NORTHERN ILLINOIS UNIVERSITY

Comparison in the ITS-2,or second internal transcribed spacer, among the different geographic isolates of *Strongyloides Stercoralis*

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by

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Introduction

The phylum Nematoda or round worms comprises numerous free-living and parasitic species. The free-living forms are distributed ubiquitously in soil and water. The parasitic species live in annelids, arthropods, mollusks, plants, and vertebrates. It is estimated that over 80,000 species are parasites of vertebrates. Species parasitic in humans range in length from 2.0 mm (*Strongyloides stercoralis*) to over a meter (*Dracunculus medinensis*). The sexes are usually separate. The male, which is smaller than the female, usually has a curved posterior end and, in some species, copulatory spicules and a bursa (Brown, 1975).

This study is focused on *Strongyloides stercoralis,* a member of the Order Rhabditata. The specific diseases that can be caused by *S. stercoralis* are Strongyloiidiasis, and Chochin-China diarrhea. The distribution of *S. stercoralis* is worldwide, but human infection is especially prevalent in tropical and warm areas. However, endemic cases of Strongyloidiasis were noted in some mental institutions of the United States. Humans are the principal host of *Strongyloides stercoralis*, but it also can infect other primates, as well as dogs, cats, and some other mammals. The males usually die early during the life cycle. The pathogenetic parasitic female, measuring around 2.20 mm by 0.04 mm, penetrates the mucosa of the intestinal villi, and lays eggs. The adult worms also can be found in the submucosa of the duodenum and jejunum (Brown, 1975).

The *Strongyloides stercoralis* is unique in that it has 3 different potential life cycles:
1. Direct cycle, 2. Indirect cycle, and 3. Autoinfective cycle. During the direct cycle, after a short feeding period of 2 to 3 days in the soil, the rhabditiform larva molts into the filariform larva. The filariform larva, which is infective, penetrates the skin of humans, and after a hookworm-like migration, reaches the intestine and matures in about 2 weeks.

In the indirect, or free-living cycle, the rhabditiform larvae mature to adult free-living males and females in the soil. After fertilization, the females produce eggs that develop into rhabditiform larvae. These may enter the host as infective filariform larvae, or may repeat the free-living generations. This switching between free-living and indirect cycles may be associated with the optimum environmental conditions, especially in tropical areas. Particular strains may choose the indirect cycle over the direct cycle. Some may revert to a parasitic phase if there is a change in the environment.

With autoinfection, the maturation of rhabditiform to filariform larvae may occur within the intestine or upon the perianal skin; thus, infection may take place without larvae leaving the body. In terms of pathogenesis and symptomatology, the following occurs:

1. Migration of larvae:

   Few pulmonary symptoms, except with heavy infective doses or autoinfection; then cough and focal pneumonitis.

2. Adult worms:

   a. Adult worms focally destroy mucosa. May be malabsorption of glucose; relationship to achlorhydria observed when larvae are in gastric aspirate.

   b. Symptoms: resemble peptic ulcer, with midepigastric pain and tenderness; may have nausea and diarrhea. This complex, plus the presence of peripheral eosinophilia, should initiate search for larvae.

3. Activation of low grade infection if host defense is altered: reports of fatal Strongyloidiasis in patients receiving corticosteroids, or otherwise immunosuppressed. The definitive diagnosis of Strongyloidiasis is the demonstration of rhabditiform or filariform larvae in feces or duodenal aspirates.

The geographic distribution of *Strongyloides* infection runs parallel to that of hookworms, but its pervasiveness is lower in the temperate zones. It is particularly pervasive in tropical and subtropical areas, where moisture, warmth, and a poor sanitary environment favor its free-living cycle (Brown, 1975). Laboratory diagnosis consists of examination of feces and duodenal constituents for larvae by direct or indirect methods.

Many human infections by *Strongyloides* are asymptomatic because they are light. Moderate infections may cause a burning, sharp or dull, midgastric pain due to the females inserted mainly in the duodenal region. When palpitated, this area may elicit pain and tenderness. Vomiting, nausea, diarrhea, and constipation may occur alternately. Heavy infection may result in weight loss, and may lead to chronic dysentery. Thus, in heavy infections, all of the symptoms are more noticeable, and death may occur.

In an autopsy of a patient who died of heavy infection, filariform larvae were seen in the liver, lungs, gallbladder, pancreas, adrenals, thyroid and parathyroid glands, lymph glands, and GI tract (Brown, 1975).

In our study the ITS-2,or second internal transcribed spacer (of the Ribosomal DNA repeat) , among the samples from four different geographic regions were compared to see how different or related they are. Also, by studying the genetic differences or similarities, we hope to eventually investigate if there is strain variation correlated with variation in pathogenicity. In addition to comparisons of ITS-2 data generated in our laboratory, we used ITS-1 data on *Strongyloides* species available from Genbank.

