Cuticular ridges |
| µm | = | Micrometer |
| PCA | = | Principal Component Analysis |
| DFA | = | Discriminant Function Analysis |
| SPSS | = | Statistical Package for Social Science |
| µL | = | Microlitre |
| bp | = | Base pair |
| dNTP | = | Dinucleotide triphosphate |
| MgCl | = | Magnesium Chloride |
| Rpm | = | Revolutions per minute |
| ND4 | = | Nicotine amide dehydrogenase subunit-4 |
| ITS-2 | = | Internal Transcriped Spacer-2 |
| AMOVA | = | Analysis of Molecular Variance |

**BANDINGAN BIONOMIK *Haemonchus contortus* DALAM BEBIRI DAN KAMBING DI ANTARA MALAYSIA DAN YEMEN DAN CIRI MORFOLOGI DAN MOLECULARNYA MASING-MASING**

## ABSTRAK

Bionomik cacing nematod parasit, *Haemonchus contortus* telah dibandingkan di antara Malaysia dan Yemen. Ciri-ciri morfologi dan molekular isolat *H. contortus* dari kedua-dua negara tersebut telah dikaji. Sampel-sampel diperolehi dari bebiri dan kambing. Tempat kajian terdiri dari dua Negeri di Malaysia (Perak dan Kelantan) dan dua Governorat di Yemen (Sana'a dan Al-Hudaydah). Tempat kajian telah dipilih berdasarkan pengasingan geografi bagi setiap negeri. Rekod jumlah total bebiri dan kambing yang terjangkit oleh cacing *H. contortus* dewasa semasa 2007-2009 di Malaysia dan Yemen telah dikumpul dari laporan tahunan perkhidmatan pusat veterinar bagi setiap negeri. Prevalens yang tinggi bagi nematod ini dalam bebiri dan kambing dilaporkan dari Yemen. Data suhu, kelembapan, dan hujan dalam tahun-tahun tersebut telah dikumpul bagi menunjukkan keadaan iklim di kawasan kajian dan untuk menilai kemungkinan hubungan faktor-faktor iklim tersebut dengen peratusan haiwan terjangkit. Kajian rumah sembelihan juga dijalankan di Yemen dalam masa dari September 2008 hingga Februari 2009. Abomasum 68 bebiri dan 33 kambing diperolehi dari Sana'a manakala 59 abomasum bebiri dan 105 abomasum kambing diperolehi dari Al-hudaydah. Data yang perolehi menunujukkan prevalens bulanan caing yang tinggi dalam bebiri dan kambing tanpa perbezaan signifikans diantara spesies perumah dalam prevalens dan bebanan cacing. Di Malaysia, amat susah untuk mendapatkan cacing dewasa dari abomasum bebiri dan kambing. Dari

itu, sampel cacing untuk kajian ini diperolehi dari Bahagian Perkhidmatan Veterinar Perak dan Kelantan.

Perbezaan taksonomi di antara populasi *H. contortus* dalam bebiri dan kambing dari Malaysia dan Yemen dikaji menggunakan teknik morfolgi dan molecular. Analisis statistik menunjukkan terdapat beberapa perbezaan signifikans di antara isolat *H. contortus* dari bebiri dan kambing di keempat-empat kawasan kajian. Juga, isolat *H. contortus* dari kedua-dua negeri menunjukkan perbezaan signifikans dalam kesemua ciri morfologi yang dikaji, kecuali kepanjangan gubernukulum cacing jantan. Ini juga disokong oleh analisis komponen utama dan analisis plot fungsi diskriminant, di mana populasi *H. contortus* bebiri dan kambing dari Malaysia dan Yemen terasing kepada dua kumpulan yang berbeza dan mempunyai sedikit pertindihan di antaranya. Ini disebabkan terutamanya oleh perpisahan geografi di antara kedua-dua negara and juga oleh pergerakan haiwan di aatara kedua-dua negara yang tidak terwujud.

Subunit-4 (ND4) dihidrogenase nikotin amid mitokondria telah diguna untuk meniliti dan membanding genetik populasi *H. contortus* bebiri dan kambing dari Malaysia dan Yemen. Keputusan menunjukkan jumlah haplotip yang tinggi (113 dari 120 individu yang disekuens) dan komposisi bes adalah kaya-AT (82.1%). Analisis filogenetik menunjukkan pola geografi yang jelas di antara isolat dari Malaysia dan Yemen, di mana populasi *H. contortus* membentuk kluster yang berasingan, tetapi tanpa pengasingan perumah. Pada amnya, kajian ini menunjukkan struktur populasi yang rendah di dalam satu negeri berbanding dengan struktur genetik yang tinggi pada skala goegrafi yang lebar. Ini telah dijelaskan dengan aliran gen yang tinggi dalam setiap negeri disebabkan kemungkinan pergerakan perumah manakala terdapat rintangan geografi kepada aliran gen. Keputusan AMOVA menunjukkan kebanyakan

varians genetik adalah di dalam populasi *H. contortus*, diwakili oleh 72.19% varians genetik total.

Tambahan, spaser-2 (ITS-2) transkrib dalaman adalah tanda nuklear yang telah digunakan untuk mengesahkan pengenalan spesies *H. contortus* dalam bebiri dan kambing dari Malaysia dan Yemen. Penjajaran sekuens pasangan bes 216 fragmen ITS-2 dalam bebiri dan kambing dari Malaysia dan Yemen dan sekuens GenBank yang tersedia menunjukkan pengenalan yang tinggi (98-100%) mengesahkan status taksonomi populasi tersebut sebagai satu spesies. Ini telah disokong lagi dengan membandingkan sekuens ITS-2 dalam kajian ini dengan spesies yang paling terdekat hubungannya iaitu *H. placei* dari GenBank, di mana tiga perbezaan nukelotid tetap dilihat di antara kedua-dua spesies (peralihan di antara purin G/A dalam tiga posisi berbeza: 9, 190 dan 204).

**COMPARATIVE BIONOMICS OF *Haemonchus contortus* IN SHEEP AND GOATS BETWEEN MALAYSIA AND YEMEN AND ITS RESPECTIVE MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION**

## ABSTRACT

The bionomics of the parasitic nematode worm, *Haemonchous contortus* were compared between Malaysia and Yemen. The morphological and molecular characterizations of *H. contortus* isolates from the two countries were investigated. Samples were obtained from sheep and goats. Study sites comprised of two States in Malaysia (Perak and Kelantan) and two Governorates in Yemen (Sana'a and Al-Hudaydah). The sites were chosen based on their geographical isolation within each country. Records of total number of infected sheep and goats by *H. contortus* adult worms during 2007-2009 in Malaysia and Yemen were collected from the annual reports of veterinary service centers in each country. High prevalence rates of this nematode in both sheep and goats were reported in Yemen. Data for temperature, humidity and rainfall during those years were collected to show the climatic conditions in the study areas and to assess a possible relation between these climatic parameters and the percentages of infected animals. Abattoir investigations were also carried out in Yemen during the period from September 2008 till February 2009. The abomasa of 68 sheep and 33 goats were collected in Sana'a while 59 abomasa of sheep and 105 abomasa of goats were collected in Al-Hudaydah. The data obtained showed high monthly prevalences of this worm in sheep and goats with no significant difference between host species in prevalences and total worm burdens. In

Malaysia, it was difficult to find adult worms in the abomasa of sheep and goats. Thus samples utilized in this study were collected from the Department of Veterinary Services in Perak and Kelantan.

