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Full Length Research Paper

Characterization of *Toxascaris leonina* and *Tococara canis* from cougar (*Panthera leo*) and common wolf (*Canis lupus*) by nuclear ribosomal DNA sequences of internal transcribed spacers

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In the present study, samples representing *Toxascaris leonina* and *Tococara canis* were collected from cougar (*Panthera leo*) and common wolf (*Canis lupus*) from Harbin Northern Forest Zoo in Heilongjiang Province, China, and they were characterized by nuclear ribosomal DNA (rDNA) sequences of internal transcribed spacers (ITS). The ITS rDNA was amplified by polymerase chain reaction (PCR), then sequenced and compared with that of other members of ascaridida published in GenBank™. Furthermore, the phylogenetic relationship was reconstructed using Maximum Parsimony and Bayesian analyses. For the *T. leonina* of cougar, the length of the ITS sequences was 698 bp (the ITS1 and ITS2 were 419 and 279 bp, respectively) and the corresponding sequences of *T. canis* from common wolf were 808 bp (the ITS1 and ITS2 were 485 and 323 bp, respectively). The identity of ITS sequences of *T. leonina* and *T. canis* from cougar and common wolf was 76%. The ITS sequences were analyzed by PCR-linked restriction fragment length polymorphism (PCR-RFLP) which was established for the unequivocal delineation of the *T. leonina* and *T. canis* with which were easily confused morphologically, using restriction endonuclease *Eco*RV and *Sal*I. The valuable tool established in this study makes the molecular identification, the ecology and genetic structure studies of these two ascaridoids possible.

Key words: *Toxascaris leonina*, *Tococara canis*, ribosomal DNA (rDNA), internal transcribed spacers (ITS), PCR-linked restriction fragment length polymorphism (PCR-RFLP).

INTRODUCTION

Toxascaris leonina is an ascarid nematode which lives in the small intestine of canids (dogs, foxes, wolves, etc.) and felines (cats, lions, etc.) with a worldwide distribution, while the paratenic hosts are usually rodents, such as, mice or rats. Infection occurs in the definitive host when the animal eats an infected rodent. While *T. leonina* can occur in either canids or felines and it is far more frequent in the latter (Sprent and English, 1958; Vanparijs et al., 1991; Awoke et al., 2011). *Toxocara canis* is also distributed worldwide, a helminth parasite of dogs and other canids occurring in the intestine of the definitive host. The other vertebrates except canids can also get infected with *T. canis*; the larvae in the organs and

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tissues can live long-term, but cannot continue to develop. In adult canids, the infection is usually asymptomatic. In contrast, massive invasion of *T. canis* can be fatal in puppies (Jacobs, 1987). As paratenic hosts, a number of vertebrates including humans and some invertebrates can become infected. Humans are infected like other paratenic hosts, by ingestion of embryonated *T. canis* eggs (Gillespie, 1988).

The infection symptoms of *T. leonina* and *T. canis* are similar. It is a common cause of diarrhea in young animals and can cause vomiting as well. For *T. canis*, extreme cases of severe infections can lead to pneumonia as the worms migrate, and for both of them if there are too many worms, the intestine can become obstructed (Webster, 1958; Sprent and English, 1958). The difference of *T. leonina* from *T. canis* is that the larvae do not migrate through the lungs, but rather, the entire developmental cycle occurs in the gut (Matoff and Komandarev, 1965).

The traditional methods to identify and differentiate the two parasites are based on host preference and morphological characteristics. But morphological approaches has limitations in identifying and distinguishing closely related species particularly at larval and egg stages (McManus Bowles, 1996). The original identification of the parasite now known as *T. canis* is attributed to Werner in 1782 (Webster, 1958), but until now, there have been confusion between it and related species, particularly with *T. leonina*, which was not clearly differentiated from *T. canis* until described by Lieper (Matoff and Wassileff, 1958). *T. leonina* and *T. canis* are very similar and both are nematodes of the small intestine. The definitive hosts of *T. leonina* include canids and felines, while the *T. canis* lives in the small intestine of dogs and other canids. The larvae of *T. canis* may invade the human body and cause the 'larva migrans' such as ocular larva migrans (OLM), visceral larva migrans (VLM), eosinophilic meningoencephalitis (EME) and/or covert toxocariasis (CT) (Despommier, 2003; Vidal et al., 2003), while there are rarely reports of human infected with *T. leonina* (Kim et al., 2008). *T. leonina* can be differentiated from *T. canis* by the cephalic alae, but this requires professional expertise and specialized skill.