MATERIALS AND METHODS

Table 1 shows the four samples of *S. stercoralis* that served as DNA sources for this study as well as the geographic origin, and host.

Table 1- Origins of the four *Strongyloides* samples examined in the study (ITS-2 Source)

Species /sample ID No.	Host	Origin
7-555 Strongyloides stercoralis	Dog	Culture derived L3i of AL3 type (Received from G. Schad)
7-556 S. stercoralis	human	AE 694 - from Honduran patient
7-557 S. stercoralis	human	AE 894 - Southeast Asian patient(human) passed 1x through Gerbil
7-560 S. stercoralis	Dog	lab strain/ from G. Schad

DNA/Nucleic acid extractions: (Nadler/Hafner protocol)

The specimens were stored at -70° C until ready for DNA extraction. A small amount (~0.2 g) of L_3 larvae was extracted using a modified proteinase K digestion /phenol:chloroform procedure. The tissue was homogenized on ice in a mixture that contained 500 µl chilled STE. The homogenate supernatant was transferred to a 1.5 ml microfuge tube. Fifty µLof 20% SDS and 20 µL of Proteinase K (10 mg/ml) were added, and then this mixture was incubated for 1 hour at 50°C. The contents were centrifuged and the supernatant added to a new tube. An equal volume of phenol was added, mixed, and the tube centrifuged for 3 min at 10K rpm (at 4° C). The aqueous layer was transferred to a new microfuge tube. The process was repeated until the phenol-supernatant interface was free of protein. Next, an equal volume of chloroform/isoamyl alcohol (24:1) was added, mixed, and the tube centrifuged for 3 min. at 10K rpm (at 4 °C). The aqueous layer was transferred to a new microfuge tube. To this, 50µL of 3M NaAc and 1 mL of ice-cold 95% EtOH was added, and this was mixed well and placed in a -20 °C

freezer overnight. Nucleic acids were pelleted by centrifugation. The pellet was washed twice with 50μL of ice-cold 70% EtOH. The pellet was dried to completion, and then resuspended in 100 μL of TE (pH 8.0).

Nucleic acid quantification:

The nucleic acid that was extracted was quantified using a spectrophotometer at A(absorbance) of 260 nm.

PCR Primer design:

PCR primer design was done as follows: First, Genbank was searched for the appropriate nematode sequences. Once the sequences were selected, these were analyzed using the Multiple alignment program, Clustal V. Then, the program Primer (v 0.5) was used to analyze the primers selected for use in the PCR reactions (see Table 2 for list of primers used).

TABLE 2. Sequences of primers used for PCR and sequencing:
(Forward primer → ; Reverse primer ←)

Primer		Sequence (5' → 3')	Remarks
91	←	GCTGGGTTCTTCATCGAT	For ITS < 5.8 S (5'); T_m = 57
92	→	ATCGATGAAGAACGCAGC	For ITS > 5.8S (5') ; T_m = 57
93	→	TTGAACCGGGTAAAAGTCG	For ITS > 18S (3') ; T_m = 58
94	←	TTAGTTTCTTTTCCTCCGCT	For ITS < 28 S (5') ; T_m = 56
156	→	GGCCAGTGAATTGTAATACGATC	
157	←	GACACTATAGAATACTCAAGCTATGC	

PCR Optimization:

Several replicate sets of PCR reactions were done to determine the optimum reaction conditions for the best specificity and yield. The contents used in PCR reactions were as follows: UV-irradiated water, 10X buffer, dNTP mix, varying amounts of MgCl2 (to get the optimum reaction conditions), TAQ polymerase, and various combinations of primers to obtain the appropriate PCR product.

TA-cloning of PCR products, Preparation of PCR product, and Ligation reaction:

PCR products were cleaned by spin filtration, 7 µl of product was used in a ligation reaction using pGEM-T vector.

Transformation of *E. coli* :

Next , transformation of *E. coli* (DH5α) was performed on ice for at least two hours. Transformation is the direct uptake of the naked plasmid DNA by cells. In some bacterial species, transformation is a significant natural process for increasing genetic variation. The capability to take up naked DNA is called competence. This often happens at a specific stage in the growth of a culture (i.e., stationary phase), and may be associated with the induction of the synthesis of a set of proteins termed the competence proteins. The degree of competence that is required experimentally for *E. coli* must be induced artificially and can be attained most easily in two ways: one involves a chemical treatment, and the other involves an electric shock (Howe, 1995). The method we used in our lab was the former. To check for positive clones, boil prep reactions were used. The *E. coli* transformation was done as follows: Thaw the competent cells on ice and then add 40 ng DNA to 100 µl of competent cells (control w/ no DNA). Incubate 2 hours or more on ice, then incubate 3 minutes at 37°C. Next, add 500 µl LB to each 100 µl, control also. Incubate 1 hour at 37°C, without shaking. Spread each 100 µl onto LB plates + selection. Incubate at 37° C overnight. Check for proper colonies and do boil prep PCR reactions were used to confirm proper insert.