Taxonomic differences of *H. contortus* populations of sheep and goats from Malaysia and Yemen were investigated using morphological and molecular techniques. Statistical analysis revealed that there were several significant differences between *H. contortus* isolates from sheep and goats in the four study areas. Also, *H. contortus* isolates from the two countries showed significant differences in all morphological characters investigated except gubernacula lengths of male worms. This was also supported by the principal component analysis and discriminant function analysis plots, where *H. contortus* populations of sheep and goats from Malaysia and Yemen were separated into two distinctive groups with very slight overlapping between them. This was attributed mainly to the geographical isolation between the two countries and thus movement of animals between countries is totally absent .

The mitochondrial nicotine amide dyhydrogenase subunit-4 (ND4) was used to explore and compare the population genetics of *H. contortus* of sheep and goats from Malaysia and Yemen. The results showed high numbers of haplotypes (113 among 120 individuals sequenced) and the base composition of ND4 was very AT-rich (82.1%). The phylogenetic analysis showed a clear geographical pattern between isolates from Malaysia and Yemen, where populations of *H. contortus* formed distinct clusters, but with no host isolation. In general, this study showed low population structure within the same country in comparison with higher genetic structuring at a wider geographical scale. This was explained by the high gene flow within the same country due to the possibility of host movement while between the

two countries, there was a strong geographical barrier to gene flow. The AMOVA results showed that the majority of genetic variance was within *H. contortus* populations, represented by 72.19% of the total genetic variance.

Furthermore, the internal transcribed spacer-2 (ITS-2) which is a nuclear marker was used to confirm the species identity of *H. contortus* in sheep and goats from Malaysia and Yemen. Sequence alignment of the 216 base pair of the ITS-2 fragment in sheep and goats from Malaysia and Yemen and available GenBank sequences revealed high identity (98-100%) confirming the taxonomic status of these populations as a single species. This was further supported by comparing the ITS-2 sequences of the present study with the most closely related species of *H. placei* from GenBank, where three fixed nucleotides differences were observed between the two species (transitions between purines G/A in three different positions: 9, 190 and 204).

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Classification and morphology of *Haemonchus contortus*

### 2.1.1 Classification of *Haemonchus contortus*

The genus *Haemonchus* belongs to the superfamily: Trichostrongyloidea (Soulsby, 1982). This superfamily includes the following genera: *Dictyocaulus, Haemonchus, Cooperia, Ostertagia, Nematodirus, Trichostrongylus, Hyostrongylus, Marshallagia and Meecistocirrus* (Urquhart *et al*., 1996). Structure-wise, members of these genera are small, often hair-like worms in the bursate group which are found in the gastro-intestinal tracts of animals and birds except for the lung worm *Dictyocaulus.* The buccal capsule is absent or weakly developed. Species identification is based on the bursa and two spicules of male worms. Their life cycle is direct and the third stage larva ($L_3$) is the infective stage (MAFF, 1986; Urquhart *et al*., 1996).

Ever since the genus *Haemonchus* was erected by Cobb (1898) with a single species *H. contortus* described by Rudolphi (1803), as many as 13 species have been listed by Skrjabin *et al*. (1954) and 12 by Yamaguti (1961). However, in some investigations where the *Haemonchus* species could not be identified, the investigators always presume that the species involved was *H. contortus* (Rahman, 1989). Previous observations have indicated that the so-called ovine and bovine strains of *H. contortus* are distinct species (Roberts *et al*., 1954; Bremner, 1955). The name *H. contortus* was retained for the species commonly found in sheep and goats, and the name *H. placei* described by Place (1893) was referred to by Ransom (1911)

as the species commonly found in cattle. However, other reports have also documented this species in goats (Fabiyi, 1970; Tongson *et al*., 1981). In a revision of the genus *Haemonchus*, nine species including *H. contortus* were identified based on the morphlogical characters of the male worms and *H. placei* was synonymized with *H. contortus* (Gibbons, 1979). However, Lichtenfels *et al*. (1986) described differences between *H. contortus* and *H. placei* in the percentage of cuticular ridges (synlophe) covering the body and observed that it was possible to differentiate between these two species if at least 10 worms were examined. Thus, considering their veterinary and economic importance (Gibbons, 1979), species of the genus *Haemonchus* should be accurately identified.

Taxonomy, Scientific and common name of *H. contortus* (Urquhart *et al*., 1996):

Kingdom: Animalia

Phylum: Nemathelminthes

Class: Nematoda

Subclass: Strongylida

Order: Strongylina

Superfamily: Trichostrongyloidea

Family: Trichostrongylidae

Sub family: *Haemonchinae*

Genus /: *Haemonchus*

Species: *contortus* (Barber's pole worm)

Gibbs and Herd (1986) stated that the large stomach worms *H. contortus, H. placei* and *H. similis* are among the most pathogenic nematodes of sheep, goats and

cattle worldwide, causing significant production losses due to morbidity, mortality and cost of treatment. Furthermore, *H. contortus* also infects other domestic and wild ruminants (Gibbs and Herd, 1986). The genus *Haemonchus* is normally an abomasal parasite, but in heavier infections, it may be found in the duodenum (Fabiyi, 1970).

### 2.1.2 Taxonomic status and basic characters of *H. contortus* and closely related species

The adult *H. contortus* worms are easily identified because of their specific location in the abomasum (Fig. 2.1) and their large size which is easily visible to the naked eye. Both sexes have a small buccal cavity containing one tooth and the cervical papillae is present (MAFF, 1986; Urquhart *et al*., 1996). Male worms are reddish in color, the lateral lobes of bursa are long, the dorsal lobe is positioned asymmetrically and barbed spicules are present (MAFF, 1986). The white uteri and ovaries of female worm winding around the red blood-filled intestine give it a twisted or barber's pole appearance (Fig. 2.2); hence *H. contortus* is popularly known as the barber's pole worm (MAFF, 1986).

Several characters have been traditionally used to study the morphology of *H. contortus* male worms such as, body length (Fig. 2.3), cervical papillae length (Fig. 2.4), sipcule length (Fig. 2.4 and 2.5), gubernaculum length (Fig. 2.5), barb length (distance from tip to hook) (Fig. 2.5) and number of cuticular ridges (synlophe) (Fig. 2.6) (Gibbons, 1979; Lichtenfels *et al*., 1986; Lichtenfels *et al*., 1994; Rahman and Hamid, 2007). For female worms, body length (Fig. 2.3), cervical papillae length, number of cuticular ridges and vulva flap morphology (Fig. 2.4) were used by several authors (Gibbons, 1979; Lichtenfels *et al*., 1994; Rahman and Hamid, 2007).

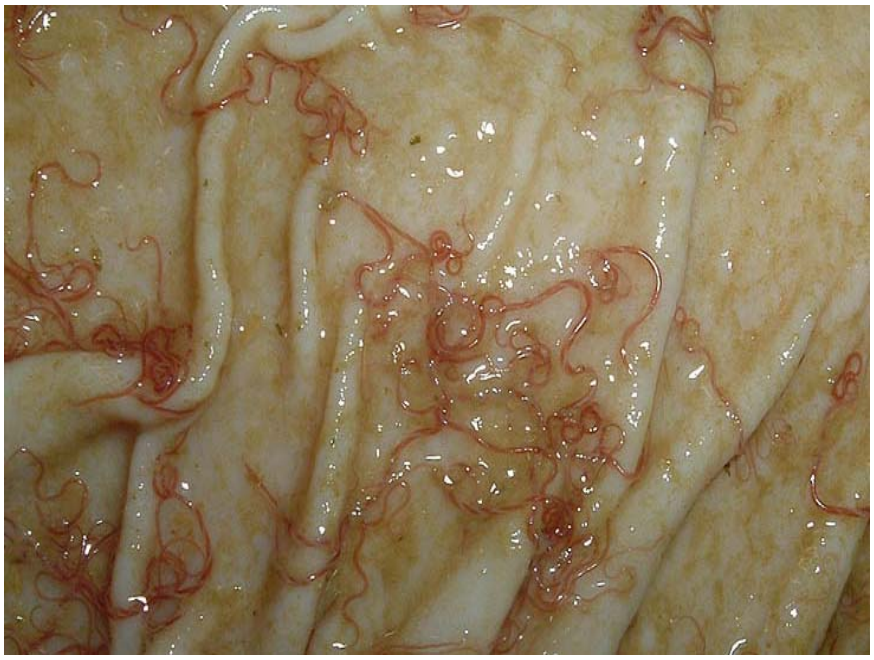

Fig. 2.1. Adults of *H. contortus* in abomasum. (Kaplan, 2004)

