

GENETIC CHARACTERIZATION OF *HAEMONCHUS CONTORTUS* IN GOATS (*CAPRA HIRCUS*) AND SHEEP (*OVIS ARIES*) IN PENANG, MALAYSIA

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Abstrak: *Haemonchus contortus* merupakan cacing nematod dalam kambing dan bebiri yang mengakibatkan kerugian besar terhadap ekonomi. Kajian terdahulu menunjukkan perbezaan yang ketara pada ciri morfologi cacing itu, dipercayai terdedah serta dipengaruhi oleh faktor persekitaran. Kajian ini dilakukan untuk mencirikan perbezaan genetik antara *H. contortus* yang dijumpai di dalam kambing dan bebiri di Pulau Pinang, Malaysia. Ruang Tertranskripsi Dalam kedua (ITS-2) daripada DNA ribosom (rDNA) nucleus *H. contortus* telah diampikasi dan diujuk DNANYa untuk tujuan perbandingan genetik. Sejumlah enam ITS-2 haplotaip telah dikenal pasti. Jujukan ITS-2 sepanjang 231 pasang bes telah dijejer dan digunakan untuk analisis. Analisis berteraskan jarak genetik dengan menggunakan kaedah Neighbor-Joining dan parsimoni telah digunakan untuk menjana pepohon filogenetik untuk mendapatkan pertalian genetik. Rajah pepohon yang dijana daripada kedua-dua kaedah menunjukkan topologi yang sama dengan membahagikan *H. contortus* dari Pulau Pinang kepada dua kumpulan yang agak berbeza. Keputusan awal ini memberikan gambaran tentang hubungan genetik *H. contortus* dari perumah berbeza serta status jujukan DNA yang digunakan dalam kajian.

Abstract: *Haemonchus contortus* is a sheep and goat parasitic nematode that causes severe economic losses. Previous studies have indicated a high degree of morphological traits difference which are believed to be subjected to environmental influences. This study was carried out to analyse the genetic difference among *H. contortus* found in sheep and goats in Penang, Malaysia. The second Internal Transcribed Spacer region (ITS-2) of the nuclear ribosomal DNA (rDNA) of *H. contortus* was amplified and sequenced. A total of six ITS-2 haplotypes were identified. The ITS-2 sequences that are 231 base pair in length was aligned and used for analysis. Distance based analysis using Neighbor-Joining method and parsimony analysis were used to construct phylogenetic trees to elucidate genetic relationships. Both set of trees showed the same topology separating Penang's *H. contortus* into two quite distinct groups. The preliminary results provide an insight on genetic relationships of *H. contortus* from different hosts and the status of the DNA sequence used in the analysis.

Keywords: *Haemonchus contortus*, Goats, Sheep, ITS-2, Genetic Characterization

INTRODUCTION

The large stomach worm, *Haemonchus contortus*, commonly known as the "barber's pole worm" is found in the abomasum of goats and sheep. It sucks blood and causes significant production losses due to morbidity and mortality. With rising problems of this worm, sheep and goats in Malaysia are heavily infected (Dzulzila & Mohd Izranuddin 2004). In Malaysia, the goat is the most

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important ruminant reared by small holder farmers of rural areas. Unhealthy goats will result in losses to these farmers either due to disease infection, poor growth and production or even death of the animals.

These infections are commonly treated with broad-spectrum anthelmintics such as benzimidazoles (BZ), levamisole (LEV) or ivermectin (IVM). However, because of their genetic heterogeneity, nematode populations are able to respond to the selective pressure of chemotherapeutics (Roos *et al.* 2004). As a result, resistance for anthelmintics has been reported all over the world (Roos *et al.* 2001, Prichard 2001; Himmelstjerna *et al.* 2002; Roos *et al.* 2004). Besides that, *H. contortus* from both hosts have shown differences in the characteristics of cuticular ridges (synlophe) (Hoekstra *et al.* 2000; Zarlenga *et al.* 2001, Roos *et al.* 2004).