Preparation of competent *E. coli*:

The competent *E. coli* was prepared as follows: Inoculate 10 ml of LB+ selection agent with *E. coli* strain, incubate at 37°C, and leave in shaker at 250 rpm, overnight. Next day, inoculate 500 ml LB+ selection with 4 ml of overnight culture. Grow these at 37°C, shake at 250 rpm, till OD 600= 0.3. Then split cells into 50 ml aliquots, pellet cells at 3500 rpm for 10 minutes. Then resuspend each pellet in 3 ml of ice-cold 50µM $CaCl_2$ + 15% glycerol. Aliquot as 1ml in eppendorfs. Store in -80°C. Do not use before 2 hr. in -80°C.

CsCl preparation of plasmids for sequencing was done ccording to a standard protocol (Lovett and Keggins ,1979).

Cycle sequence reactions; gel reading/ assembly:

CsCl sample was sequenced several times for each strand to resolve sequence ambiguities. For the cycle sequencing reactions, we varied the annealing temperature to get the optimum reaction conditions.

Next, the gels were read using the EasyReader Digitizer. Once the gels were read, the Assemgel program in PC Gene was used to assemble individual primer reads into a single sequence. The assembled gel information was checked for conflicts, and this sequence information was aligned using the program Clustal.

Finally, the results were analyzed using the programs PAUP (Phylogenetic Analysis Using Parsimony, version 3.1.1 Mac version) and PHYLIP (Phylogeny Inference Programs) programs. Maximum parsimony is one of the most widely used method of inferring phylogenetic relationships (Nadler , 1990). The gaps were treated as fifth base for analysis of the ITS-2 data. The ITS-1 data (Accession numbers: U43576-U43581; U43962) were obtained from Genbank (Ramachandran et al. 1996) and included in our analysis. The same methods used to analyze the ITS-2 data were used to analyze the ITS-1 data.

RESULTS AND DISCUSSION: **ITS-2:** Multiple alignment of the ITS-2 sequences (Fig. 1) yielded the following results.

Figure 1. Multiple alignment, pairwise 5,1,5,5 of STS ITS-2 region
CLUSTAL V multiple sequence alignment (Refer to table 1 for sample numbers)
555STS5 = clone 5 of 7-555 isolate,555STS7 = clone 7 of 7-555 isolate
556STS2 = clone 2 of 7-566 isolate,557STST = clone of 7-557 isolate
560STS4 = clone 4 of 7-560 isolate

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555STS5         GTAAGGTGAATTGCTAAGCAGAGCC-TTAAATTCTTGAATGCAGATGGCG
555STS7         GTAAGGTGAATTGXTAAGCAGAGCC-TTAAATTCTTGAATGCAAATGGCG
556STS2         GTAAGGTGAATTGXTAAGXAGAGCXCTTAAATTCTTGAATGCAAATGGCG
557STST         GTAAGGTGAATTGCTAAGCAGAGCC-TTAAATTCTTGAATGCAXATGGCG
560STS4         GTAAGGTGAATTGXTAAGCAGAGCC-TTAAATTCTTGAATGCAAATGGCG
                ************* **** *****  ***************** ******

555STS5         CTAATAGTATTTTATACTATTAGCATATATAATTGAGGGTGATAACAAGT
555STS7         CTAATAGTATTTTATACTATTAGCATATATAATTGAGGGTGATAACAAGT
556STS2         CTAATAGTATTTTATACTATTGGCATATATAATTGAGGGTGATAACAAGT
557STST         CTAATAGTATTTTATACTATTXGCATATATAATTGAGGGTGATAACAAGT
560STS4         CTAATAGTATTTTATACTATTAGCATATATAATTGAGGGTGATAACAAGT
                ********************* ****************************

555STS5         AGTATTGTATATTATTATTTTTATATTAAAAATAATAAATATATTAATAA
555STS7         AGTATTGTATATTATTATTTTTATATTAAAAATAATAAATATATTAATAA
556STS2         AGTATTGTATATTATTATTTTTATATTAAAAATAATAAATATATTAATAA
557STST         AGTATTGTATATTATTATTTTTATATTAAAAATAATAAATATATTAATAA
560STS4         AGTATTGTATATTATTATTTTTATATTAAAAATAATAAATATATTAATAA
                **************************************************