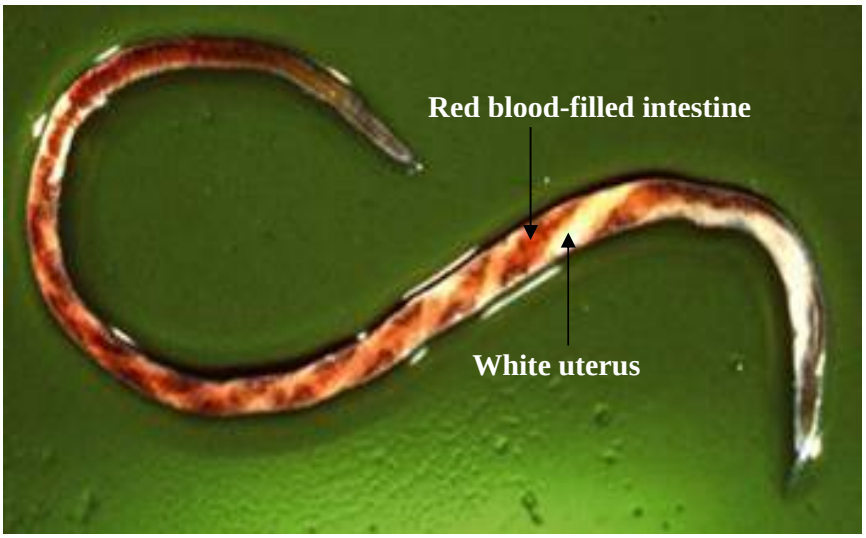


Fig. 2.2. Barber's pole appearance of *H. contortus*. (Miller & Zajac, 2009)

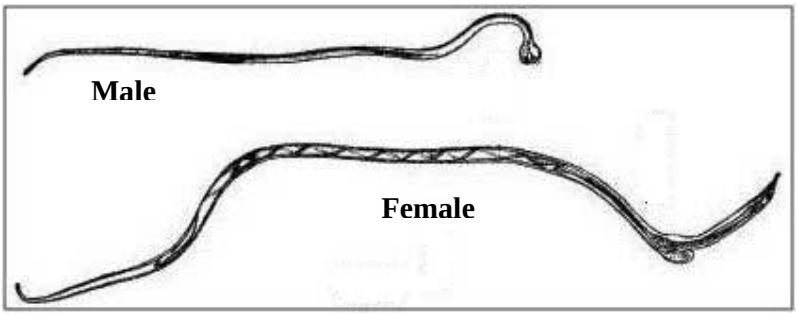


Fig. 2.3. Adults of *H. contortus*. (Kirchhoff & Katahdins, 1998)

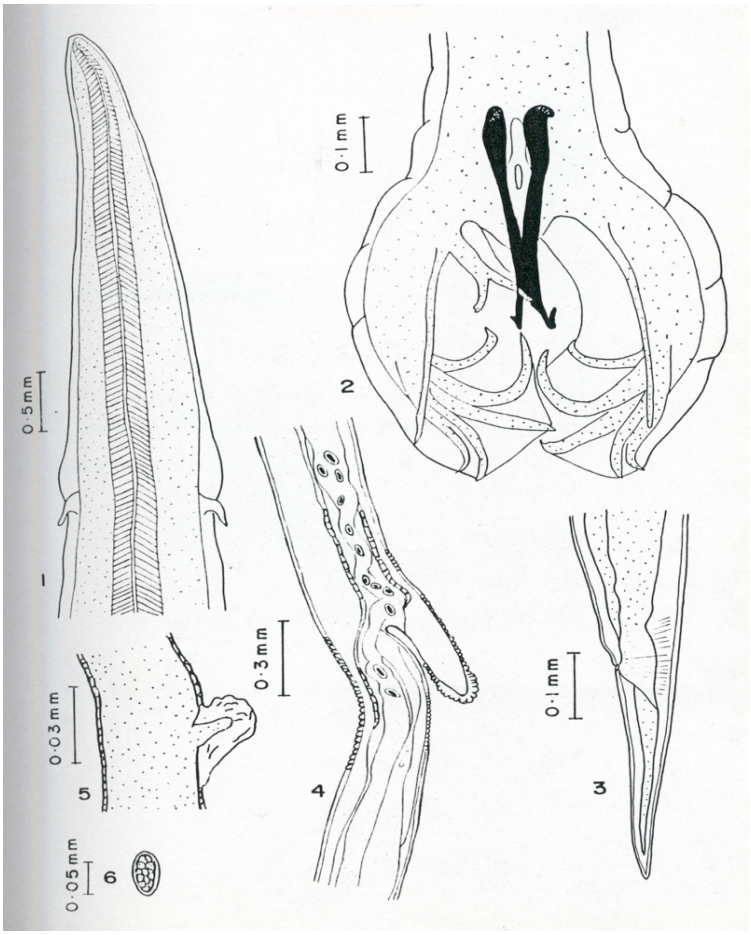


Fig. 2.4. Morphological characters of *H. contortus*. **1**. Anterior end, showing a pair of cervical papillae. **2**. Posterior end of male, showing spicules and arrangement of rays in bursa. **3**. Posterior region of female, showing tail. **4**. Vulva region of linguiform female. **5**. Vulva region of knobbed female. **6**. Egg. (http://www.nehu.ac.in)

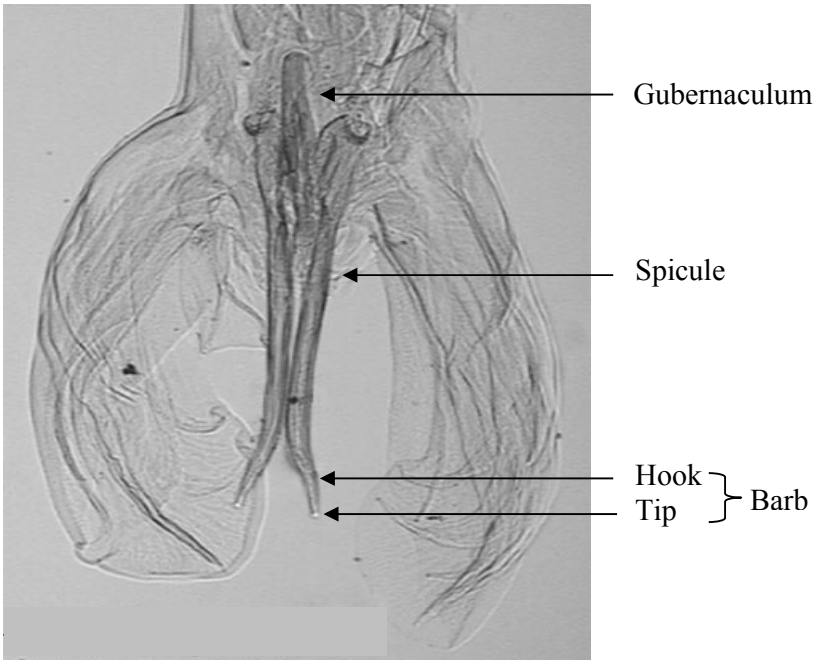


Fig. 2.5. Male bursa of *H. contortus* showing spicules, gubernaculum and barbs. (Eslami *et al*., 2007)

