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Genetic markers in ribosomal DNA for hookworm identification

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Abstract

Polymerase chain reaction-linked restriction fragment length polymorphism (PCR-RFLP) analysis of ribosomal (r) DNA was conducted on *Uncinaria stenocephala*, *Ancylostoma caninum*, *A. tubaeforme* and *A. ceylanicum*. The rDNA region spanning the first and second internal transcribed spacers (ITS1 and ITS2) plus the 5.8S (ITS^+) gene was amplified by PCR from each of the species, digested separately with 9 restriction endonucleases and the fragments separated by agarose gel electrophoresis. Characteristic PCR-RFLP patterns were produced for each morphologically defined species using some of the endonucleases. The present study demonstrated that the ITS^+ provides genetic markers for the delineation of each species examined and suggests that this region of rDNA will be useful for the identification of other hookworms from a range of hosts. The results are likely to have important implications for studying the genetic structure of hookworm populations, the systematics and the epidemiology of hookworm infections.

Keywords: Hookworm; *Ancylostoma* spp.; *Uncinaria* spp.; Ribosomal DNA; Internal transcribed spacer; Species identification; PCR-RFLP

1. Introduction

There are many species of hookworms which infect humans and other mammals, some of which are of clinical significance (see Yamaguti, 1961; Schad and Warren, 1990). Hookworms are thought to be host-specific (e.g., Biocca, 1954; Burrows, 1962). However, in northern Australia the "dog hookworm", *Ancylostoma caninum*, can infect humans and cause eosinophilic enteritis (Croese, 1988; Prociv and Croese,

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1990; Croese et al., 1994) and *A. ceylanicum*, a parasite commonly found in humans in parts of Asia, has been found in cats in this region of Australia (Stewart, 1994).

Detailed morphological examinations of adult worms of *Ancylostoma* from dogs and cats have revealed variation in morphology (=morphotypes) within a parasite species (Stewart, 1994), so that there can be confusion as to the taxonomic status of such morphotypes. Such morphotypes may represent either morphologically similar but genetically distinct species (i.e., cryptic species), subspecies or population variants that are capable of infecting heterologous host species. Several studies have already demonstrated the existence of cryptic species in bursate nematodes with slight or no morphological differentiation (Chilton et al., 1992, 1993; Beveridge et al., 1993). Alternative techniques (to morphological examination) are required for the accurate identification of parasite species.

DNA technology and biochemical methods (e.g., allozyme electrophoresis) have proven to be valuable tools for the identification of parasite species (e.g., Bowles and McManus, 1993; Chilton et al., 1993, 1995; Stevenson et al., 1995). Recent studies have shown that internal transcribed spacer ribosomal (r)DNA (ITS) provides reliable genetic markers for the identification of nematode species belonging to the Order Strongylida (Campbell et al., 1995; Chilton et al., 1995; Hoste et al., 1995; Stevenson et al., 1995). Given that hookworms also belong to this Order, it was anticipated that spacer rDNA would be useful for the accurate identification of hookworm species and therefore detect the presence of cryptic species. In this study, we evaluate whether the rDNA internal transcribed spacers (ITS1 and ITS2) provide genetic markers for the delineation of morphologically defined hookworm species from carnivores.

2. Materials and methods

2.1. Morphological identification of parasites and morphometry

Adult worms of *Uncinaria stenocephala*, *Ancylostoma caninum*, *A. tubaeforme* and *A. ceylanicum* were collected at necrospy from the small intestine of their hosts (Table 1), washed 5 times in physiological saline and examined live with a compound microscope. *Uncinaria stenocephala* was identified according to Skrjabin et al., 1952). For *Ancylostoma*, worms were identified individually to species using morphological and morphometric criteria employed by Stewart (1994).

2.2. Isolation of parasite DNA and amplification of rDNA

Genomic DNA was isolated from worms by sodium dodecyl-sulphate and proteinase K treatment, phenol/chloroform extraction, ethanol precipitation and purification with PrepaGene® (Biorad) (Gasser et al., 1993). The rDNA region comprising the first and second internal transcribed spacers plus the 5.8S gene (=ITS$^+$) was amplified from 10 to 20 ng template by PCR (Mullis et al., 1986), using oligonucleotide primers NC5>: 5′-GTAGGTGAACCTGCGGAAGGATCATT-3′ (forward) and <NC2: 5′-TTAGTTTCTTTTCCTCCGCT-3′ (reverse) designed to regions of