555STS5         AATATTTTTAATTTTTTAAATAAAAGTATTTTTATTGTATATTTTTTTTT
555STS7         AATATTTTTAATTTTTTAAATAAAAGTATTTTTATTGTATATTTTTTTTT
556STS2         AATATTTTTAATTTTTTAAATAAAAGTATTTTTATTGTATATTTTTTTTT
557STST         AATATTTTTAATTTTTTAAATAAAAGTATTTTTATTGTATATTTTTTTTT
560STS4         AATATTTTTAATTTTTTAAATAAAAGTATTTTTATTGTATATTTTTTTTT
                **************************************************

555STS5         TTT-ATTATATAAAAAAAAAATAAAATATCATGTTTGATATATATTTATA
555STS7         TTTTATTATATAAAAAAAAAATAAAATATCATGTTTGATATATATTTATA
556STS2         TTT-ATTATATAAAAAAA--ATAAAAAATCATGTTTGATATATATT-ATA
557STST         TTT-ATTATATAAAAAAA--ATAAAATATCATGTTTGATATATATT-ATA
560STS4         TTTTATTATATAAAAXXAAAATAAAATATCATGTTTGATATATATTTATA
                *** ************  *  ****** ******************** ***

555STS5         TTTTTTTTTAATAAAAAAAAAAAAATCATTAATAATTTTTTTAAATTT---
555STS7         TTTTTTTTTAATAAAAAAAAAAAAATCATTAATAATTTTTTTAAATTT---
556STS2         TTTTTTTTTAATAAAAAAA---AATCATTAATAATTTTTTTAAATTTATT
557STST         TTTTTTTTTAATAAAAAAA---AATCATTAATAATTTTTTTAAATT---T
560STS4         TTTTTTTTTAATAAAAAAAXAAAATCATTAATAATTTTTTTAAATTT---
                ********************   ************************

555STS5         -TTTTTGCCTCAATTATGTATGATTACCCGCTGAACTTAAGCATATCGTT
555STS7         -TTTTTGCCTCAATTATGTATGATTACCCGCTGAACTTAAGCATATCGTT
556STS2         TTTTTTGCCTCAATTATATATGATTACCCGCTGAACTTAAGCATATCGTT
557STST         TTTTTTGCCTCAATTATGTATGATTACCCGCTGAACTTAAGCATATCGTT
560STS4         -TTTTTGCCTCAATTATGTATGATTACCCGCTGAAC------------TT
                 **************** ******************           **

555STS5         AAGCGGAGGAAAAGAAACTAA
555STS7         AAGCGGAGGAAAAGAAACTAA
556STS2         AAGCGGAGGAAAAGAAACTAA
557STST         AAGCGGAGGAAAAGAAACTAA
560STS4         AAGCGGAGGAAAAGAAACTAA

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From the pair wise comparisons (Fig. 2) we can see that there is not much variability in the ITS-2 region of *S. stercoralis* from different geographic regions.

FIGURE 2.
ITS-2 SEQUENCE COMPARISONS
(PERCENT SIMILARITY OF ITS-2 SEQUENCES)

	555Sts5001	555Sts7001	556Sts2001	557Sts001	560Sts4001
555Sts5001		363/366 (99.2%)	353/365 (97.0%)	357/365 (97.8%)	348/365 (95.3%)
555Sts7001			355/365 (97.0%)	356/366 (97.3%)	351/366 (96.7%)
556Sts2001				358/364 (98.4%)	345/360 (94.8%)
557Sts001					346/360 (96.1%)
560Sts4001					

(Refer to **table 1** for sample/ID numbers) :

555 Sts5001 = clone 5 of 7-555 , 555 Sts7001 = clone 7 of 7-555, 556 Sts2001 = clone 2 of 7-556
557 Sts001 = clone of 7-557, 557 Sts4001 = clone of 7-557

As expected, the highest similarity was between the two 555Sts clones, each representing a single isolate. The most interesting finding was from the comparison of clones representing 560Sts and 555Sts. These two strains (from G. Schad's lab) presumably have a common origin, but differ in that they are maintained via different life cycles. The 560 Sts strain has autoinfective life cycle, whereas the 555Sts represents larvae obtained from culture (direct cycle). The difference between the two strains is suggestive of species-level differentiation. However, more study such as their pathogenicity in relation to their different life cycles may allow us to make a conclusion .

The data obtained in our experiment agrees closely with the published data (Hoste et al 1995) for differences in the ITS-2 region (r DNA) of *Trichostrongylus* nematodes. The GC content of our data was 18% (Fig. 3) in comparison to published data for *Trichostrongylus*, which is 30 %.