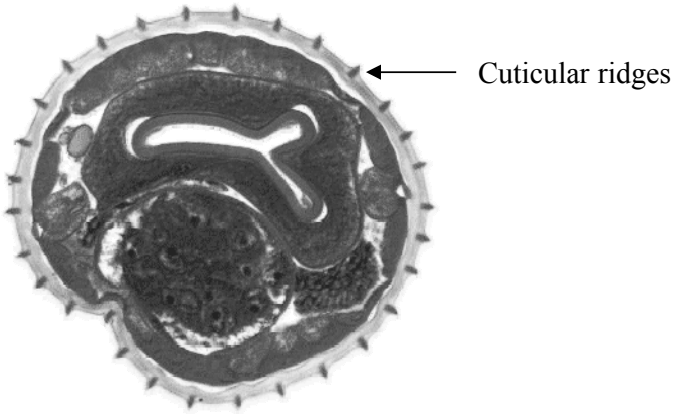


Fig. 2.6. Cross section of *Haemonchus* showing cuticular ridges (synlophe). (http://www.anri.barc.usda.gov)

Previous reports found that male characters of the genus *Haemonchus*, especially the spicules, gubernaculum and dorsal ray were the main characters to differentiate between species. Gibbons (1979) identified nine species of *Haemonchus* based on only the morphological characteristics of spicule, gubernaculum and dorsal ray in the male worms. Spicule length was also found to be a discriminant character to distinguish between most populations of *H. contortus* and *H. placei* (Lichtenfels *et al*., 1988; Jacquiet *et al.*, 1997).

Lichtenfels *et al*. (1994) found the spicule length in *H. contortus* to be shorter than *H. placei* which ranged between 383-475 μm and 438-511 μm respectively. On the other hand, the right/left barb lengths were found to be shorter in *H. contortus* than *H. placei* and *H. similis* (Lichtenfels *et al*., 1994; Jacquiet *et al.*, 1997; Achi *et al*., 2003). Lichtenfels *et al*. (1994) found the right/left barb lengths in *H. contortus*, *H. placei* and *H. similis* ranged between 37-48/19-24 μm, 45-60/22-32 μm and 62-81/41-65 μm respectively. Jacquiet *et al.* (1997) found the mean of the right/left barb lengths in *H. contortus* from sheep was 41.1/20.9 μm, from goat was 43.8/22.3 μm and *H. placei* from zebu was 50.7/28.2 μm respectively.

Durette-Desset (1983) identified the synlophe as the system of cuticular ridges distributed longitudinally on the surface of most nematodes in the Trichostrongyloidea. This character had been used by Durette-Desset (1983) to classify genera and higher taxa of the Trichostrongyloidea. The use of the number and pattern of ridges on the anterior half of the nematode had been found to be one of the most sensitive and useful characters for identifying species of stomach worms of ruminants (Gibbons, 1979, Measures and Anderson, 1983, Lichtenfels and Hoberg, 1993; Lichtenfels *et al*., 1994). Lichtenfels *et al*. (1994) found the synlophe of *H. contortus*, *H. placei* and *H. similis* is bilaterally and dorsoventrally symmetrical,

resulting in the left and right, and also dorsal and ventral halves being mirror images of each other. They also found that *H. placei* and *H. similis* bore 17 dorsal and 17 ventral (total 34) longitudinal cuticular ridges in the posterior half of esophagus while *H. contortus* bore 15 dorsal and 15 ventral ridges (total 30) (Lichtenfels *et al*., 1994). Gibbons (1979) also found that the number of longitudinal cuticular ridges in *H. contortus* to range between 22-30.

The vulva is an opening covered by cuticle in the hypodermis from which eggs are laid at the ventral exterior of adult females of nematode (Carta *et al*., 2009). Several variations in the shape of vulva were reported in earlier studies (Roberts *et al*., 1954; Rao and Ghafoor, 1968) while Chitwood (1957) reduced these variations in the vulva flap of *Haemonchus* females to three basic morphological types with a linguiform flap, with knoblike projection (Fig. 2.4) and with no projection (smooth). On the basis of the proportion of these three shapes, *H. contortus* had been described to the following subspecies and varieties: *H. contortus contortus* (Das and Whitlock, 1960), *H. contortus cayugensis* (Das and Whitlock, 1960), *H. contortus* var. *uktalensis* (Das and Whitlock, 1960), *H. contortus bangalorensis* (Rao and Rahman, 1967), *H. contortus hispanicus* (Martínez Gómez, 1968) and *H. contortus kentuckiensis* (Sukhapesna, 1974). For example, if the proportion of linguiform type is > 80, knobbed < 10 and smooth < 10 then the species is described as *H. contortus contortus* subspecies (Das and Whitlock, 1960).

Daskalov (1971) noted that on the other hand, the variation in vulva flap morphology of female *Haemonchus* worms was found to be of no taxonomic importance, attributable to geographical and ecological factors. However, Daskalov (1972) later considered that the development of the various vulva types may be related to age and reproductive activity of the worms. Veglia (1915) suggested that

the vulva flap morphs are accessary organs in the copulatory act. Michel (1967) studied the effect of the host's resistance on the development of the vulva flaps of *Ostertagia ostertagi* and found that the absence of the flaps was not due to the increase in the number of worms with a certain morphological type, but due to the acquired resistance of the host. In the same manner, Michel *et al*. (1972) considered the development of the flaps in *O. ostertagi* to be influenced by the enviromental conditions, in which the worms develop, which was, in turn, affected by the acquired resistance of the host. Considering these reports, Gibbons (1979) rejected the establishment of *H. contortus* subspecies and varieties based on the vulva flap morphology and considered these subspecies and varieties as synonyms of *H. contortus*.

### 2.1.3 Qualititative and quantitative morphology of adult *H. contortus* worms

Gibbons (1979) found the measurements of *H. contortus* male worm as follows: body 8.21-19.00 mm long; 0.183-0.337 mm wide just anterior to the bursa; head diameter 0.019-0.033 mm; cervical papillae 0.259-0.475 mm from the anterior end; longitudinal cuticular ridges 22-30; spicule 0.381-0.550 mm long; each spicule with a single barb 0.014-0.038 mm and 0.029-0.065 mm from the distal end respectively; gubernaculum 0.179-0.451 mm long. In females, Gibbons (1979) found the measurements as follows: body 12.34-30.5 mm long; 0.216-0.664 mm wide in vulva region; head diameter 0.025-0.035 mm; cervical papillae 0.216-0.480 mm from the anterior end; longitudinal cuticular ridges 22-30; tail 0.310-0.719 mm long; vulva flap, linguiform process and knobs present or absent.

Lichtenfels *et al*. (1994) studied the morphology of 23 male and 22 female worms of *H. contortus* in North America and found the morphometrics of male

worms as follows: body 11-17 mm long; cervical papillae 271-462 μm from the anterior end; spicule 383-475 μm long; spicule barb right/left 37-48 and 19-24 μm from the tip to hook respectively; gubernaculum 195-255 μm long. For females, the morphometrics were as follows: body 14.8-27.2 mm long; cervical papillae 243-484 μm from the anterior end; tail 251-530 μm long; longitudinal cuticular ridges 22-30.

