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Full Length Research Paper

Phylogenetic studies of *Oesophagostomum asperum* from goats based on sequences of internal transcribed spacers of ribosomal deoxyribonucleic acid (DNA)

San-Ke Yu[1,3], Bing Hu[1,3], Yan Deng[2,3], Hong-Mei Li[1,3], Wan-Xin Ren[1], Qing-Qing Bian[1], Man Gao[1], Xing-Ye Wang[1], Mei-Mei Cong[1], Jun-Ke Song[1], Qing Lin[1], Min-Jun Xu[3] and Guang-Hui Zhao[1,3]**

[1]College of Veterinary Medicine, Northwest A and F University, Yangling, Shaanxi Province, P. R. China.
[2]Guangzhou Airport Entry-Exit Inspection and Quarantine Bureau, Guangzhou, Guangdong province, P. R. China.
[3]State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, Gansu Province 730046, P. R. China.

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In the present study, the phylogenetic position of *Oesophagostomum asperum* was examined based on the sequences of internal transcribed spacer (ITS) of ribosomal DNA. The DNA fragment spanning 3' end of 18S rDNA, complete ITS-1, 5.8S, ITS-2 rDNA, and 5' end of 28S rDNA was amplified from six nematodes morphologically identified as *Oesophagostomum asperum* from 3 infected Cashmere goats (one pair parasites for each animal) in Shaanxi province, China. The amplicons were sequenced, and compared with corresponding sequences of other *Oesophagostomum* species available in GenBank™. The ITS-1, 5.8S, and ITS-2 sequences of all the parasites were 374, 153 and 259 bp in length, respectively, with no sequence difference in 5.8S rDNA and only very subtle sequence heterogeneity in ITS-1 and ITS-2 rDNA. The interspecies difference in ITS-1 and ITS-2 rDNA between *O. asperum* and other *Oesophagostomum* species showed that *O. asperum* is closely related to *O. venulosum*. Phylogenetic studies using Bayesian analysis revealed that all the *O. asperum* isolates clustered together sistered to *O. venulosum*. Our study characterized the *O. asperum* at molecular level for the first time, which would have important implications for further molecular identification and differentiation, and the genetic analysis of this nematode between specimens and hosts and the haplotypic variation and within regions in Shaanxi province and other locations.

Key words: Phylogenetic study, *Oesophagostomum asperum*, internal transcribed spacer (ITS), Cashmere goat.

INTRODUCTION

Oesophagostomiasis, caused by "node worms" of the genus *Oesophagostomum*, is one of the important emerging helminth zoonoses worldwide (McCarthy and Moore, 2000). *Oesophagostomum* spp. commonly inhabits in the intestines of ruminants (Cutillas et al., 1999; Wang et al., 2006; Jas et al., 2010; Ranjan et al., 2010), pigs (Weng et al., 2005; Lin et al., 2007, 2008), dogs (Boes et al., 2000) and non-human primates (de Gruijter et al., 2006; Gasser et al., 2006), occasionally in humans (Polderman et al., 1999; de Gruijter et al., 2005; Ziem et al., 2006). Like other helminth infections, asymptomatic infection (subclinical and chronic in nature) is common in animals and humans (Gasser et al., 2006; Jas et al., 2010). Major pathogenicity of these nematode parasites is caused by the migratory or immature stages of the parasites (Jas et al., 2010). *Oesophagostomum* species also can cause serious and frequent diseases in the host particularly when associated with hypoimmunity,

*Corresponding author. E-mail: zgh083@163.com

overcrowding and stress, resulting in the formation of granulomata, caseous lesions or abscesses around encysted larvae in the small and large intestinal walls, and even life-threatening (Stewart and Gasbarre, 1989; Gasser et al., 1999, 2006).