Table 1
Hookworm specimens used in this study

Species	Specimens	Sex	Host	Location in Australia	Reference no.[a]
Ancylostoma caninum	Ac1	male	dog	Townsville	AHC 30164
	Ac2	male	dog	Townsville	AHC 30165
Ancylostoma tubaeforme	At1	male	cat	Townsville	AHC 30166
	At2	male	cat	Townsville	AHC 30167
Ancylostoma ceylanicum	Acey1	female	cat	Townsville	AHC 30168
Uncinaria stenophala	Us1	mixed	fox	Victoria	

[a]Anterior and posterior ends of each *Ancylostoma* adult have been deposited in the Australian Helminthological Collection (AHC), South Australian Museum, Adelaide.

the 18S and 28S genes, respectively, and found to be conserved across a range of strongylid nematodes (unpublished). PCR reactions (100 μl) were performed using the following PCR mix: 10 mM Tris-HCl, pH 8.4; 50 mM KCl; 3.5 mM $MgCl_2$; 200 μM each of dATP, dCTP, dGTP and dTTP; 100 pmol of each primer and 1 unit *Taq* polymerase (Promega) under the following conditions: 94°C, 30 s (denaturation); 55°C, 30 s (annealing); 72°C, 30 s (extension) for 30 cycles (480 Thermocycler, Perkin Elmer Cetus). Samples without DNA were included in each amplification run. Also, to confirm specificity of the PCR and conditions, genomic DNA from a dog was subjected to the same amplification procedure as for nematode DNA. No amplification products were detected on agarose gels in these control samples (data not shown). Partial DNA sequencing (Gasser et al., 1993) confirmed the identity of PCR products as ITS^+ by comparison with the sequences of a wide range of species of strongylid nematodes (unpublished data).

2.3. PCR-RFLP

ITS^+ PCR products (2.5 μl) were digested directly with restriction endonucleases for 16 h in a total volume of 20 μl using 8–12 units (1 μl) of each endonuclease according to the manufacturer's protocols (Promega). Nine endonucleases (*Alu*I, *Cfo*I, *Dde*I, *Dra*I, *Hae*III, *Hin*fI, *Msp*I, *Rsa*I and *Vsp*I) were selected based on known restriction maps of the ITS2 sequence of a range of strongylid nematodes (Hoste et al., 1995; Stevenson et al., 1995). No inhibitory effect of the PCR buffer on digestion with these enzymes was detected. Restriction fragments were separated in 1.5% agarose-TBE (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 7.4) gels (Sambrook et al., 1989), stained with ethidium bromide and photographed using Polaroid 667 film (Kodak). Size of fragments was estimated by comparison with pGEM® and ΦX174-*Hae*III markers (Promega).

3. Results and discussion

For each hookworm species examined, the undigested ITS^+ PCR product was approx. 860 bp in size (not shown; same size as bands in *Dra*I and *Vsp*I, Fig. 1).