Traditional procedures for diagnosis involve cultivation of larvae and microscopic differentiation of third-stage larvae (L3) based on morphology (Himmelstjerna *et al.* 2002). However, this approach is tedious and not always reliable due to minor morphological characteristics. 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). rDNA genes and their associated spacer regions provide suitable targets for developing diagnostic probes or makers for species identification (Hoste *et al.* 1995).

Much research had been carried out on the differences in the Internal Transcribed Spacer (ITS) among nematodes (Zarlenga *et al.* 2001; Gasser & Newton 2000) but until today, systematic studies on morphologically and a proficient DNA fingerprinting techniques to determine the differences of *H. contortus* among goats and sheep especially in Malaysia or throughout the world is lacking.

rDNA sequences at the ITS-2 regions of *H. contortus* were used in this study using specific PCR primers. The aim of the present study was to determine phylogenetic relationships among *H. contortus* derived from goats and sheep using molecular technique.

MATERIALS AND METHOD

Contortus from abomasums of goats and sheep were obtained from the Georgetown City Abattoir, Penang. Abomasums were taken directly to the laboratory and individual adult worms were collected.

DNA isolation and amplification

The techniques used follow those described by Taggart *et al.* (1992) with slight modifications. Genomic DNA was extracted from 15 to 20 pooled worms. The ITS-2 plus primer flanking sequence was amplified by PCR from genomic DNA (10–20 ng template). PCR was performed in a 25 µl reaction volume for 30 cycles at 95°C for 2 minutes (heating), 1 minute (denaturation), 55°C for 1 minute (annealing) and 72°C for 1 minute (extension) using conserved oligonucleotide primers ITS-2, NC1 (forward: 5'-ACGTCTGGTTCAGGGTTGTT-3') and NC2(reverse:5-TTAGTTTCTTTTCCTCC-GCT-3'). Negative (without DNA)

controls were included in each set of reactions. PCR products were detected on 0.8% TBE (0.45 M Tris Borate, 0.01M EDTA, pH 8) agarose gels (Sambrook, Fritsch & Maniatis 1989), and purified using columns (Qiagen, Diagen). Both strands of the ITS-2 were purified using NC1 and NC2 in separate reactions and the sequences were aligned using the computer program AssemblyLIGN (Kodak).

Cloning and DNA sequencing

The ITS-2 PCR products were ligated directly into the plasmid vector pGEM-T (Promega) and transformed into JM109 competent cells (Promega). Transformants (white colonies) were selected from LB plates containing IPTG (0.1mM), X-Gal (20 µl 50 mg/ml) and ampicillin (100 µgm⁻¹) and were confirmed to contain ITS-2 inserts by PCR using primers NC1 and NC2. Three individual clones of *H. contortus* from sheep (HcOvMlys2a1, HcOvMlys2b and HcOvMlys2d) and seven individual clones from goats (HcChMlys, HcChMlys4a HcChMlys4a2, HcChMlys4a3, HcChMlys4b2, HcChMlys4b1 and HcChMlys4b3) were selected for DNA sequencing. Cycle sequencing used in this research was a modified protocol described in Perkin Elmer's ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit manual. For each cycle sequencing reaction, the following reagents were added into a 0.2 ml microfuge tube: 30–90 ng of cleaned PCR product; 4 µl Terminator Ready Reaction Mix (Perkin Elmer); 5 ng of oligonucleotide primer; and sterile distilled water to a final volume of 10 µl. Tubes with cycle sequencing reaction were subjected to a cycle sequencing thermal profile consisting of 26 cycles of 10 denaturation at 94°C, 5 annealing at 50°C and 4 min extension at 60°C. The cycle sequencing product was cleaned by ethanol precipitation to remove any excess of dye terminator, and subsequently sent to a private company to run on an automated DNA sequencer.