Oesophagostomum asperum, *Oesophagostomum columbianum* and *Oesophagostomum venulosum* are considered as the three main species in goats. The specific identification and differentiation of each species has important implications in studying their epidemiology, population biology, and systematics and is also central to the diagnosis and control of goat oesophagostomiasis. Although, adult parasites of these species have characteristic morphological features, it is difficult for non-experts to recognize each species, especially for the differentiation of *Oesophagostomum* spp. at egg and larval stages to the species level (Lin et al., 2007, 2008). Recently, DNA technology based on ribosomal DNA (rDNA) has been proved to be an alternative in addressing taxonomic and epidemiological problems related to parasites (Romstad et al., 1997; Gasser et al., 1999; Lin et al., 2008). The first internal transcribed spacer (ITS-1) rDNA provides a reliable genetic marker for the identification of *O. dentatum* and *O. quadrispinulatum* in pigs (Lin et al., 2007). The second internal transcribed spacer (ITS-2) rDNA could effectively differentiate five species of *Oesophagostomum* found in livestock, and is also a suitable marker for genetic analysis of *Oesophagostomum* spp. in humans and non-human primates (Romstad et al., 1997; Newton et al., 1998; Gasser et al., 1999).

O. asperum is an important nematode in goats and sheep, and has been reported in several provinces of China, with infection rates of 47.36 and 37.7% in goats in Guangxi (Tao et al., 2010), and sheep in Heilongjiang (Zhang et al., 2002) province, respectively. However, prior to the present study, no molecular study has been carried out to characterize *O. asperum* and to determine its phylogenetic position in relation to other *Oesophagostomum* species.

The objectives of this study was to characterize the ITS rDNA sequences of adult male and female *O. asperum* isolates from cashmere goats, and examine the phylogenetic position of *O. asperum* based on ITS-1 and ITS-2 rDNA sequences using Bayesian analysis.

MATERIALS AND METHODS

Parasites and isolation of genomic DNA

A total of 6 adult nematode isolates (3 male and 3 female worms) were collected from the large intestine of 3 infected cashmere goats (one pair parasites for each animal) in Shaanxi province, China. Individual worms were washed extensively in physiological saline and identified as *O. asperum* according to their predilection site and morphological characters using existing keys and description (Rao and Venkataratnam, 1977; Lu et al., 2002). The male and female adult parasites were separately stored in 90% molecular grade ethanol, and stored at -20°C before extraction of genomic DNA. Total genomic DNA from individuals was extracted using Wizard® SV Genomic DNA Purification System (Promega) and eluted into 40 µl elution buffers according to the manufacturer's recommendations (Zhao et al., 2009, 2010, 2011a, b; Lin et al., 2011).

Polymerase chain reaction (PCR) amplification and sequencing of the ITS rDNA

The rDNA fragment spanning 3' end of 18S rDNA, complete ITS-1, 5.8S, ITS-2 rDNA, and 5' end of 28S rDNA of *O. asperum* was amplified by polymerase chain reaction (PCR) from single adult using universal primers NC5 and NC2 described previously (Zhu et al., 1999; Lin et al., 2007). PCR reactions (25 µl) were performed in 2 mM of $MgCl_2$, 0.5 mM of each primer, 2.5 µl of 10× Ex*Taq* buffer, 0.2 mM of each dNTPs, 1.25 U of ExTaq DNA polymerase (TAKARA), and 1 µl of DNA sample in a thermocycler (Eppendorf) under the following conditions: after an initial denaturation at 94°C for 5 min, then 94°C for 45 s (denaturation), 50°C for 30 s (annealing), 72°C for 45 s (extension) for 35 cycles, followed by a final extension at 72°C for 10 min. Samples without genomic DNA (no DNA controls) and cashmere goat genomic DNA (host DNA controls) were included in each amplification run, and in no case were amplicons detected in the no DNA and host DNA controls (not shown). Each amplicon (5 µl) was examined by agarose gel electrophoresis to validate amplification efficiency according to previous studies (Zhao et al., 2009, 2010, 2011a, b; Lin et al., 2011).

Positive amplicons were selected, purified and sequenced using an ABI 377 automated DNA Sequencer (using BigDye Terminator Chemistry), employing the same primers (individually) used in the PCR. All the amplicons were sequenced from both sides (forward and reverse). The ITS-1 and ITS-2 rDNA sequences are available from DDBJ, EMBL, and GenBank™ under the accession numbers shown in Table 1.