Previous studies have shown that the sequences of the first and/or second internal transcribed spacers (ITS) of ribosomal DNA (rDNA) provide genetic markers for molecular systematic studies of many parasite groups (Zhu et al., 2007; Wang et al., 2009). Before the present study, there had been no reports characterizing *T. leonina* and *T. canis* from cougar and common wolf using well-defined DNA sequences. Furthermore, it is yet to know whether ITS rDNA can provide a genetic marker for the accurate identification and differentiation of *T. leonina* and *T. canis*. Accordingly, the aim of this study was to characterize samples representing *T. leonina* and *T. canis* from cougar and common wolf using ITS rDNA as genetic marker, and to establish a PCR-linked restriction fragment length polymorphism (PCR-RFLP) approach for their unequivocal differentiation.

MATERIALS AND METHODS

Parasites and DNA extraction

Ten adult nematodes representing *T. leonina* and ten adult nematodes representing *T. canis* were collected from the small intestines of infected cougar and common wolf respectively, from Harbin Northern Forest Zoo in Heilongjiang Province, China. Individual worms were washed thoroughly in physiological saline, and were then identified morphologically as *T. leonina* and *T. canis* according to existing keys and descriptions (Matoff and Wassileff, 1958; Webster, 1958) based on the different shape of the cephalic alae. The samples were fixed in 70% ethanol and frozen at -20°C until extraction of genomic DNA. Total genomic DNA was extracted from a portion of each individual adult worms according to the instructions of TIANamp Genomic DNA Kit (commercially available) produced by TIANGEN (Beijing, China). Genomic DNA was stored at -20°C for further experiments.

PCR amplification, cloning and sequencing of the ITS rDNA

The ITS rDNA plus primer flanking 18S and 28S sequences were amplified by PCR from each nematode DNA sample using primers 1 (forward; 5'- GGAAACCGCCTTAATCGCAGT -3') and 2 (reverse; 5'- TTCGCTTCGCCACCCGTACA -3') designed by the authors. PCR (25 µl in volume) were performed in 2.5 µl 10×EX *Taq* Buffer; 2 µl dNTPs; 0.5 µl of each primer, 0.2 µl EX *Taq* polymerase (TaKaRa) and 1 µl of genomic DNA and add deionized water up to 25 µl in a thermocycler under the following conditions: 10 min of initial denaturation at 94°C, followed by 36 cycles of 94°C for 1 min (denaturation); 55°C for 1 min (annealing); 72°C for 1.5 min (extension), followed by 72°C for 10 min (final extension). Five microliter of each reaction product was analyzed by electrophoresis on a 1% (w/v) agarose gel, stained with ethidium bromide and photographed using a gel documentation system (UVItec).

The PCR products were purified using the AxyPrep^{TM} DNA gel extraction Kit (Axygen) following by the manufacturer's recommendations. The purified products were ligated with pMD18-T vector (TaKaRa) and transformed into the DH5α competent cells. The recombinant bacterium was screened, identified by enzyme digestion with restriction endonuclease *Eco*RI and *Hind*Ⅲ, and then sequenced by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). Three clones for each of the two representative nematodes representing each species were sequenced.

RFLP analysis of the ITS amplicons

Analysis of the ITS sequences for *T. leonina* and *T. canis* obtained in the present study using the software DNAStar (Ver 4.0) revealed that, these two species could be delineated by using the restriction endonucleases *Sal*I and *Eco*RV. Based on this information, a PCR-RFLP method was established to distinguish *T. leonina* and *T. canis*. 10 µl column-purified (AxyPrep^{TM} DNA gel extraction Kit) ITS PCR products were digested directly with 18 µl deionized water, 2 µl buffer and 1 µl of the restriction endonuclease *Eco*RV or *Sal*I (Fermentas), respectively, at 37°C for 3 to 4 h in a volume of 31 µl. Restriction fragments were separated on 1% (w/v) agarose gels, stained with ethidium bromide and photographed. The DL2000 DNA size marker (TaKaRa) was used to estimate the sizes of fragments.

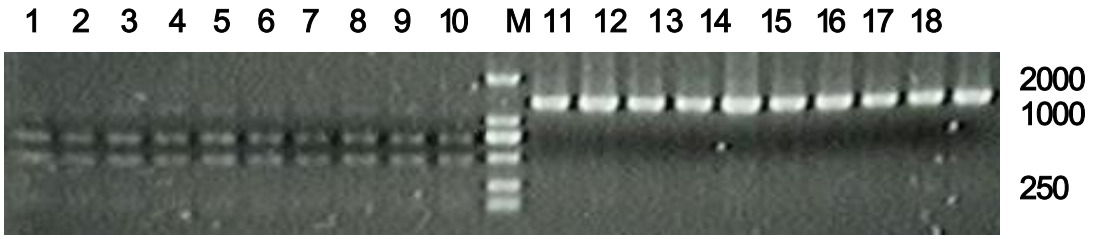

Figure 1. The PCR products of ITS from *T. leonina* and *T. canis* samples were analyzed by the method of RFLP using restriction enzyme *Eco*RV. Lanes 1 to 10, represent *T. leonina* samples; lanes 11 to 20, represent *T. canis* samples; lane M, represents DL2000 DNA marker.