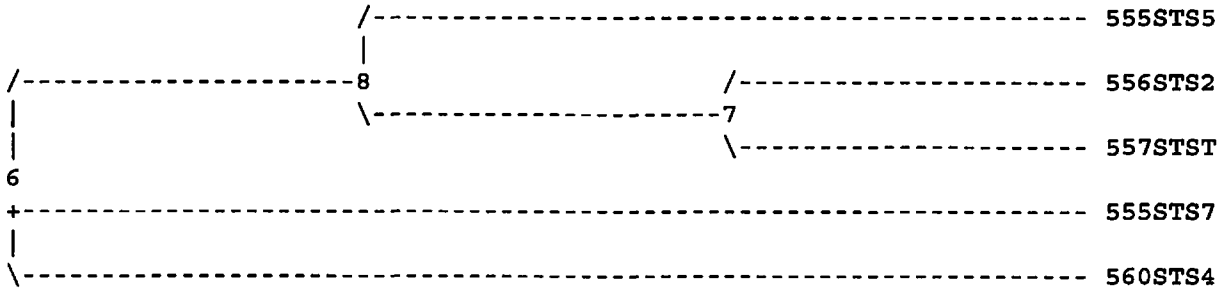
FIGURE 3.
ITS-2 SEQUENCES
SUMMARY OF GC CONTENT
(% GC content)

	Tot. bp 's	total A's	Total C's	Total G's	Total T's	% GC
555Sts5001	365	145	26	41	154	18.1
555Sts7001	366	146	24	40	155	17.5
556Sts2001	364	143	23	40	155	17.3
557Stst001	360	139	25	40	154	18.1
560Sts4001	354	139	22	38	151	16.9

For the ITS-2 data, maximum parsimony (MP) analysis, with gaps treated as "fifth base", using the branch-and bound algorithm of PAUP yielded a single most parsimonious tree with 29 steps and CI (excluding uninformative characters) of 1.0. The midpoint rooting also gave us a CI of 1.0 (Fig. 4).

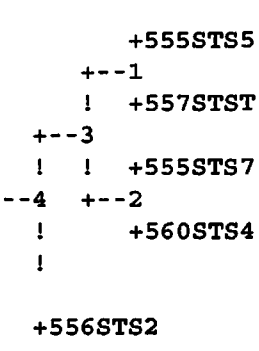
Figure 4. Maximum parsimony analysis of ITS-2 nucleotide data with gaps as a "fifth base." This tree was rooted at midpoint.Branch-and-bound search recovered a single shortest tree of 29
steps with a C.I. of 1.0.

Gaps identified by '-', treated as "fifth base"



The distance method, UPGMA, was used to analyze the similarity of the clones (Fig. 5).

Figure 5. UPGMA phenogram of Kimura distances inferred for ITS-2 data. The tree is rooted at the midpoint of the longest branch.



The distance methods do not use discrete character state data. This method assumes rate constancy. It transforms the original data into a pairwise matrix of distances, and then fit a phylogenetic tree to this matrix. A second assumption of this method is that the expected distances between taxa represent sums of branch lengths along the tree. The main purpose of the distance method is to minimize the differences between the observed and expected distances calculated for the resulting tree (Nadler 1990). Looking at the consensus tree from UPGMA output (Fig. 6), we see, as expected, the two 555Sts clones being most similar and the 556 clone as most dissimilar, a slightly different result from the pair-wise comparison.

Figure 6. Consensus of 100 UPGMA phenograms, ITS-2 data, default Kimura distance. Majority-rule and strict consensus tree program, version 3.57c
Species in order: 555STS5,555STS7,557STST,560STS4,556STS2

Sets included in the consensus tree

Set (species in order)	How many times out of 100.00
****.	97.00
.*.*.	62.00
...	62.00

Sets NOT included in consensus tree:

Set (species in order)	How many times out of 100.00
**...	38.00
***..	37.00
.*.**	2.00
***.*	1.00
**..*	1.00

Figure 6 (Continued).

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CONSENSUS TREE:
the numbers at the forks indicate the number
of times the group consisting of the species
which are to the right of that fork occurred
among the trees, out of 100.00 trees

            +----557STST
      +-62.0
      !     +----555STS5
  +-97.0
  !     !     +----555STS7
  !     +-62.0
  !           +----560STS4
  !
  +--------------556STS2
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The main purpose of the parsimony analysis is to find the arrangement of taxa and branch points that minimizes the number of character state changes required to account for observed variation in the data set. This process is often called " character optimization." The main purpose of parsimony for the nucleic acid sequence data, is to find the network (unrooted tree) with the minimum number of substitutions. The network is rooted by reference to one or a series of outgroups (Nadler 1990). Our data indicates that the 560Sts is most different from other clones which is in agreement with our pair-wise comparison data.

