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Characterization of *Bunostomum trigonocephalum* and *Bunostomum phlebotomum* from sheep and cattle by internal transcribed spacers of nuclear ribosomal DNA

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ABSTRACT

In the present study, samples representing *Bunostomum trigonocephalum* and *Bunostomum phlebotomum* from sheep and cattle in Heilongjiang Province, China, were characterized and grouped genetically by the first (ITS-1) and second (ITS-2) internal transcribed spacers (ITS) of nuclear ribosomal DNA (rDNA). The rDNA region including the ITS-1, 5.8S, ITS-2, and flanking 18S and 28S rDNA sequences was amplified by polymerase chain reaction (PCR), then sequenced and compared with that of other members of the hookworms available in GenBank™, and phylogenetic relationships between them were reconstructed using the Maximum-Parsimony method. The ITS-1, 5.8S, and ITS-2 sequences of the sheep hookworm were 381, 153, and 231 bp in length, respectively, and the corresponding sequences of the cattle hookworm were 392, 153, and 240 bp in length. The identity of ITS sequences of *B. trigonocephalum* and *B. phlebotomum* from sheep and cattle was 87.4%. A PCR-linked restriction fragment length polymorphism (PCR-RFLP) assay using restriction endonuclease *Nde* I was established for the unequivocal differentiation of the two hookworm species. Phylogenetic analyses based on the ITS sequences revealed that *B. trigonocephalum* and *B. phlebotomum* were closely related, but they represent two different species.

1. Introduction

Members of the Family Ancylostomatidae are commonly called hookworms and are parasitic nematodes of humans and animals, causing the death of humans and animals, as well as considerable economic losses to the livestock industries. *Bunostomum trigonocephalum* and *Bunostomum phlebotomum* are two such hookworms, with the former mainly parasitizing in the small intestine of sheep, and the latter residing in the cattle's small intestine (Tang and Tang, 1987). Larval penetration of the lower limbs may cause uneasiness and stamping, particularly in grazing cattle and sheep. Adult worms attach to the mucosa of the small intestine and suck blood, causing anemia and rapid weight loss. Diarrhea and constipation may alternate. Hypoproteinemic edema may be present.

Sheep and cattle infection with *B. trigonocephalum* and *B. phlebotomum* has a worldwide distribution (Lima, 1998; Borgsteede et al., 2000; Makovcová et al., 2008; Tariq et al., 2008, 2010). Sheep infection with *B. trigonocephalum* has a high prevalence, especially in India, where prevalence in sheep and goat were 37.7% and 30.1%, respectively (Tariq et al., 2008, 2010). In China, hookworm infection in sheep and cattle is distributed nationwide, with prevalence in sheep ranging between 14.7% and 100%, and in cattle from 7.6% to 40%. (Laboratory of Parasitic Diseases, Veterinary Research Institute of Jilin Province, 1984; Huang and Xie, 1994; Du et al., 1995; Li et al., 1999; Wang et al., 2006). *B. trigonocephalum* and *B. phlebotomum* are among the major parasites of sheep and cattle in Heilongjiang Province with severe pathogenicity, and the prevalence were 14.7% and 29.6%, and the intensity of infection were 1–1114 and 1–152, respectively (The investigation group for parasitic diseases of domestic animals and poultry in Heilongjiang Province, 1986; Wang et al., 2006), causing significant economic losses to the livestock industry. Therefore, effective control and prevention of these two nematodes is important for the livestock industry.

The traditional approaches for the identification and differentiation of parasites have been based on morphological features of parasites. But morphological approaches can have limitations in identifying and distinguishing closely related species, in particular at larval and egg stages (McManus and Bowles, 1996). *B. trigonocephalum* and *B. phlebotomum* are very similar and both are nematodes of the small intestine. *B. trigonocephalum* resides in the small intestine of sheep and goats, sometimes in cattle (Tang and Tang, 1987; Du et al., 1995), while *B. phlebotomum* lives in the

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small intestine of cattle, sometimes in sheep and goats (Laboratory of Parasitic Diseases, Veterinary Research Institute of Jilin Province, 1984; Huang and Xie, 1994). *B. phlebotomum* can only be differentiated from *B. trigonocephalum* by the presence of two pairs of subventral lancets in the buccal capsule and the longer male spicules, which measure 3.5–4 mm, but this requires expertise and skill.

Previous studies have shown that the first (ITS-1) and/or second (ITS-2) internal transcribed spacers (ITS) of nuclear ribosomal DNA (rDNA) provide genetic markers for the accurate identification of many parasite groups (e.g., Wu et al., 2005; Li et al., 2006; Lin et al., 2007; Zhu et al., 2000, 2007a; Wang et al., 2009). But before the present study, there had been no reports characterizing *B. trigonocephalum* and *B. phlebotomum* from sheep and cattle 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 *B. trigonocephalum* and *B. phlebotomum*. Therefore, the objectives of the present study were to characterize samples representing *B. trigonocephalum* and *B. phlebotomum* from sheep and cattle using ITS rDNA as genetic markers, and to establish a PCR-linked restriction fragment length polymorphism (PCR-RFLP) approach for their unequivocal differentiation.