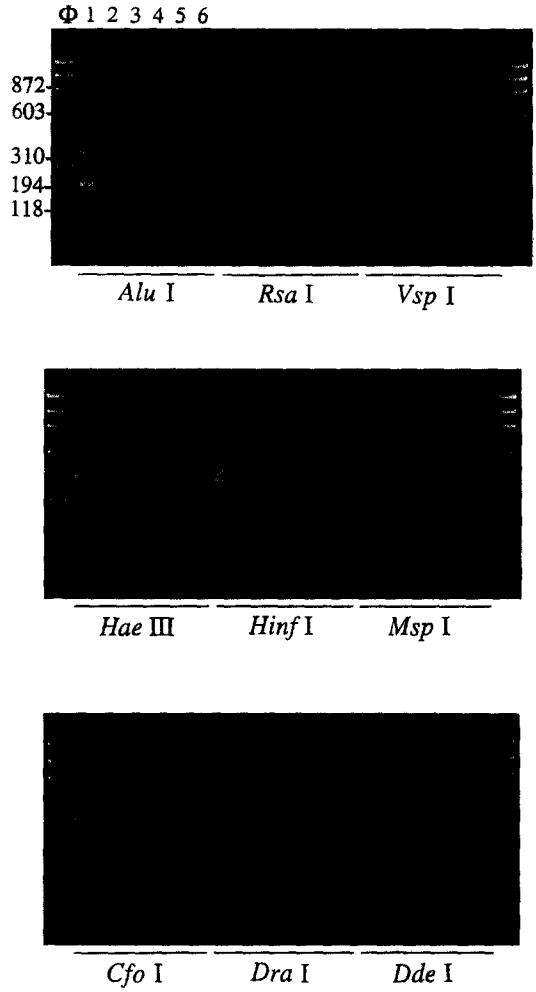

Fig. 1. PCR-RFLP analysis of the ITS$^+$ of 4 hookworm species. Panels: agarose gel separation of PCR products digested with *Alu*I, *Rsa*I, *Vsp*I, *Hae*III, *Hin*fI, *Msp*I, *Cfo*I, *Dra*I or *Dde*I. Lanes: *Uncinaria stenocephala* (1); *Ancylostoma caninum* – Ac1 (2); *A. caninum* – Ac2 (3); *A. tubaeforme* – At1 (4); *A. tubaeforme* – At2 (5); and *A. ceylanicum* (6). Ordinate values indicate approx. size in base pairs.

While the ITS$^+$ remained undigested in PCR-RFLP with *Dra*I or *Vsp*I, there were distinct differences in restriction patterns between taxa using the remaining 7 endonucleases. Representative RFLP patterns of the ITS$^+$ for the hookworm species examined are shown in Fig. 1. Patterns produced by digestion of ITS$^+$ with *Hae*III, *Hin*fI, *Rsa*I and *Alu*I allowed the delineation of *Ancylostoma* species and *Uncinaria stenocephala*. The *Ancylostoma* species examined could be differentiated by digestion with *Rsa*I and *Cfo*I. *A. caninum* and *A. tubaeforme* could be differentiated from *A. ceylanicum* based on the restriction patterns produced with *Alu*I, *Rsa*I, *Hin*fI, *Cfo*I and *Dde*I.

An interesting finding was the difference in RFLP patterns between the *A. caninum* samples, Ac1 and Ac2, with *Rsa*I (Fig. 1, lanes 2 and 3). The (reproducible) presence of an additional band of 600 bp in Ac1 indicated the existence of two or more sequence types in the ITS$^+$ region in a single organism (=intraindividual variation). Intraindividual variation has recently been demonstrated for the ITS2 sequence of another bursate nematode, *Haemonchus contortus* (see Stevenson et al., 1995). There was also evidence for the existence of different sequence types in both *A. tubaeforme* worms, At1 and At2 (e.g., Fig. 1, *Msp*I, lanes 4 and 5, faint bands at 700 bp) and the *U. stenocephala* isolate Us1 (e.g., Fig. 1, *Rsa*I, lane 1) based on their restriction patterns. Recent reports describe that different sequence types are most likely to be a result of base changes at certain positions in the sequence of a proportion of rDNA repeats as a consequence of mutational exchange during DNA replication (Hancock and Dover, 1990; Schlötterer and Tautz, 1992), the extent of which appears to differ depending on the taxonomic group (e.g., Wesson et al., 1992; Campbell et al., 1995; Hoste et al., 1995; Gasser et al., 1996). Given that sequence variation in ITS within a parasite species can be associated with "strain" variation in cestodes (Bowles and McManus, 1993; Gasser and Chilton, 1995), it is possible that the difference in RFLP patterns between single worms of *Ancylostoma* species found in this study reflects similar variation in hookworms. Nonetheless, based on a number of studies of strongylid nematodes (Hoste et al., 1993; Campbell et al., 1995; Gasser and Hoste, 1995; Stevenson et al., 1995), the degree of sequence variation among geographical isolates of a species is expected to be low.

In spite of the small number of samples examined, the results of this study clearly indicate that ITS$^+$ region of rDNA provides genetic markers for some hookworm species. Although applied to hookworms from dogs and cats, it is likely that this region will also provide species markers for a range of hookworm species from other hosts, because previous studies have consistently demonstrated that even very closely related strongylid nematodes can be identified reliably to species using ITS sequences (e.g., Chilton et al., 1995; Gasser and Hoste, 1995; Hoste et al., 1995; Stevenson et al., 1995). Such markers may have important implications for studying the systematics of hookworms and the epidemiology/population biology of hookworm infections. However, prior to their application, further work is required (1) to determine the quantitative differences in ITS$^+$ rDNA sequence (or parts thereof) among hookworm species, (2) to critically assess levels of sequence variation within individual species (=intraspecific variation) using a wide range of geographical isolates, and (3) to attempt to quantify the number of ITS$^+$ sequence types in the different geographical isolates of each species in order to determine whether different ITS sequence types relate to the morphotypes within a species.

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