Sequence comparison and phylogenetic analyses

The 5' and 3' ends of the ITS-1 and ITS-2 rDNA of *O. asperum* were determined by comparison with that of *O. venulosum* (GenBank™ accession number HQ283349) and *Oesophagostomum* sp. NMG-2010 (HQ844232). Sequences of the ITS-1 and ITS-2 rDNA of different isolates were separately aligned using the computer program Clustal X 1.83 (Thompson et al., 1997) and modified by eye. Sequence difference (D) in ITS rDNA among *O. asperum* isolates was examined using the formula $D = 1-(M/L)$, where M is the number of alignment positions at which the two sequences have a base in common, and L is the total number of alignment positions over which the two sequences are compared (Chilton et al., 1995; Zhao et al., 2009, 2011a; Lin et al., 2011). Meanwhile, Megalign procedure within the DNAStar 5.0 (Burland, 2000) was used to analyze sequence similarity of different species in the genus *Oesophagostomum*, and Mega 4.0 (Tamura et al., 2007) was used to calculate base composition, transition, and transversion.

To study the evolutionary position of *O. asperum* compared with the relevant *Oesophagostomum* species available in GenBank™ (Table 1), phylogenetic analyses were performed using MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001), with *Bunostomum* sp. (HQ844234) as out-group. The ambiguously aligned regions for each gene were excluded using Gblocks online server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html), using the options for a less stringent selection. Models for Bayesian analysis were independently chosen for each partition using jModeltest v.0.1.1 (Posada, 2008). Bayesian analysis was run with

Table 1. Geographical origins and genders of nematode samples used in the present study, as well as their GenBank™ accession numbers for sequences of the internal transcribed spacer of nuclear ribosomal DNA.

Species/Sample codes	Gender	Host	Geographical origin	GenBank™ accession number		Reference
				ITS-1	ITS-2	
Oesophagostomum asperum/OeASYM1	Male	Cashmere Goat	China (Shaanxi)	JN835417	JN835417	The present study
O. asperum/ OeASYM2	Male	Cashmere Goat	China (Shaanxi)	JN835418	JN835418	The present study
O. asperum/ OeASYM3	Male	Cashmere Goat	China (Shaanxi)	JN835419	JN835419	The present study
O. asperum/ OeASYF1	Female	Cashmere Goat	China (Shaanxi)	JN835420	JN835420	The present study
O. asperum/ OeASYF2	Female	Cashmere Goat	China (Shaanxi)	JN835421	JN835421	The present study
O. asperum/ OeASYF3	Female	Cashmere Goat	China (Shaanxi)	JN835422	JN835422	The present study
Oesophagostomum venulosum/-	-	Sheep	Ireland (Dublin)	HQ283349	HQ283349	Unpublished
Oesophagostomum columbianum/Oec05	-	Sheep	Australia (New South Wales)	-	AJ006150	Newton et al., 1998
Oesophagostomum radiatum/-	-	Cattle	USA(Beltsville)/Australia (Victoria)	AF344881	AJ006149	Unpublished/Newton et al., 1998
Oesophagostomum dentatum/-	-	Pig	China (Guangdong)	AJ619979	AJ619979	Unpublished
Oesophagostomum quadrispinulatum/-	-	Pig	China (Guangdong /Chongqing)	AJ889567	AJ889568	Lin et al., 2007
Oesophagostomum stephanostomum/-	-	chimpanzee	Tanzania (Mahale)	-	AF136576	Gasser et al., 1999; Schindler et al., 2005
Oesophagostomum bifurcum/-	-	Human	Australia (Togo)	-	Y11733	Romstad et al., 1997
Oesophagostomum bifurcum/-	-	*Cercopithecus mona*	Ghana (Fiema)	-	AF136575	Gasser et al., 1999
Bunostomum sp.	-	Sheep	China	HQ844234	HQ844234	Unpublished

four chains of 10,000,000 (for ITS-1 rDNA) or 15,000,000 (for ITS-2) generations, sampling each 10,000 (for ITS-1 rDNA) or 15,000 (for ITS-2) generations for a total of 1,000 trees. The first 250 trees were omitted as burn-in and the remaining trees were used to calculate Bayesian posterior probabilities (PP).