Sequence comparison and phylogenetic analyses

The combined ITS1 and ITS2 sequences of *T. leonina* and *T. canis* were compared with the relevant sequences of other members of ascaridida available in GenBank™. Phylogenetic relationship was reconstructed using Bayesian and maximum parsimony (MP) analyses. Bayes analyses were performed using Mr Bayes 3.1 programme, MP analyses performed by MEGA ver. 3 (Kumar et al., 2004). *Necator americanus* was used as outgroup.

RESULTS

A total of 20 nematode samples were used to extract genomic DNA, 10 represented *T. leonina* and the others 10 represented *T. canis*. The length of the amplified ITS fragments (including partial 18S and 28S rDNA sequences) from these samples were approximately 1200 bp (*T. leonina*) and 1300 bp (*T. canis*) respectively. All samples were presented as a single band (not show). The length of the ITS sequences of *T. leonina* from cougar was 698 bp (the ITS1 and ITS2 were 419 bp and 279 bp, respectively) and the corresponding sequences of *T. canis* from common wolf were 808 bp (the ITS1 and ITS2 were 485 bp and 323 bp respectively). There were a total of 117 nucleotide differences in ITS sequences between the *T. leonina* and *T. canis*. Compared with the two ITS sequences, the identity between them was 76%.

Based on the restriction maps generated for the ITS sequences of the *T. leonina* and *T. canis* (not show), a PCR-RFLP approach was established to differentiate them using restriction endonuclease *Eco*RV and *Sal*I, respectively. The undigested ITS PCR products of *T. leonina* was approximately 1200 bp and that of *T. canis* was approximately 1300 bp in length. When the purified ITS PCR products were digested by restriction endonuclease *Eco*RV, the *T. leonina* and *T. canis* produce distinct restriction profiles allowing the identification and differentiation of the two ascarid species (Figure 1). The ITS PCR products of *T. canis* samples remained undigested (1300 bp in length), but that of *T. leonina* samples were digested into two bands of approximately 480 and 720 bp respectively (Figure 1). When the purified ITS PCR products were digested by restriction endonuclease *Sal*I, The ITS PCR products of *T. leonina* samples remained undigested (approximately 1200 bp in length), but that of *T. canis* samples were digested into two bands of 550 and 750 bp respectively (Figure 2). The lengths of all the digestion fragments were accordant with calculations based on the restriction maps (not shown).

The phylogenetic relationships of *T. leonina* and *T. canis* with other members of ascaridida nematodes were reconstructed by the MP utilizing the combined ITS1 and ITS2 sequences shown in Figure 3, and the Bayesian analyses results is the same as MP (not shown).

DISCUSSION

For the metazoan parasites, ITS1 and ITS2 are located between the 18S, 5.8S and 28S coding regions of the nuclear ribosomal DNA, and have proven useful for diagnostic purposes at the species level (Morgan and Blair, 1995; Nolan and Cribb, 2005). As useful molecular markers, ITS sequences have been demonstrated for other parasitic helminths which can serve as an effective genetic marker for identification (Zhu et al., 2007; Wang et al., 2009).

The ITS sequences obtained from the present study were compared with those published in GenBank and the results indicated that the ITS1 and ITS2 sequences of ascarids from common wolf was identical to that of *T. canis* published previously (Ishiwata et al., 2004; Wickramasinghe et al., 2009). The ITS sequence of ascarid from cougar was identical to that of *T. leonina* (Accession Nos. HM800922 and HM800923) in GenBank.

The phylogenetic tree was reconstructed based on the sequences of *T. leonine*, *T. canis* and other members of ascaridida. The results showed that the roundworm from common wolf represented *T. canis* belonging to *Toxocara* and the roundworm from cougar represented *T. leonina* belonging to *Toxascaris*. In brief, the roundworm from common wolf and cougar represent two different genera, but they were extremely similar morphologically.