ITS-1 :

In addition to comparisons of ITS-2 data generated in our laboratory, we used the ITS-1 data on *Strongyloides* species from Genbank (Figs 7 & 9A): three different *Strongyloides* species, and *S. stercoralis* from 5 different geographic regions were compared.

FIGURE 7.
SOURCES FOR ITS-1 SEQUENCES (FROM GENBANK)

Species	Source	Accession Number (From Genbank)	Remarks (File name)
S. fulleborni	Rheusus monkey	U43581	NSFULL
S. ratti	Rat	U 43580	NSRATTI
S. stercoralis	Carribean	U43578	NSSTCA
S. stercoralis	Dog	U 43962	NSSTDO
S. stercoralis	S.E. Asia	U43576	NSSTSE
S. stercoralis	W.est Virginia	U43579	NSSTWV
S. stercoralis	Unknown	U43577	NSSTUNK

From the pair-wise comparison (Figs. 8) of the ITS-1 data, the results show the greatest similarity among the *S. stercoralis* isolates from the different geographic regions, and the most difference was between the different species, as expected.

FIGURE 8. PERCENT SIMILARITY OF ITS-1 SEQUENCES

	NSFULL	NSRATTI	NSSTCA	NSSTDO	NSSTSE	NSSTWV	NSST UNK
NSFULL		196/245 (80 %)	201/245 (82 %)	191/245 (78 %)	203/245 (83%)	184/245 (75%)	203/245 (83%)
NSRATTI			218/266 (82%)	211/268 (79%)	218/268 (80%)	201/249 (80%)	219/268 (82%)
NSSTCA				246/266 (92%)	265/266 (99.6 %)	249/249 (100%)	266/266 (100%)
NSSTDO					242/268 (90.3%)	236 /249 (95%)	242/268 (90.3%)
NSSTSE						248/249 (99.6%)	266/268 (99.3%)
NSSTWV							268/268 (100%)
NSST UNK							

Multiple alignment for the ITS-1 region for the data from Genbank is yielded the following results (Fig. 9A).

Figure 9A. Multiple sequence alignment for the ITS-1 region for the different species of Strongyloides and geographic isolates for the S. Stercoralis (Also refer to fig. 7).

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SFULL           GGTGAACCTGCAGAAGGATCATTATGAT-------------TTTTA----
SRATI           GGTGAACCTGCAGAAGGATCATTTTGAAAAAAATATA-TTATAATAAAAA
SSTCA           GGTGAACCTGCAGAAGGATCATTATGATAAAAATATAATTATTTTA----
SSTDO           GGTGAACCTGCAGAAGGATCATTATGATTA---TA-AATTATTTTA----
SSTSE           GGTGAACCTGCAGAAGGATCATTATGATAAAAATATAATTATTTTA----
SSTUNK          GGTGAACCTGCAGAAGGATCATTATGATAAAAATATAATTATTTTA----
SSTWV           GGTGAACCTGCAGAAGGATCATTATGATAAAAATATAATTATTTTA----
                ********************** ***              *  **

SFULL           T-------TTT-----------TTTTA--ATAAA---ATAAAAAAA----
SRATI           TAATTTTTTTTTATATATATATATTTTTTTTTAAAAATATATTATAATAAA
SSTCA           TAATATA---TAAA-----ATATTTTATAATAAACACATAAGATGGTACG
SSTDO           TAATATAATTTAAA-----ATATTTTATAATAAACACATAAGATGGTACG
SSTSE           TAATATA---TAAA-----ATATTTTATAATAAACACATAAGATGGTACG
SSTUNK          TAATATA---TAAA-----ATATTTTATAATAAACACATAAGATGGTACG
SSTWV           TAATATA---TAAA-----ATATTTTATAATAAACACATAAGATGGTACG
                *         *           ****    ****   ***  *

SFULL           -AAAAAATTTT-TATAATAAACACATTGT------AATGGTACGTAATTT
SRATI           CACATTGAGTGGTACGTATTCTTAAAATTAAATTAAAATAAATTTATATT
SSTCA           TAAAAAAA-TT-TAAAAAAA-TAAATTTTT-ATTTATT-ATTTTT-----
SSTDO           TAAAAAAAATT-TAAAAAAAATAAATTTTTTATTTATTTATTTTTTTTTT
SSTSE           TAAAAAAA-TT-TAAAAAAA-TAAATTTTT-ATTTATT-ATTTTT-----
SSTUNK          TAAAAAAA-TT-TAAAAAAA-TAAATTTTT-ATTTATT-ATTTTT-----
SSTWV           TAAAAAAA-TT-TAAAAAAA-TAAATTTTT-ATTTATT-ATTTTT-----
                 * *     *  **          *   *      *        *