In a previous study in Malaysia, Rahman and Hamid (2007) reported that measurements of male worms in goats and sheep respectively, were as follows: body lengths 11.8 and 12.3 mm, cervical papillae 319.2 and 335.0 μm from the anterior end, left spicule lengths 446.0 and 483.2 μm, right spicule lengths 453.2 and 489.3 μm, gubernaculum lengths 234.0 and 231.0 μm, cuticular ridges 26 and 24 at 4 mm from anterior end, cuticular ridges 20 and 22 at 8 mm from anterior end. In females the measurements in goats and sheep respectively were as follows: body lengths 18.8 and 17.8 mm, cervical papillae 319.7 and 333.2 μm from the anterior end. They (Rahman and Hamid, 2007) also showed the presence of linguiform, knobbed and smooth vulva flap morphs and reiterated that their distribution in the two hosts was similar.

## 2.2 Life cycle of *H. contortus* and climatic conditions

### 2.2.1 Life cycle

The life cycle of *H.* contortus was first described in detail by Veglia (1915). It consists of two phases, a free-living phase on pasture, and a parasitic phase inside the host (Fig. 2.7). Eggs are passed in faeces of animals, and hatch to first stage larvae ($L_1$), which then develop to the second larval stage ($L_2$), then to third stage infective larvae ($L_3$). This development may take a period of five days or may be delayed for weeks or months under cool conditions (Urquhart *et a*l., 1996). Animals become

infected during feeding on pasture contaminated with $L_3$ and then the $L_3$ travels to the predilection site in the abomasum, where the larva moult twice to $L_4$ and late $L_4$, then develop into the sexually mature adult stage. The late $L_4$ developed piercing lancet before moulting to adult stage to enable them to obtain blood from the mucosal vessels. The prepatent period is approximately 2-3 weeks in sheep (Urquhart *et a*l., 1996).

It is estimated that 2,000 *Haemonchus* worms suck a minimum of 29 ml of blood per day from the host (Martin and Clunies Ross, 1934). The minimum withdrawal of blood from the host by these worms is itself, sufficient proof to explain the severe anaemia, progressive debility and other pathogenic effects (Rahman, 1989). The establishment and development of *H. contortus* in the goat is similar to that described in sheep (Al-Quaisy *et al*., 1987; Rahman and Collins, 1990). Twelve goats were inoculated with 40,000 third-stage *H. contortus* larvae, then two were slaughtered and abomasa were examined on days 4, 7, 11, 14, 18 and 21 after inoculation. In day 4 after inoculation, all $L_3$ had developed to the $L_4$ stage and at day 11 the majority of the worms were adults (Rahman and Collins, 1990).

### 2.2.2 The optimal conditions for development of *H. contortus*

Climatological factors such as temperature, humidity and rainfall have a major impact on the development of *H. contortus* from eggs to $L_3$ in the free living pastoral phase. In general, warm and humid climate is favourable for *H. contortus* development. Previous studies have investigated the optimum environmental conditions that help in the development and survival of *H. contortus*. Rossanigo and Gruner (1995) found that the optimal condition for development from egg to $L_3$ is 28°C with humidity > 70% while Gordon (1948) found a mean monthly temperature

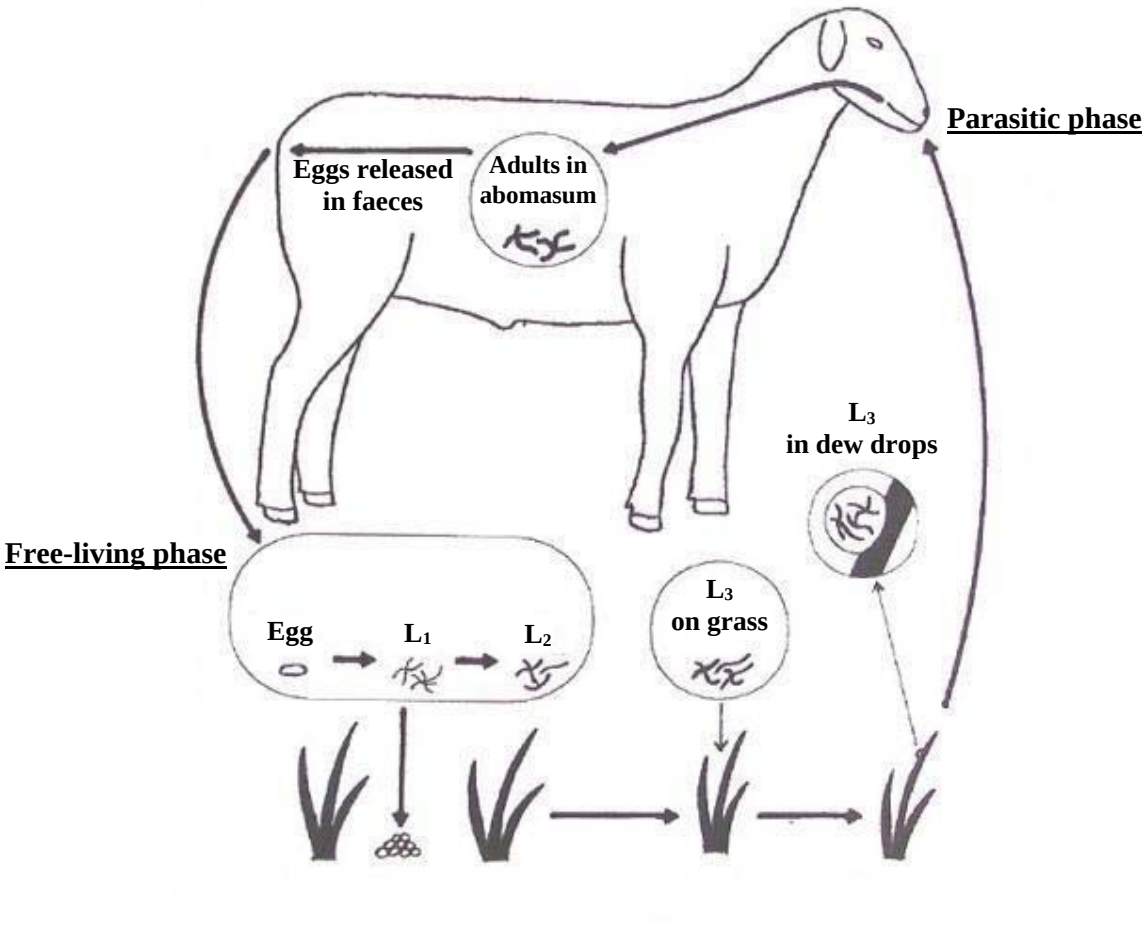


Fig. 2.7. Life cycle of *Haemonchus contortus*.
(Machen *et al.*, 1993)

of 18°C and a minimum monthly rainfall of 50 mm as the lower environmental limits for infection by this worm.

It was suggested that changes in climatic conditions can cause inhibition for larval stages of *H. contortus* but they become active again with the onset of the rainy season and continue their development (Muller, 1968; Connan, 1971; Vercruysse, 1985). This phenomenon which is known as hypobiosis, is considered as an adaptation to survive the low temperature in winter (Blitz and Gibbs, 1972; Waller and Thomas, 1975) and high temperature in summer (Eysker and Kooyman, 1993). Some authors suggested that inhibition in *H. contortus* is an obligatory genetic strategy to avoid unfavourable conditions (Waller and Thomas, 1975; Waller *et al*., 2004).

Malaysia has a tropical rainy climate with two distinct seasons, dry and rainy seasons. The dry season occurs during the south-west monsoon between May and September. The north-east monsoon brings the rainy season to the country during mid-November until March. Throughout the year the average temperature is constantly high (26°C) and the average humidity is also high (between 70% - 90%) with rainfall exceeding 2500 mm per year (http://www.met.gov.my; Chandrawathani, 2004). The humid and tropical environment of Malaysia was found to be favourable for the development of various species of trichostrongylid nematodes (Rahman, 1995; Cheah and Rajamanicam, 1997).