RESULTS AND DISCUSSION

The rDNA fragment spanning 3' end of 18S rDNA, complete ITS-1, 5.8S and ITS-2 rDNA, and 5' end of 28S rDNA was amplified from the three pairs of *O. asperum* isolates. Four different ITS sequences for the *O. asperum* were obtained after sequencing and gene identification.

The ITS-1, 5.8S, and ITS-2 sequences of *O. asperum* were 374, 153, and 259 bp in length, respectively. Pair-wise comparison showed that no sequence variation was detected in 5.8S rDNA and only very subtle sequence heterogeneity in ITS-1 and ITS-2 rDNA was observed among 6 *O. asperum* isolates (Table 2). Sequence variations among all the *O. asperum* isolates were 0.0-1.6% for ITS-1 rDNA and 0.0-1.5% for ITS-2 rDNA. The inter-species differences in ITS-1 and ITS-2 rDNA among species of the genus *Oesophagostomum* ranged from 2.5% (between *O. asperum* and *O. venulosum*) and 31.6% (between *O. asperum* and *O. radiatum*), from 2.4% (between *O. bifurcum* and *O. stephanostomum*) and 33.9% (between *O. radiatum* and *O. quadrispinulatum*) (Table 2), suggesting that *O. asperum* is closely related to *O. venulosum*. Then, the phylogenetic relationships of *Bunostomum* species was reconstructed using Bayesian analysis (Bayes) based on ITS-1 (Figure 1A) and ITS-2 (Figure 1B) rDNA sequences. For

Table 2. Pairwise comparison of sequence difference (in %) in the first (ITS-1, above the diagonal) and second internal transcribed spacer (ITS-2, below the diagonal) ribosomal DNA.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	-	0.8	1.6	1.1	0.8	0.8	13.0	-	-	-	-	3.3	10.8	11.5
2	0.0	-	0.8	0.5	0.0	0.0	11.9	-	-	-	-	2.5	10.1	10.5
3	1.2	1.2	-	1.3	0.8	0.8	12.7	-	-	-	-	3.3	11.1	11.5
4	0.4	0.4	1.5	-	0.5	0.5	12.7	-	-	-	-	3.0	10.1	11.1
5	0.0	0.0	1.2	0.4	-	0.0	11.9	-	-	-	-	2.5	10.1	10.5
6	0.0	0.0	1.2	0.4	0.0	-	11.9	-	-	-	-	2.5	10.1	10.5
7	31.8	31.8	31.1	31.2	31.8	31.8	-	-	-	-	-	11.9	10.9	10.8
8	13.1	13.1	12.5	12.5	13.1	13.1	23.8	-	-	-	-	-	10.1	11.8
9	12.7	12.7	12.1	12.1	12.7	12.7	24.0	0.0	-	-	-	-	-	8.6
10	12.4	12.4	11.8	11.9	12.4	12.4	23.8	2.4	2.5		-	-	-	-
11	15.7	15.7	15.1	15.2	15.7	15.7	29.3	7.5	7.6	8.1	-	-	-	-
12	7.9	7.9	7.4	8.3	7.9	7.9	31.8	13.1	12.7	12.4	16.9	-	-	-
13	14.4	14.4	13.9	13.9	14.4	14.4	24.8	8.0	8.1	8.5	10.1	16.9	-	-
14	22.8	22.8	22.1	22.2	22.8	22.8	33.9	15.2	15.4	17.0	14.3	25.0	11.3	-

Samples of 1-14 represent OeASYM1, OeASY1M2, OeASYM3, OeASYF1, OeASYF2, OeASYF3, *O. radiatum*, *O. bifurcum* (*Cercopithecus mona*), *O. bifurcum* (Human), *O. stephanostomum*, *O. columbianum*, *O. venulosum*, *O. dentatum*, *O. quadrispinulatum*.