SFULL           TAATTTAAATAA---CGTTTAAATAG-ATATTTTAAATAATATCAGCCAT
SRATI           TATTTTATTTTAATACGTTTAAATAGTAAGAAGTAATTTTTA-CAGCCAC
SSTCA           ------AAATAAA--CGTTTAAATAG-ATATTTTAATTAATATCAGCCAT
SSTDO           TTTTTTAAATAAA--CGTTTAAATAG-ATATTTTAATTAATATCAGCCAT
SSTSE           ------AAATAAA--CGTTTAAATAG-ATATTTTAATTAATATCAGCCAT
SSTUNK          ------AAATAAA--CGTTTAAATAG-ATATTTTAATTAATATCAGCCAT
SSTWV           ------AAATAAA--CGTTTAAATAG-ATATTTTAATTAATATCAGCCAT
                      *  * *   *********** *     *** *  ** ******

SFULL           TAAATTTTATTCTTA-TACAAATTTTTTTGTTATCTATTATTTATAATAA
SRATI           TTGAAACT-TTTATACAATACATTTTTTTTTTAT-AATTAAAAATA-TAA
SSTCA           CAAATATTATTTT-AATATATAATTTTTT-TTATTAAATAAAAATAA-AA
SSTDO           CAAATATTATTTT-AATACATAATTTTTT-TTATTAAATAAAAATAA-AA
SSTSE           CAAATATTATTTT-AATATATAATTTTTT-TTATTAAATAAAAATAA-AA
SSTUNK          CAAATATTATTTTTAATATATAATTTTTT-TTATTAAATAAAAATAA-AA
SSTWV           CAAATATTATTTT-AATATATAATTTTTT-TTATTAAATAAAAATAA-AA
                   *   * **   *  * * * ****** ****  * **   ***  **
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Figure 9A continued.

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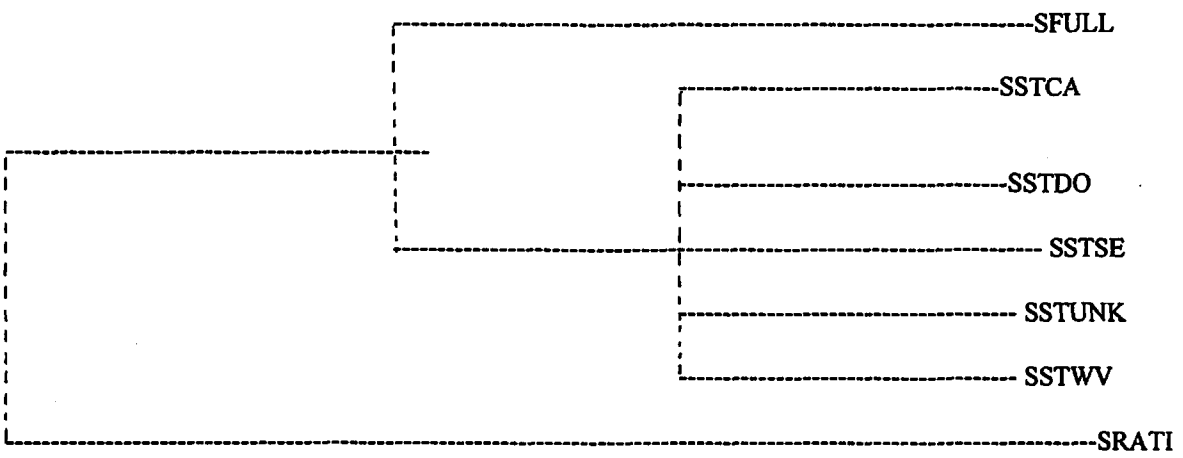
SFULL           AATAATATATAAAATCGTGTCGGTGGATCATTCGGTTCATAGGTCGATGA
SRATI           A------TATAAAATCGTGTCGGTGGATCATTCGGTTCATAGGTCGATGA
SSTCA           ATTTTTTTATAAAATCGTGTCGGTGGATCATTCGGTTCATAGGTCGATGA
SSTDO           ATTTTTT-ATAAAATCGTGTCGGTGGATCATTCGGTTCA----------
SSTSE           ATTTTTT-ATAAAATCGTGTCGGTGGATCATTCGGTTCATAGGTCGATGA
SSTUNK          ATTTTTTTATAAAATCGTGTCGGTGGATCATTCGGTTCATAGGTCGATGA
SSTWV           ATTTTTTTATAAAATCGTGTCGGTGGATCATTC----------------
                *       *************************

