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Sequences of the second internal transcribed spacer of ribosomal DNA for three species of *Trichostrongylus* (Nematoda: Trichostrongylidae) from sheep in Russia

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Summary

For the first time, DNA sequence data were obtained for three species of *Trichostrongylus* from Russia. Internal transcribed spacer (ITS-2) of ribosomal DNA was sequenced for *T. axei, T. colubriformis* and *T. probolurus* from sheep from the Moscow region. ITS-2 rDNA length was estimated as 238 nucleotides for *T. colubriformis* and *T. probolurus* and 237 nucleotides for *T. axei*. The G+C content of the ITS-2 sequences of *T. colubriformis, T. axei* and *T. probolurus* were 31 %, 32 % and 34 % respectively. The level of interspecific differences in ITS-2 of rDNA of *T. axei, T. probolurus* and *T. colubriformis* ranged from 3 to 4 %. The ITS-2 sequences from the Russian specimens were compared with those of *T. axei*, *T. probolurus* and *T. colubriformis* from Australia and Germany. Intraspecific variation ranged from 0 % in *T. colubriformis* to 3.0 % in *T. axei*.

Key words: *Trichostrongylus* spp.; ITS-2; interspecific differences; intraspecific variation; sheep; Russia

Introduction

Trichostrongyle nematodes are widespread in mammals (artiodactyles, tylopoda, rodents, lagomorphs, primates) in many countries throughout the world, including Russia (Asadov, 1960; Govorka *et al*., 1988; Pryadko, 1976; Skrjabin *et al*., 1954). Infection of the gastrointestinal tract by trichostrongyle nematodes frequently causes a detrimental effect to cattle breeding because of the impact on productivity slowdown in adults and young animals' survivability (Akbaev *et al.,* 1998; Boev *et al.,* 1963; Skrjabin & Petrov; 1964). Quite often the same host can harbour several species of trichostrongyle nematodes, which differ in some details of their life cycle (Boev *et al.,* 1963; Ivashkin *et al.,* 1989). Reliable species differentiation of *Trichostrongylus* spp. is essential in effective control of these parasites. Specific differentiation based on morphology is quite secure for trichostrongyle males but not possible for females and eggs. Existing methods of differentiation based on larvae morphology are still quite laborious (Hoste *et al*., 1995, 1998; McMurtry *et al.*, 2000).

It has been shown that the second internal transcribed spacer (ITS-2) of ribosomal DNA is a valuable target region for species differentiation within the genus *Trichostrongylus* because of a lack or low levels of intraspecific variation compared with the level of interspecific sequence differences (Chilton *et al.*, 1998; Heise *et al.*, 1999; Hoste *et al*., 1993, 1995; Wimmer *et al.,* 2004). However, there is no DNA data available for *Trichostrongylus* spp. found in the territory of Russia. The study of ITS-2 rDNA data for *Trichostrongylus* spp. from Russia is adding new data to the knowledge of this rDNA domain in trichostrongyle nematodes and can reveal fixed genetical differences. The aim of the present study was to compare the ITS-2 sequences of species of *Trichostrongylus* from ruminants from the territory of Russia with those previously published from other regions of Europe and from Australia.

Material and Methods

Male worms of *Trichostrongylus axei* (Cobbold, 1879)*, T. colubriformis* Giles, 1892 and *T. probolurus* (Railliet, 1896) were removed from the abomasum of a spontaneously infected sheep originating from a private farm in Moscow region. The material was not exposed to any preservative solution. Nematodes were examined using a microscope and identified according to the key of Ivashkin *et al.* (1989). A single specimen of each species was used. Genomic DNA was isolated from nematodes using the procedure as described by Dallas *et al.* (2000), but with minor changes. Each worm was placed in 100 µl of 10 mM-Tris-Hcl, 0.1 mM-EDTA, pH 8.0, containing 20 µg/

ml-proteinase K in Eppendorf ™ 0.5 ml tubes. Samples were incubated in a thermostat for 18 h at 55°C. Then the homogenate was heated at 95°C for 10 min and stored at -20°C.

The ITS-2 was amplified by polymerase chain reaction (PCR) using primers NC1 (forward; 5'-ACGTCTGGTT CAGGGTTGTT-3') and NC2 (reverse; 5'-TTAGTTTCT TTTCCTCCGCT-3') (Gasser *et al.,* 1993). PCR was performed using Sileks M ™ DNA-amplification kit (Moscow, Russia) in a 25 µl reaction volume according to the manufacturer's protocol. PCR was performed according to the scheme: 1st step – denaturation of the DNA at 90°C for 2 min, 2nd step - denaturation of the DNA at 90°C for 15 s, 3rd step – annealing of the primers at 55°C for 15 s, 4th step – extension of the chain at 72°C for 30 s, 5th step – extension of the chain at 72°C for 5 min. Steps 2nd to 4th were repeated 30 times. PCR products were detected using electrophoresis on 1 % agarose gel. The DNA isolation from the agarose gel was carried out using Sileks M ™ DNA-isolation kit according to the manufacturer's protocol.