Data Analysis

The sequences obtained were aligned by eye. Two ITS-2 sequences, one from *H. contortus* in sheep from the USA and the other of *H. contortus* in sheep from Australia we obtained from gene bank and used for comparative purposes. Phylogenetic analysis was performed both using distance and parsimony methods as implemented in the software PAUP (Swofford 2002). Under the distance method, Neighbor-Joining procedure was carried out to construct a phylogenetic tree. In addition bootstrap analysis of 100 replicates was performed to find supports for tree branches. Using parsimony method, heuristic searches were conducted with RANDOM addition sequence strategy. Descriptive statistics that reflect the amount of phylogenetic signal in the parsimony analysis were computed as consistency index (CI) and rescaled consistency index (RC). Sets of equally parsimoniously trees were summarized using 50% majority rule consensus tree. Bootstrap analysis was also conducted using 100 replicates of heuristic searches with RANDOM addition.

RESULTS

PCR amplification of *H. contortus* yielded a fragment of 321 bp in size which included 231 bp of the ITS-2 plus 90 bp of flanking regions (20 bases from the 3' end of the 5.8S gene and 70 bases from the 5' end of the 28S gene). The identity and source of *H. contortus* sequenced are provided in Table 1. There were no insertion or deletion in all sequences analysed. The ITS-2 sequence of *H. contortus* from goats have approximately 52% G+C content while from sheep is 61%. Only 231 bp of the ITS-2 sequences were used for phylogenetic studies.

Table 1: Identity and sources of *H. contortus* used for ITS-2 DNA sequencing analysis.

| Label name | Host | Origin | Clone identity |
|-------------|-------|----------------------|----------------|
| HcOvAust | Sheep | Australia (Genebank) | - |
| HcOvUSA | Sheep | USA (Genebank) | - |
| HcOvMlys2a1 | Sheep | Penang, Malaysia | 2a1 |
| HcOvMlys2b | Sheep | Penang, Malaysia | 2b |
| HcOvMlys2d | Sheep | Penang, Malaysia | 2d |
| HcChMlys | Goat | Penang, Malaysia | 1 |
| HcChMlys4a | Goat | Penang, Malaysia | 4a |
| HcChMlys4a2 | Goat | Penang, Malaysia | 4a2 |
| HcChMlys4a3 | Goat | Penang, Malaysia | 4a3 |
| HcChMlys4b2 | Goat | Penang, Malaysia | 4b2 |
| HcChMlys4b1 | Goat | Penang, Malaysia | 4b1 |
| HcChMlys4b3 | Goat | Penang, Malaysia | 4b3 |

Table 2: Data matrix of the ITS-2 sequences of *H. contortus* used in the study.