SFULL           AG------
SRATI           --------
SSTCA           --------
SSTDO           --------
SSTSE           AGAGCGCA
SSTUNK          A-------
SSTWV           --------
```

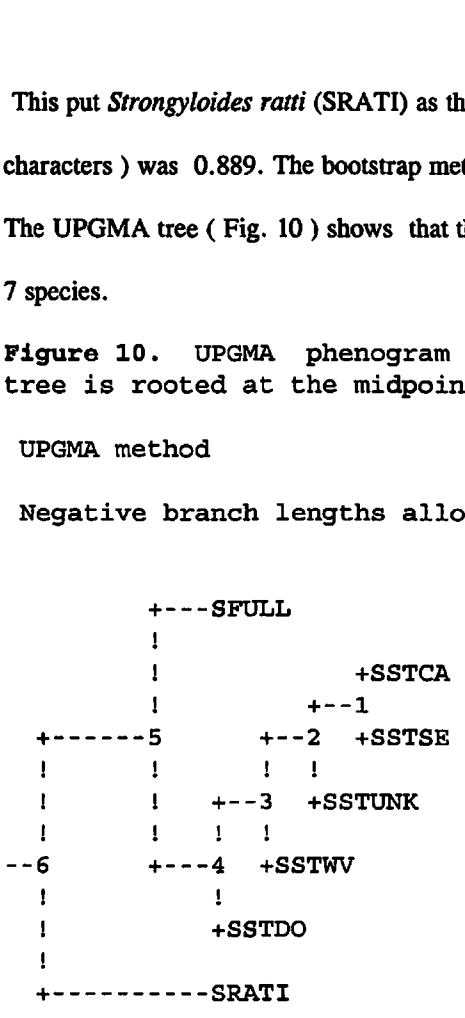
The analysis of the ITS-1 data was done with the same two methods used in ITS-2 data analysis. The parsimony method yielded shortest trees of 99; the number of trees retained = 75, and midpoint rooting gave us the strict consensus tree seen in Fig. 9B.

FIGURE 9B. Maximum parsimony analysis of ITS-1 nucleotide data. This tree was rooted at midpoint of the longest tree. Branch-and-bound method recovered the tree of 75 steps with C.I.(excluding uninformative characters) of 0.889.



This put *Strongyloides ratti* (SRATI) as the most different from the other taxa. The CI (excluding uninformative characters) was 0.889. The bootstrap method gives us SRATI and SFULL as basal to the *S. stercoralis* taxa. The UPGMA tree (Fig. 10) shows that the five *S. stercoralis* species are the most similar among the group of 7 species.

Figure 10. UPGMA phenogram of Kimura distances inferred for ITS-1 data. The tree is rooted at the midpoint of the longest branch.



The consensus tree (Fig. 11) is consistent with the UPGMA method. This is also expected from our pair-wise comparison analysis.

Figure 11. Consensus of 100 UPGMA phenograms of default Kimura distance of ITS-1 data. The tree is rooted at the midpoint of the longest branch.

Species in order:

SFULL
SSTCA
SSTSE
SSTUNK
SSTWV
SSTDO
SRATI

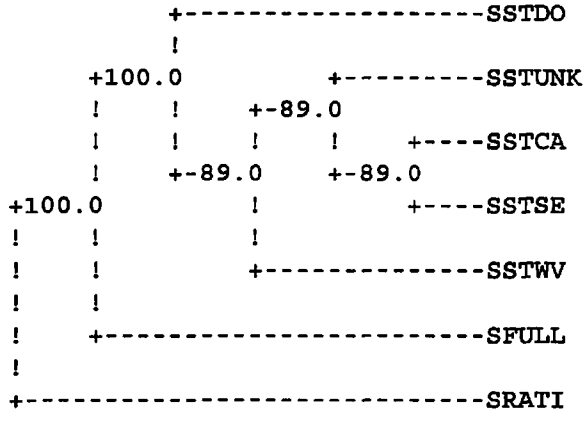
Sets included in the consensus tree

Set (species in order)	How many times out of 100.00
.*****.	100.00
******.	100.00
.****..	89.00
.***...	89.00
.**....	89.00

Sets NOT included in consensus tree:

Set (species in order)	How many times out of 100.00
.*...*.	11.00
.***.*.	11.00
.**..*.	11.00

CONSENSUS TREE:
the numbers at the forks indicate the number of times the group consisting of the species which are to the right of that fork occurred among the trees, out of 100.00 trees



Conclusion:

In observing the sequence data of the ITS-2 region of *S. stercoralis* from four different geographic regions, our study indicates there is very little variability among the four geographic isolates. However, an interesting observation was made in that the two strains from G. Schad's labs showed the most variability. This was as a result of the two strains having 2 different life cycles: 1. autoinfective, and 2. free-living. The observed level of difference may be consistent with species-level differences. However, further investigative study is needed to confirm this hypothesis.

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