The ITS-2 of each worm was sequenced twice in both directions using ABI "Prism"™ automatic sequencer. The sequences alignment was conducted using the Clustal V program (DNAstar ™ computer application package).

The ITS-2 sequences for three *Trichostrongylus* species from Russia were compared with those of *T. axei* from bison in Australia (Hoste *et al.,* 1995) and from ruminants in Germany (Heise *et al.,* 1999), *T. colubriformis* from sheep in Australia (Hoste *et al.,* 1993) and from ruminants in Germany (Heise *et al.,* 1999), and *T. probolurus* from

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                    1                   20                  40                  60
                    |--------|---------|---------|---------|---------|---------|
T.axei              AATGAATTTCTACAGTGTGGCTAACTCTAACACTGTTTGTCGAATGGTCATTGTCAAATA
T.axei*             ............................................................
T.axei**            ............................................................
T.colubriformis     ............................................................
T.colubriformis*    ............................................................
T.colubriformis**   ..............................................W.............
T.probolurus        ............................................................
T.probolurus*       ............................................................

                    61                  80                  100                 120
                    |--------|---------|---------|---------|---------|---------|
T.axei              TTGTGATAATTCCCATTTCAGTTTAAGAATAATACATGCAACATGATGTTAATGTTG-AA
T.axei*             ..................T.......................................-..
T.axei**            ..................T.......................................-..
T.colubriformis     .......G.............C.................................T..
T.colubriformis*    .......G.............C.................................T..
T.colubriformis**   .......G.............C.................................T..
T.probolurus        ......................CC.......C.........................T..
T.probolurus*       ......................C.......C.........................T..

                    121                 140                 160                 180
                    |--------|---------|---------|---------|---------|---------|
T.axei              CGACATTAATRTCCCTGTATGATGTGAACGTGTTG-TCACTGTTTGAATGTACTCAGTGA
T.axei*             ...................................-........Y..........W....
T.axei**            ..........G.............-.C...C.A..A.....................
T.colubriformis     T.........G.T.......................-.T.....................
T.colubriformis*    T.........G.T.......................-.T.....................
T.colubriformis**   T.........G.T.......................-.T...R.................
T.probolurus        ..........G.T.......................-.....................
T.probolurus*       ..........G.T.......................-.....................

                    181                 200                 220                 240
                    |--------|---------|---------|---------|---------|---------|
T.axei              ATTTGAGATTGATTTAAACAGGGACATGTATGACAATAATGTTCAATTATCATTTGTAT
T.axei*             ............................................................
T.axei**            ..C............T.............................................
T.colubriformis     ...............................A............................
T.colubriformis*    ...............................A............................
T.colubriformis**   ...............................A............................
T.probolurus        .G............C..T.....................................
T.probolurus*       .G..............G.....................................
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Fig. 1. Alignment of the ITS-2 sequences (5' to 3') of *T. axei, T. colubriformis* and *T. probolurus* according to our results, Hoste *et al.* (1995) for *T. axei**, Hoste *et al.* (1993) for *T. colubriformis**, Chilton *et al.* (1998) for *T. probolurus**, Heise *et al.* (1999) for *T. axei*** and *T. colubriformis***. (.) – same base as *T. axei* sequence, (-) – base deletion

sheep in Australia (Chilton *et al.,* 1998).
The sequence of rodent parasite *T. retortaeformis* (Hoste *et al.,* 1995) was used as outgroup in the phylogenetic analysis. The dendrogram was generated using program PAUP 4.0b 10 (Swofford, 1998) using maximum parsimony algorithm (MP).
Sequences obtained in the course of this study were deposited in the NCBI GenBank: *T. axei* - EF427622; *T. probolurus* - EF427623; and *T. colubriformis* - EF427624.

Results

The ITS-2 sequences for three *Trichostrongylus* species are shown in Fig. 1. The length of ITS-2 rDNA fragments was 238 nucelotides for *T. colubriformis* and *T. probolurus* and 237 nucleotides for *T. axei*.
The G+C content of the ITS-2 sequences of *T. colubriformis, T. axei* and *T. probolurus* were 31 %, 32 % and 34 % respectively.
The ITS-2 fragments for three *Trichostrongylus* species studied identical in 92 %: the sequences coincided for 218 out of 238 nucleotides. Five basic regions of interspecific homology (positions 1 – 67, 93 – 117, 134 – 144, 199 – 211 and 213 – 239; Fig. 1) can be distinguished. In most cases variable positions demonstrated single base substitutions (point mutations). Single base deletion was reported for positions 118, 145 and 156.
Nine of the point mutations involved substitutions between pyrimidines (i.e. C with T). Six substitutions between a purine and a pyrimidine were discovered. In 198 position in *T. axei* and *T. colubriformis*, there was always a purine (A), whereas in *T. probolurus*, there was a pyrimidine (T) according to our data and a purine (G) according to Chilton *et al.* (1998).
The level of interspecific differences in the ITS-2 of *Trichostrongylus* spp. of our collection was at 3.5 % for *T. axei* and *T. probolurus*, 3.0 % for *T. axei* and *T. colubriformis,* and 3.9 % for *T. probolurus* and *T. colubriformis*.
The dendrogram (Fig. 2) represents the similarity level among the ITS-2 sequences of three species of *Trichostrongylus* and the intraspecific isolates. It is possible to clearly mark only two clusters at the phylogenetic tree, *T. colubriformis* and *T. probolurus*. These two close species differ well, and their geographical isolates group closely with high bootstrap-support.
Another species, *T. axei*, particularly the Russian isolate, is characterized by relatively high level of interspecific and intraspecific differences.