| <u>Taxon</u> | 111111111122222222223333333333444444444455555555556 123456789012345678901234567890123456789012345678901234567890 |
|--------------------|---|
| <u>HcOvAust</u> | AACCATATACTACAATGTGGCTAATTTCAACATTGTTTGTCAAATGGCATTGTCTTTTA |
| <u>HcOvUSA</u> | AACCATATACTACAATGTGGCTAATTTCAACATTGTTTGTCAAATGGCATTGTCTTTTA |
| <u>HcOvMlys2b</u> | AACCATATACTACAATGTGGCTAATTTCAACATTGTTTGTCAAATGGCATTGTCTTTTA |
| <u>HcOvMlys2a1</u> | AACCATATACTACAATGTGGCTAATTTCAACATTGTTTGTCAAATGGCATTGTCTTTTA |
| <u>HcOvMlys2d</u> | AACAATATACTACAATGTGGCTAATTTCAACATTGTTTGTCAAATGGCATTGTCTTTTA |
| <u>HcChMlys</u> | AACCATATACTACAATGAGGGCAATTTCAACATTGTTTGTCAAATGGCATTGTCTTTAA |
| <u>HcChMlys4a</u> | AACCATATACTACAATGAGGGCAATTTCAACATTGTTTGTCAAATGGCATTGTCTTTTA |
| <u>HcChMlys4a2</u> | AACCATATACTACAATGTGGCTAATTTCAACATTGTTTGTCAAATGGCATTGTCTTTTA |
| <u>HcChMlys4a3</u> | AACCATATACTACAATGTGGCTAATTTCAACATTGTTTGTCAAATGGCATTGTCTTTTA |
| <u>HcChMlys4b2</u> | AACCATATACTACAATGAGGGCAATTTCAACATTGTTTGTCAAATGGCATTGTCTTTAA |
| <u>HcChMlys4b1</u> | AACCATATACTACAATGTGGCTAATTTCAACATTGTTTGTCAAATGGCATTGTCTTTTA |
| <u>HcChMlys4b3</u> | AACCATATACTACAATGTGGCTAATTTCAACATTGTTTGTCAAATGGCATTGTCTTTTA |
| <u>Taxon</u> | 111111111111111111111111111111111111 6666666667777777777888888888899999999990000000000111111111112 123456789012345678901234567890123456789012345678901234567890 |
| <u>HcOvAust</u> | GACAATTCATTTTCAGTTCAAGAACATATACATGCAACGTGATGTTATGAAATTGTAAC |
| <u>HcOvUSA</u> | GACAATTCATTTTCAGTTCAAGAACATATACATGCAACGTGATGTTATGAAATTGTAAC |
| <u>HcOvMlys2b</u> | GACAATTCATTTTCAGTTCAAGAACATATACATGCAACGTGATGTTATGAAATTGTAAC |
| <u>HcOvMlys2a1</u> | GACAATTCATTTTCAGTTCAAGAACATATACATGCAACGTGATGTTATGAAATTGTAAC |
| <u>HcOvMlys2d</u> | GACAATTCATTTTCAGTTCAAGAACATATACATGCAACGTGATGTTATGAAATTGTAAC |
| <u>HcChMlys</u> | GATAATTCATTTTCAGTTCAAGAACATATACATGCAACGTGATGTTATGAAATTGTAAC |
| <u>HcChMlys4a</u> | GACAATTCATTTTCAGTTCAAGAACATATACATGCAACGTGATGTTATGAAATTGTAAC |
| <u>HcChMlys4a2</u> | GACAATTCATTTTCAGTTCAAGAACATATACATGCAACGTGATGTTATGAAATTGTAAC |
| <u>HcChMlys4a3</u> | GACAATTCATTTTCAGTTCAAGAACATATACATGCAACGTGATGTTATGAAATTGTAAC |
| <u>HcChMlys4b2</u> | GATAATTCATTTTCAGTTCAAGAACATATACATGCAACGTGATGTTATGAAATTGTAAC |
| <u>HcChMlys4b1</u> | GACAATTCATTTTCAGTTCAAGAACATATACATGCAACGTGATGTTATGAAATTGTAAC |
| <u>HcChMlys4b3</u> | GACAATTCATTTTCAGTTCAAGAACATATACATGCAACGTGATGTTATGAAATTGTAAC |

The ITS-2 Neighbor-Joining tree clearly separated *H. contortus* in sheep from the USA and Australia from those from Penang (88% bootstrap – Fig. 1). *H. contortus* from Penang can be divided into two major groups that are supported by 83% and 72% bootstrap values respectively. The clade with 83% support was made up of *H. contortus* found both in sheep (HcOvMlys2b, HcOvMlys2a1 and HcOvMlys2d) and goats (HcChMlys4a2, HcChMlys4a3, HcChMlys4b1 and HcChMlys4b3). Within this clade, there are two ITS-2 haplotypes, i.e. one made up of HcOvMlys2a1, HcChMlys4a3, HcChMlys4b1 and HcChMlys4b3 while the second haplotypes was made up of HcOvMlys2b, HcOvMlys2d and HcOvMlys2b. The second clade with 72% support was made up of *H. contortus* found only in goats (HcChMlys, HcChMlys4b2 and HcChMlys4a). However there are two ITS-2 haplotypes, i.e. one made up of HcChMlys and HcChMlys4b2, and the other is HcChMlys4a.

Three most parsimonious trees with 12 tree length were obtained from parsimony analysis. There are 10 variable characters with 7 characters being parsimony informative from the total 231 characters studied. The data has relatively low homoplastic characters having CI and RC values of 0.837 and 0.714 respectively. Tree topology and information on genetic relationship from parsimony analysis are similar to the Neighbor-Joining tree whereby the *H. contortus* from Penang can be partitioned into two clades (Fig. 2). The clade that contain *H. contortus* found in sheep and goats was supported with only 67% bootstrap values while the clade that contain *H. contortus* found only in goats was supported with only 100% bootstrap values.