According to Yemen Meteorological Service, the climate in Yemen is dependant on altitudes of the regions (highlands, coasts, deserts) (http://www.yms.gov.ye). Generally, rainfall is limited, with variations based on elevations where the rainfalls in highlands is more than in the coastal and desert areas. The highlands enjoy a temperate, rainy summer with an average high

temperature of 21°C and a cool, moderately dry winter with temperatures occasionally dipping below 0°C. The highest mountainous areas of Yemen receive an average annual rainfall of 750 mm. The climate of the coastal regions is tropical where the average maximum temperatures is 28°C in winter and 37°C in summer, and the humidity ranges from 50 to 70%. The annual average of rainfall in this area is not more than 100 mm. The eastern part of Yemen (desert area) is arid and hot, and the humidity ranges from 35% in June to 64 % in January. In general, Yemen is dry in the east and humid in the west. A study on the seasonal changes of abomasal nematodes in Saudi Arabia which has almost similar environment as Yemen showed a high prevalence of nematodes in sheep and goats especially in summer (El-Azazy, 1995).

### 2.3 Prevalences of *H. contortus* in sheep and goats and climatic conditions

Infection by *H. contortus* depends on factors that affect the free-living stages, such as temperature, humidity and rainfall. Thus, seasonal variations and prevalences of infection are due to the changes in these factors. The development of free-living stages of *H. contortus* occurs at relatively high temperature, high humidity and severity of outbreaks of disease is largely depending on the rainfall in any particular area (Urquhart *et al*., 1996). In the tropics and subtropics, *H. contortus* has a major economic importance to the small ruminants industry (Allonby and Urquhart, 1975; Schillhorn van Veen, 1978).

Pneumonic pasteurellosis and endoparasitism are the two main diseases that cause high morbidity and mortality in both sheep and goats in Malaysia (Sani and Chandrawathani, 1996; Dorny *et al*., 1995). A number of epidemiological and

bionomic studies had been carried out in Malaysia on the prevalence of nematode parasites in sheep and goats and the possible relation with the climatic factors. Daud *et al*. (1991) monitored 46 goats and found 32% of death were due to worm infection mainly *H. contortus* and *Trichostrongylus colubriformis*, while 30% of deaths were due to pasteurellosis pneumonia. Daud *et al*. (1991) also found that the goats that died due to pneumonia were also infected by *H. contortus* and *Trichostrongylus*. Goats experimentally infected by larvae of *H. contortus* were immunosuppressed which allowed the development of the experimentally induced pneumonic pasteurellosis (Zamri-Saad *et al*., 1994).

Shanta (1982) reported *Haemonchus* spp., *Trichostrongylus* spp., *Oesophagostomum* spp. as the predominant species in Malaysian goats. Dorny *et al*. (1995) studied the trichostrongyle infections in sheep and goats on traditional farms in West Malaysia and found *H. contortus* and *Trichostrongylus* spp. were the most important strongyles in these ruminants. They also found that the faecal egg counts of sheep and goats were not influenced by the small seasonal climatic variations (Dorny *et al*., 1995). A similar finding was observed by other authors who recovered larvae of trichostrongylids on pasture throughout the year (Ikeme *et al*., 1986; Cheah and Rajamanicam, 1997). Cheah and Rajamanicam (1997) found that monthly populations of *H. contortus* in sheep farms in Malaysia fluctuated slightly except in May and August during which relatively more worms were found in tracer animals. However, these authors (Cheah and Rajamanicam, 1997) concluded that the humid tropical environment of Malaysia was favourable for the development of pre-parasitic stages of this worm on pasture throughout the year.

Seasonal variations of trichostrongylid nematodes were investigated in Penang and the faecal egg counts of these worms in goats were found to be

correlated to the total rainfall (Rahman, 1992; 1995). The humid tropical environment of Pulau Aman and Penang Island were found to be favourable for the survival and development of various species of trichostrongylid nematodes, namely *H. contortus*, *Trichostrongylus* spp., *Oesophagostomum* spp. and *Cooperia* spp (Rahman and Adanan 1991; 1992). In general, *H. contortus* was observed to be the predominant species where comprised 43% of the nematode population in Pulau Aman and 45.7% in Penang Island (Rahman and Adanan, 1991; 1992).

In Yemen Arab Republic (recently Yemen Republic), there was only one survey carried out on the prevalence of ovine internal parasites from October 1978 to November 1979 (Hunter and Heath, 1984), where five flocks of ewes and lambs were selected from deferent areas in Yemen, the results showed high prevalence rates of *H. contortus* in four of these flocks (between 83.2% and 93.4%). Their findings indicated the prevalence of haemonchosis all the year round in the Tihammah coastal plain while haemonchosis and trichostrongylosis prevailed in the periparturient periods in the Southern Uplands and Western Foothills of the country.

A number of studies in the Arabic Peninsular and Africa also showed a high prevalence of this worm. In Saudi Arabia, *H. contortus* was found to be the most common abomasal nematode in sheep and goats where 19.8% of the examined animals were infected by this worm. The worm counts and infection rates in Saudi Arabia were lowest in the winter as reported by El-Azazy (1995). In Nigeria, higher worm burdens in sheep and goats were evident during the rainy rather than the dry season where *H. contortus* survived in the host during the unfavorable dry season (November to March) as adults (Fakae, 1990a). In a study by Fakae (1990a), monthly incidences of *H. contortus* in West African dwarf sheep and goats in the Nigerian derived savanna was high, ranging between (77.8 to 100%) and female

worms burden per animal was higher than that of males. Also, in a subsequent study carried out by the same author, *H. contortus* was found to be the most prevalent helminth in small ruminants of eastern Nigeria during August 1987 to July 1988 (87.1%), followed by *Trichostrongylus* spp. (63.8%) (Fakae, 1990b).

In the same context, Jacquiet *et al*. (1992) examined sheep and goats in Mauritania and found *H. contortus* was the most prevalent worm in the small ruminants. They also noticed that young small ruminants became infected during the rainy season. Jacquiet *et al*. (1995) also studied the seasonal patterns of helminth infections in sheep and goats in the dry areas of southern Mauritania. They found that adults survived longer in the dry season and suggested the ability of these worms to survive from one rainy season to the next. *Haemonchus contortus* was also the most prevalent species in this study, where the mean number of worms was 291 in sheep and 332 in goats (Jacquiet *et al*., 1995).

Similar findings were observed in eastern Ethiopia where mean adult nematode counts in sheep and goats followed the rainfall patterns (Sissay *et al*., 2007). The highest worm burden in this area was during the rainy seasons and lowest was in dry seasons. *Haemonchus contortus* was the most common nematode species in this study, represented by more than 50% of the total worm burdens recorded in the sheep and goats at any time of the study period (Sissay *et al*., 2007). In Ethiopia, Thomas *et al*. (2007) examined 180 abomasa of sheep and 132 of goats. Their results showed that *H. contortus* was the most prevalent nematode in the two hosts represented by 81.1% in sheep and 76.8% in goats of which the highest worms count was recorded during the rainy season. In Sudan, the prevalence of some gastrointestinal parasites of sheep and goats based on postmortem examinations showed that the prevalence rates of *H. contortus* was 53.4% in sheep and 26% in

goats with a significant positive correlation between rainfall, humidity and worm burdens (Almalaik *et al*., 2008).

### 2.4 Molecular characterization of *Haemonchus contortus*

One of the most active areas of molecular biology is the identification of a species using molecular techniques (Turelli *et al*., 2001). Morphological, physiological, biochemical or behavioral characters are not enough to define a species, and can lead to wrong identification of different organisms that are not related to each other (Kunz, 2002). For example, *H. contortus* and *H. placei* are two of the most important veterinary nematodes (Hoberg *et al*., 2004) and it has been suggested for a long time that they were a single species because there were only few morphological differences between them (Gibbons, 1979; Urquhart *et al*., 1996). However, Stevenson *et al*. (1995) differentiated these two species using molecular methods and concluded that these were two different species.