Discussion

Sequence data of the ITS-2 rDNA for three *Trichostrongylus* spp. from the territory of Russia have been obtained for the first time. A comparison of the ITS-2 sequences of *T. axei, T. colubriformis* and *T. probolurus* from sheep from Moscow region revealed interspecific differences ranging from 3 % to 4 %. When compared with the ITS-2 sequences of *Trichostrongylus* spp. from Australia and

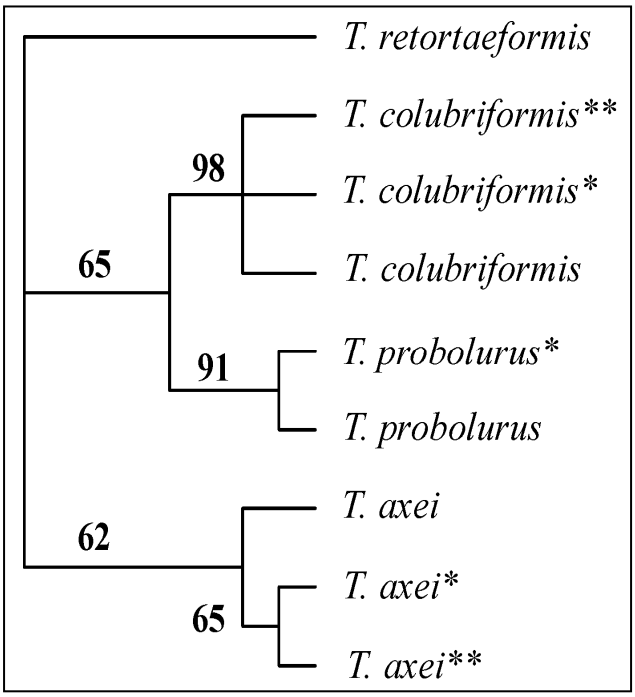

Fig. 2. Consensus tree depicting the ITS-2 similarity of three *Trichostrongylus* species according to our results, Hoste *et al.* (1995) for *T. axei**, Hoste *et al.* (1993) for *T. colubriformis**, Chilton *et al.* (1998) for *T. probolurus**, Heise *et al.* (1999) for *T. axei*** and *T. colubriformis***

Germany, the extent of the intraspecific variation ranged from 0 % in *T. colubriformis* to 3.0 % in *T. axei*.
Similar results were obtained in ITS-2 studies of *Trichostrongylus* spp. from ruminants collected in different geographic areas (Chilton *et al.,* 1998; Heise *et al.,* 1999; Hoste *et al.,* 1993, 1995). Thus, differences between *T. axei* from bison and *T. colubriformis* from sheep from Australia made 3.4 % (Hoste *et al.*, 1995). The level of homology between *T. axei* and *T. colubriformis* from ruminants from Germany was at 95.0 % (Heise *et al.*, 1999). Hoste *et al.* (1995) noted the absence of the ITS-2 intraspecific variations in *T. colubriformis*, whereas Heise *et al.* (1999) registered variations at 1.26 % level.
For analysis of intraspecific differences, comparisons were made between our data and previously published data for *T. axei* from bison and *T. probolurus* and *T. colubriformis* from sheep from Australia (Chilton *et al.,* 1998; Hoste *et al*., 1993, 1995). The 0.9 % differences of sequences in *T. axei* and 1.3 % in *T. probolurus* were found. Intraspecific differences in the ITS-2 of *T. axei* from Russia (our data) and Germany (Heise *et al.,* 1999) were found at 3.0 % level. The same level of interspecific variations in the ITS-2 was found for *T. axei* and *T. colubriformis* (our data, Hoste *et al.,* 1993) which exceeds slightly the differences between *T. axei* and *T. probolurus* from sheep from Australia.
The dendrogram (Fig. 2) represents the high level of similarity between two geographic isolates of *T. probolurus* and lack of intraspecific differences in the ITS-2 of *T. colubriformis*. Relatively high genetic divergence among isolates of *T. axei* can be possibly explained by host-speci-

ficity effect due to presence of intraspecific differences in the ITS-2 of *T. axei* parasitic in bison and in sheep. As Heise *et al.* (1999) did not give the species name of ruminants in Germany from which the material was obtained, it could not be decided whether host specificity effect was present.

Study of the ITS-2 sequences of three species of *Trichostrongylus* from Russia confirmed the low level of genetic differences within each of these species and provided additional information with respect to genetic variation within these parasites from different hosts and geographical regions.

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