2. Materials and methods

2.1. Parasites

Ten adult nematodes representing *B. trigonocephalum* and 10 adult nematodes representing *B. phlebotomum* were collected from the small intestines of infected sheep and cattle, respectively, in Heilongjiang Province, China, in 2009. Individual worms were washed extensively in physiological saline, identified morphologically as *B. trigonocephalum* or *B. phlebotomum* according to existing keys and descriptions (Mönnig, 1950; Lu et al., 2002), mainly based on the presence of two pairs of subventral lancets in the buccal capsule and the longer male spicules in *B. phlebotomum* (Fig. 1). These samples were fixed in 70% ethanol and stored at −20 °C before DNA extraction.

2.2. DNA extraction

Individual nematodes from each host were treated with sodium dodecyl sulfate/protease K [0.5 M NaCl, 0.1 M Tris–HCl, pH 8.0, 50 mM EDTA, 10% (w/v) SDS, 20 mg/ml protease K] at 37 °C overnight, and then de-activated at 95 °C for 15 min (Zhu et al., 2007b). Then, the genomic DNA was extracted by phenol/chloroform, precipitated by ethanol, and then dissolved into ddH_2O in a volume of 30 μl. The DNA samples were stored at −20 °C until they were analyzed.

2.3. PCR amplification, cloning and sequencing of the ITS rDNA

The ITS rDNA was amplified by polymerase chain reaction (PCR) from each nematode DNA sample using primers p1 (forward; 5′-GATTACGTCCCTGCCATTTGT-3′) and p2 (reverse; 5′-GTTCACT CGCCGTTACTAAGG-3′) designed based on the ITS sequence of *Ancylostoma caninum* available in GenBank™ (Accession No. AJ920347). PCR reactions in a volume of 25 μl were performed in 10 mM Tris–HCl, pH 8.4, 50 mM KCl, 3 mM $MgCl_2$, 250 μM each of dNTP, 100 pmol of each primer, and 1 U La *Taq* polymerase (TaKaRa) in a thermorcycle (Biometra) under the following conditions: 94 °C for 10 min (initial denaturation), followed by 30 cycles of 94 °C, 1 min (denaturation), 50 °C, 1 min (annealing), 72 °C, 1.5 min (extension), and a final extension of 72 °C for 10 min. Two microliters of genomic DNA was added to each PCR reaction. Five microliters 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).

PCR products were purified using the BioSpin gelextraction kit (Bioer Technology) according to the manufacturer's recommendations. The purified products were ligated with pMD 18-T vector (TaKaRa) and transformed into the JM109 competent cells. The recombinant bacterium was screened, identified by PCR amplification, and then sequenced by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. Three clones for each of the three representative nematodes representing each species were sequenced.

2.4. RFLP analysis of the ITS amplicons

Theoretical restriction mapping of the ITS sequences for *B. trigonocephalum* and *B. phlebotomum* obtained in the present study (available in the GenBank™ database, Accession Nos. GQ859496 and GQ859497) using the software DNAStar (Ver 4.0) revealed that these two species could be delineated by using the restriction endonuclease *Nde* I. Based on this information, a

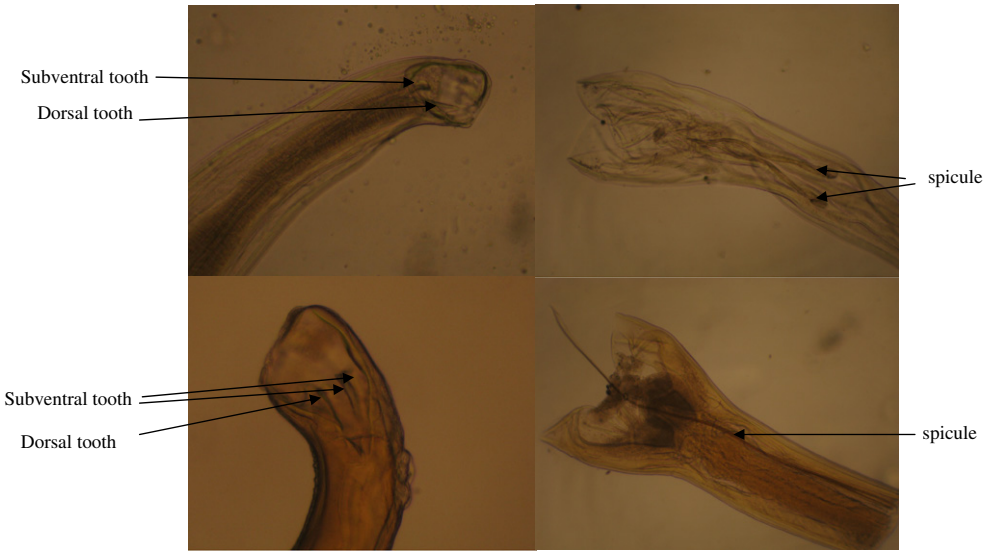

Fig. 1. The buccal capsule (left) and the male spicules (right) of *Bunostomum trigonocephalum* (top) and *B. phlebotomum* (bottom).