Molecular genetics on parasitic nematodes (Trichostrongyloidea) is of major significance in veterinary parasitology and had led to some progress in nematode investigations especially in studying parasite systematics, population genetics, drug resistance and vaccine development. *Haemonchus contortus* is considered as one of the major pathogens of small ruminants with worldwide distribution (Gibbs and Herd, 1986; O'Connor *et al*., 2006). The economic importance of *H. contortus* stimulated a lot of research interest in various approaches, including the utilization of different molecular methods for genetic characterization of this worm, such as mitochondrial DNA (Blouin *et al*., 1995; Cerutti *et al*., 2010) and ribosomal DNA

(Stevenson *et al*., 1995; Troell *et al*., 2003) where the accurate diagnosis in turn can lead to effective control for this worm.

### 2.4.1 Mitochondrial DNA

Mitochodria are subcellular organelles responsible for cellular respiration and energy production in many eukaryotic organisms. Mitochondria are believed to originate from free living eubacteria undergoing endosymbiosis with eukaryote hosts (Kita and Takamiya, 2002). Within these organelles is the mitochondrial genome which is usually a circular genome (~13-20 kb in size) and is separate to, but cooperates with the nuclear genome (Boore, 1999; Le *et al*., 2002; Hu *et al*., 2004). Mitochondria contain 12-13 protein genes (*cox*1 – *cox*3, *nad*1-*nad*6 or ND1-ND6, *nad*4L or ND4L, *cob*, *atp*6 and/or *atp*8) encoding for enzymes involved in oxidative phosphorylation, two ribosomal RNA genes and 22 transfer RNA genes (Hu and Gasser, 2006). The complete mitochondrial genome of *H. contortus* was sequenced by Jex *et al*. (2008) (Fig. 2.8).

Mitochondrial DNA (mtDNA) is an ideal molecular marker for studies of evolutionary, population structure and history of animals (Avise, 2004). Silva and Russo (2000) found that 54% of DNA-based studies used mtDNA especially in population genetics study of animals. This is because unlike nuclear DNA, mtDNA is generally maternally inherited without recombination and has a relatively rapid base substitution rate (Avise *et al*., 1987; William *et al*., 2005). Thus, it is sensitive for detecting population structure as the nucleotide and haplotype diversities allow exploration of some parameters such as subdivision and gene flow (Wang *et al*., 2000).

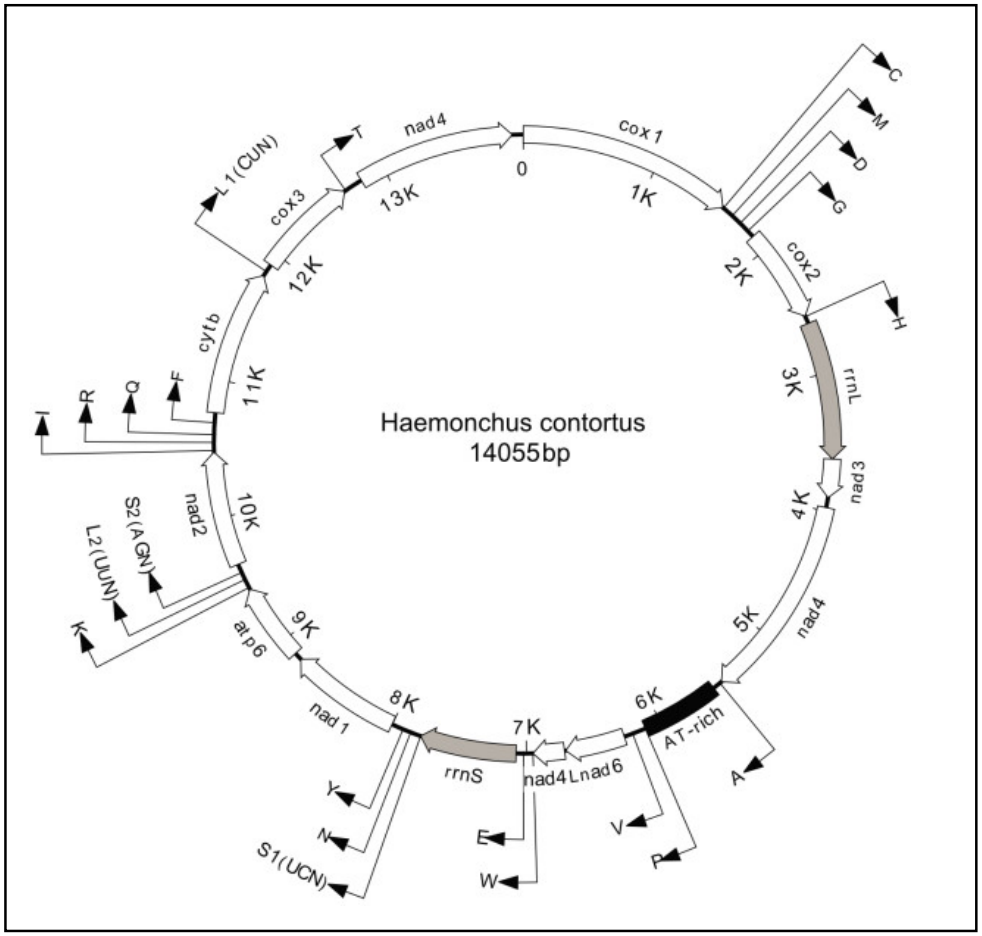


Fig. 2.8 Complete circular mitochondrial genome map for *H. contortus*. All 12 protein coding genes and the large and small mitochondrial ribosomal subunit genes are indicated in italics. All *trn* genes are indicated by their corresponding single letter amino acid code. Distinctions between the two Leucine *trn* genes and between the two Serine *trn* genes are indicated by the corresponding anticodon for each *trn* (in brackets). Direction of transcription is indicated by arrow. Diagram is presented to scale (Jex *et al*., 2008).

Generally, the high rate of substitution in mtDNA makes it useful to resolve differences between closely related individuals (Blouin, 2002) and thus useful for the study of population genetics of trichostrogylids nematodes. Sometimes it is difficult to obtain sufficient parasitic materials and these minute organisms cannot be cultivated *in vitro*. Thus an addition advantage of mtDNA is that as most cells contain hundred or thousand copies of mtDNA (Randi, 2000), which make it suitable with small sample size.

The mtDNA of nematodes are usually smaller in size (~13.6 to 14.3 kb) than other metazoan groups (Hu *et al*., 2004). Nematode mtDNA is highly AT rich, between 75 and 80% (Okimoto *et al*., 1992; Anderson *et al*., 1998; Blouin *et al*., 1998) and mtDNA was found to evolve very quickly in nematodes (Blouin *et al*., 1995; Blouin *et al*., 1998; Denver *et al*., 2000). The nicotine amide dyhydrogenase subunit-4 (*nad*4 or ND4) and cytochrome oxidase 1 (*cox*1) genes of the mtDNA have been widely used for population genetic studies of different parasitic nematodes as reviewed by (Höglund *et al*., 2006).