DISCUSSION

The ITS-2 sits within the 18S-5.8S-26S genes of the nuclear rDNA. The genes together with ITS-1 and ITS-2 are present in multiple copies arranged in tandem repeats (sometime hundred to thousands of repeat units). Sequence homogeneity among repeat units within a genome are thought to be due concerted evolution via gene duplication or unequal crossing over (Li, 1997). This work is the first study on the ITS-2 sequences of *H. contortus* from sheep and goat in Malaysia. As a matter of fact, very limited work is done on the genetics of *H. contortus* found in goat anywhere around the globe. Our results provide a preliminary insight on the genetic differences of *H. contortus* found in the two ruminants.

In this study the ITS-2 sequences of *H. contortus* from Penang can be divided into six haplotypes i.e., six individual sequences. The first four haplotypes (1–4) are those forming the first clade in Figure 1 and Figure 2. Haplotype 1 and 2 are made up of *H. contortus* from sheep i.e., HcOvMlys2d and HcOvMlys2b respectively while haplotype 3 comes from goats i.e., HcChMlys4a2. Meanwhile, haplotype 4 is made up of *H. contortus* from both sheep (HcOvMlys2a1) and goats (HcChMlys4a3, HcChMlys4b1 and HcChMlys4b3). These haplotypes differ from one another by a single nucleotide difference.

The other two haplotypes (5 and 6) formed a distinct clade by sharing 3 distinct nucleotides (position 18, 21 and 22) that were different from the rest. These haplotypes are made up of *H. contortus* from goats. Haplotype 5 was made up of HcChMlys4a while haplotype 6 is made up by HcChMlys and HcChMlys4b2. HcChMlys4a is different from the other two by four nucleotides and this resulted in a high bootstrap support in separating haplotype 5 and 6. *H. contortus* from Australia and the US made up a single haplotype, distinctly different from any of the Penang's *H. contortus*.

The presence of six ITS-2 *H. contortus* haplotypes might be due to interspecific variation within *H. contortus* as opposed intraspecific variation. Although intraspecific ITS variation (non-concerted evolution) has been reported, these are considered the exception. However, studies in plants (Angiosperms) seem to suggest there are more incidents whereby non-concerted evolution is being reported (Harpke & Peterson 2006).

Our result suggested that *H. contortus* found in sheep varies very little genetically i.e., 100% similar or differ either by one or two nucleotides. The same is also true for *H. contortus* found in goat. However, *H. contortus* in goat showed more differences in their ITS-2 sequence by differing up to four nucleotides. In this study we only obtained *H. contortus* from a single goat and sheep from Penang. The close affinity in term of locality of the two host used in this study might have resulted in the lower genetic variation observed in *H. contortus*. It is tempting to assume that if sheep especially goats were to be obtained from other areas from the Peninsular Malaysia for analysis, higher ITS-2 sequence variability may be observed.