PCR-RFLP is a useful molecular technique that exploits variations in homologous DNA sequences, and can provide reliable tool for identification purposes

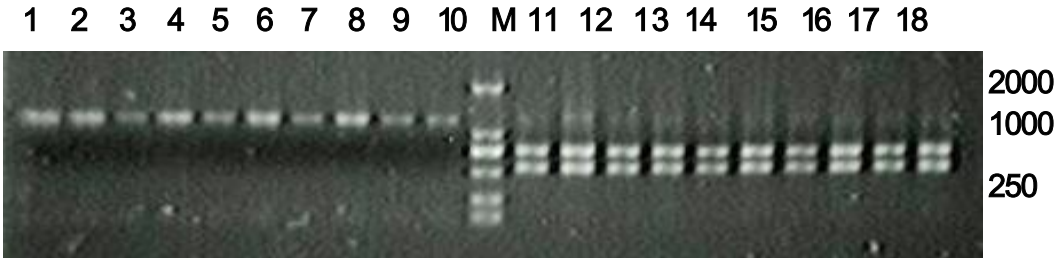

Figure 2. The PCR products of ITS from *T. leonina* and *T. canis* samples were analyzed by the method of RFLP using restriction enzyme. Lanes 1 to 10, represent *T. leonina* samples; lanes 11 to 20, represent *T. canis* samples; lane M, represents DL2000 DNA marker.

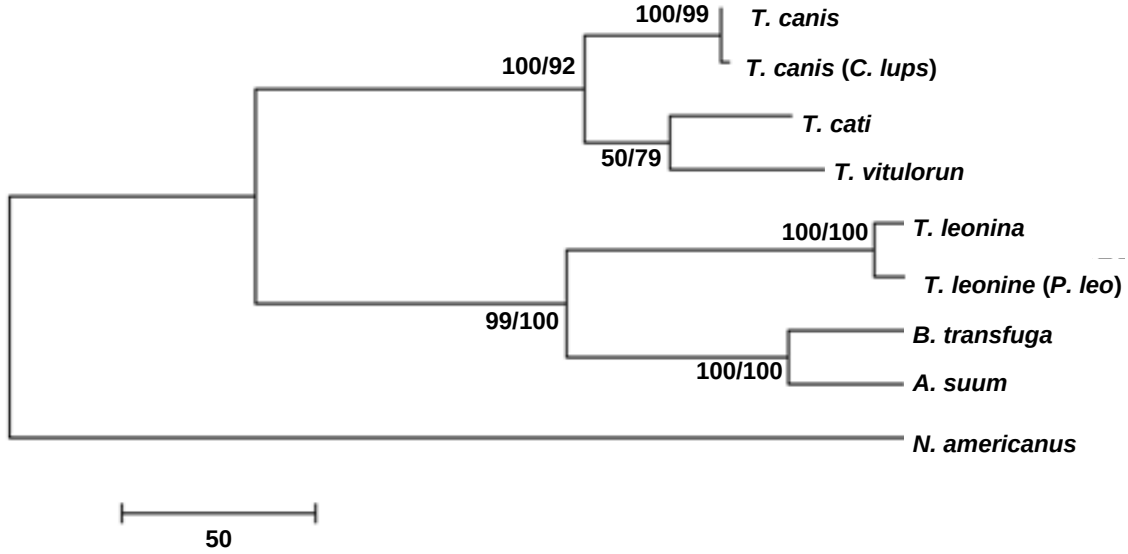

Figure 3. Phylogenetic relationships of *T. leonina* and *T. canis* with other members of the ascaridida reconstructed by maximum-parsimony method based on the ITS sequence or combined ITS-1 and ITS-2 sequences, using *Necator americanus* (GenBank™ Accession Nos. AF217891) as the outgroup. The following roundworms are used: *Toxocara canis* (AB110024 and AB110032), *Toxocara cati* (AB571303), *Toxocara vitulorum* (FJ418784 and AJ007455), *Toxascaris leonina* (HM800922 and HM800923), *Baylisascaris transfuga* (AB571304) and *Ascaris suum* (AB571302). Numbers at the branch nodes indicate percentage bootstrap support for 3000 replicates.

(Nuchprayoon et al., 2006; Wang et al., 2012). PCR-RFLP approach established in the present study using ITS sequences as genetic marker provides a simple but yet powerful tool for the unequivocal and accurate identification and differentiation of *T. leonina* and *T. canis*.

T. leonina and *T. canis* cause significant economic losses in wild animals and *T. canis* is a potential threat to the health of human beings (Despommier, 2003; Vidal et al., 2003; Kim et al., 2008; Li et al., 2008). The identification and differentiation of *T. leonina* and *T. canis* in the past has been based on morphological characteristics (Matoff and Wassileff, 1958; Webster, 1958), and both of them can make cross-infection on wild animals. It increased the difficulties to identify and discriminate *T. leonina* and *T. canis* by morphology, particularly at the larval and egg stages. In the present study, sequence analysis of ITS rDNA from the two nematodes revealed that the ITS sequences provide reliable genetic markers for the identification and differentiation of the two nematodes accurately. For the unequivocal and accurate identification and differentiation of *T. leonina* and *T. canis*, a PCR-RFLP method established using the ITS sequences as the genetic markers provides a simple but yet practical tool.

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