Previous population genetic studies on *H. contortus* (Blouin *et al*., 1997; Troell *et al*., 2006a) and other trichostrogylids (Braisher *et al*., 2004; Leignel *et al*., 2002) using ND4 gene revealed a high number of haplotypes with high within population diversity which is considered as a general phenomenon of these groups of nematodes (Blouin *et al*., 1995). For example, 42 haplotypes were obtained from 50 individuals of *H. contortus* sequenced for ND4 gene in United States (Blouin *et al*., 1997) and 94 haplotypes were observed among 150 *H. contortus* worms from different continents (Troell *et al*., 2006a). Similarly, 77 haplotypes were identified within the 85 individuals of *Teladorsagia* sequenced for ND4 gene in New Zeland (Braisher *et al*., 2004). The geographical isolation especially between different

continents or countries was also found to result in high genetic differentiation in this gene between trichostrogylid populations, because of the strong barriers to host movement and thus low gene flow between these populations (Leignel and Humbert, 2001; Troell *et al*., 2006a).

The study of haplotypes is an important method in DNA analysis (Vishwanathan *et al*., 2003), which refers to a haploid combination of genes at more than one locus (Ridley, 2004). Normally, organisms that share the same haplotype, have the same mtDNA sequence or the same composition of a restriction fragment pattern (Barnes and Gray, 2003). Thus the analysis of haplotype frequency and lineage distance disequilibrium can be calculated if different haplotypes appear in a population (Vishwanathan *et al*., 2003).

Relationships among haplotypes and their evolutionary distance can be examined by reconstruction of a phylogenetic tree. There are two methods to reconstruct a phylogenetic tree, the distance based method (Unweighted Pair Group Method with Arithmetic Mean - UPGMA and Neighbor Joining) and character based method (Maximun Parsimony, Maximum Likelihood and Bayesian analysis). The UPGMA, Neighbor Joining and Maximun Parsimony methods are widely used in the study of phylogenetic relationships among trichostrogylid nematodes (Blouin *et al*., 1995; Braisher *et al*., 2004; Cerutti *et al*., 2010).

The aim of population genetic studies is to describe the pattern of diversity within and between populations based on variety of markers such as mtDNA (Excoffier *et al*., 2005). Intraspecific genetic structure among populations, estimation of population differentiation and estimation of distance between haplotypes can be revealed through Analysis of Molecular Variancs (AMOVA) (Excoffier *et al*., 1992). This analysis is also widely utilised in population genetic studies of nematodes to

show differences within and between populations (Leignel and Humbert, 2001; Troell *et al*., 2006a; Cerutti *et al*., 2010).

### 2.4.2 Ribosomal DNA

In eukaryotic organisms the ribosomal DNA (rDNA) is a multigene family which consists of nuclear copies arranged in tandem arrays. The location of the internal transcribed spacers (ITS) in these arrays is between the small subunit (SSU) and the large subunit (LSU) rRNA genes, and is composed of two regions (ITS-1 and ITS-2) separated by the 5.8S RNA gene (Fig. 2.9) (Schlötterer *et al*., 1994). Several studies of molecular phylogenetics of strongylid nematodes have applied comparisons of nuclear rDNA sequence, especially from the ITS sequences (Gasser *et al*., 2004; Gasser and Newton, 2000) to investigate evolutionary relationships. The amplification of ITS-1 and ITS-2 can be done using universal primers which target the conserved genes flanking the spacers (Heise *et al*., 1999).

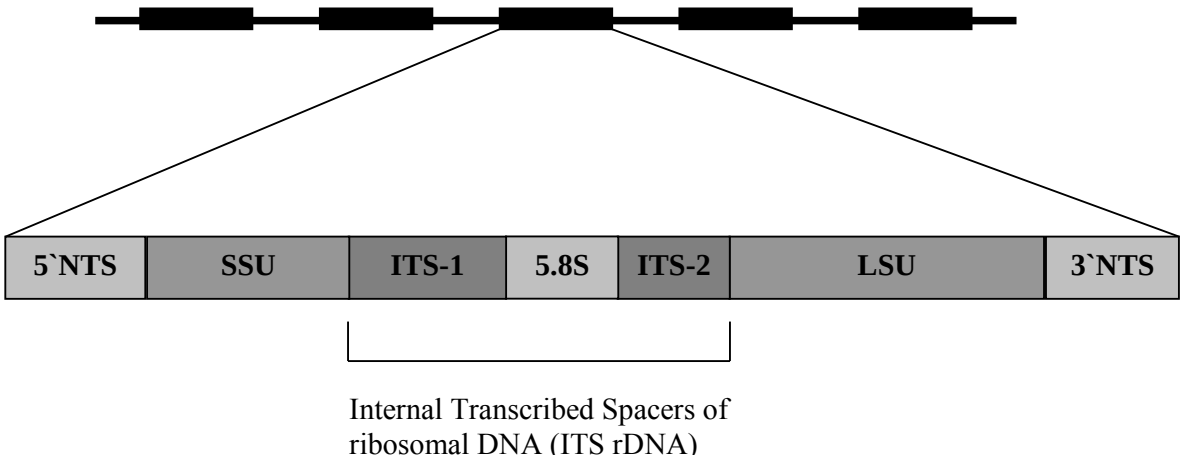


Fig. 2.9. Arrangement of ribosomal DNA. The tandem repeats are drawn as black boxes. They are composed of the non-transcribed spacers (NTS) and the transcription unit including the small subunit (SSU), the large subunit (LSU), and the 5.8S genes and two internal transcribed spacers (ITS1 and ITS2) (figure not to scale) (Troell, 2006).

Ribosomal DNA genes exhibit patterns of concerted evolution resulting in limited sequence divergence within a species (Anderson *et al*., 1998), thus rDNA can provide useful markers for parasite identification. Ribosomal DNA markers have been mainly used as diagnostic tools for studying parasite systematics (Gasser and Newton, 2000). Identification of strongylid nematodes based on morphological characters and their pathological effects are often insufficiently informative, especially for identification of several developmental stages (Lichtenfels *et al*., 1997; Andrews and Chilton, 1999). Thus, the accurate identification of these worms though molecular markers can help in the diagnosis and control of diseases caused by these nematodes.

The sequences of the internal transcribed spacers (ITS-1 and ITS-2) of rDNA provide useful genetic markers for identification of strongylid nematodes to species level because they evolve at an almost neutral rate of nucleotide substitution, usually not internally repetitive (Schlötterer *et al*. 1994) and the intraspecific variation in the sequences is usually low (<1%) compared with higher levels (>1.5%) of interspecific variation (Gasser and Newton, 2000).

Ribosomal DNA sequence had been used in previous studies to resolve taxonomic problems (Chilton *et al.*, 1995; Stevenson *et al*. 1995; Hung *et al*., 1996). One of these was the species status of *H. contortus* and *H. placei* (Bremner, 1954; Lichtenfels *et al*., 1994). Stevenson *et al*. (1995) sequenced 231bp of ITS-2 from multiple isolates of each species from different geographical areas. The result of this study showed no intraspecific variation in the sequence while three consistent nucleotide differences (1.3%) were detected between the two species. This result confirmed that *H. placei* is a separate species to *H. contortus* and supported the results of previous genetic (Zarlenga *et al*., 1994), biological (Le Jambre, 1979) and morphological (Lichtenfels *et al*., 1994) studies.

In another study, the presumed *Teladorsagia circumcincta*, *T. davtiani* and *T. trifurcate* were found to represent a single species (*T. circumcincta*) since no unequivocal nucleotide differences were detected in the ITS-2 sequences of these species (Steveson *et al*., 1996) and this was in agreement with previous studies using other techniques (Andrews and Beveridge, 1990; Lichtenfels and Hoberg, 1993). In Malaysia, the ITS-2 sequence of *H. contortus* was used to determine the phylogenetic relationships among *H. contortus* from sheep and goat (Rahman *et al*., 2007). They found that the ITS-2 sequences of *H. contortus* from sheep were 100% similar or only differed either by one or two nucleotides while in goat they differed by up to four nucleotides, however sequences from both types of hosts belonged to the same species.