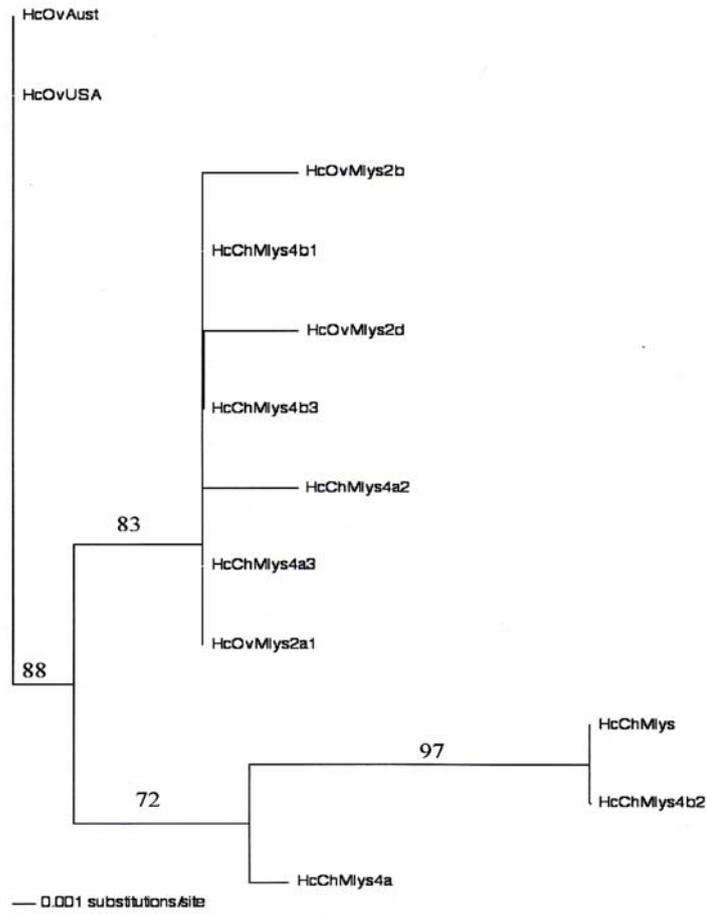


Figure 1: A Neighbor-Joining tree of *H. contortus* from Penang including those from Australia and the US. Penang's *H. contortus* can be divided into two separate clades. The values above branches are bootstrap values. Refer to text for explanation.

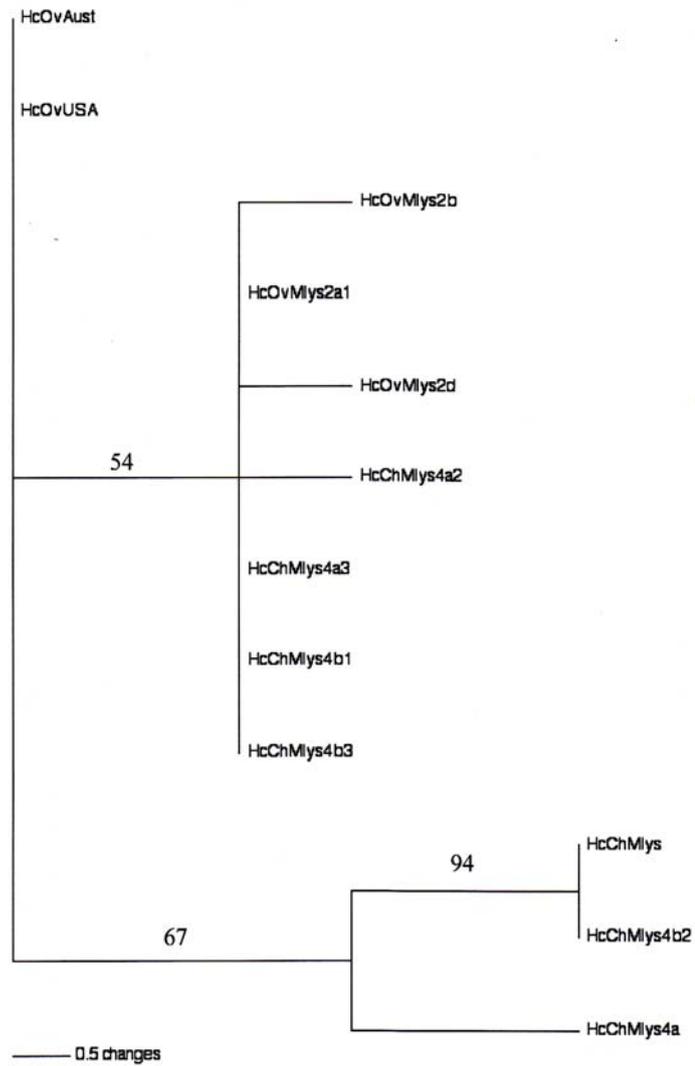


Figure 2: One of the three most parsimonious trees with branch length of 12 and CI = 0.837 and RC = 0.714. Bootstrap percentages are assigned on respective branches. The tree topology and genetic relationships elucidated are the same as those of Neighbor-Joining tree.

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