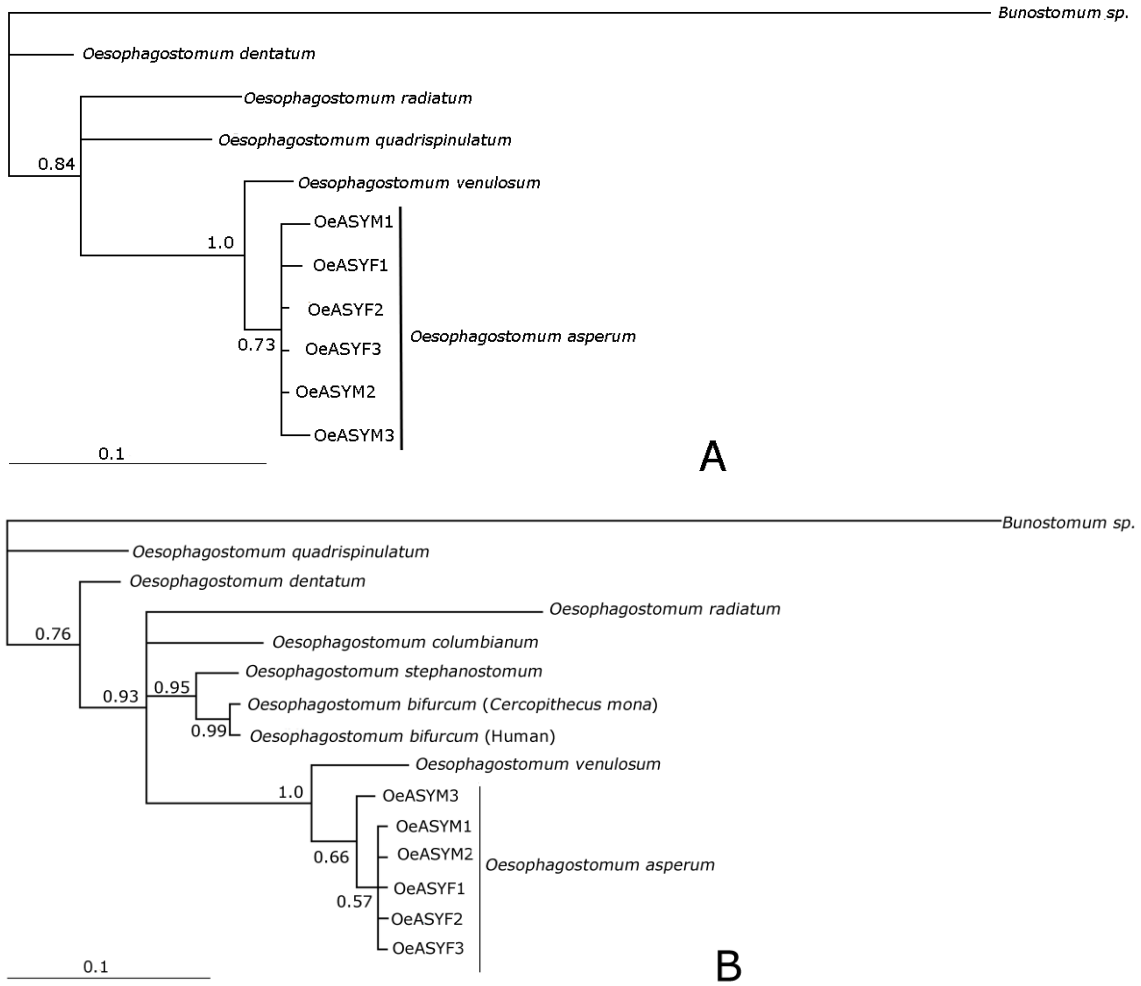

Figure 1. Bayesian maximum likelihood tree of *Oesophagostomum* worms based on ITS-1 (A) and ITS-2 rDNA (B). Values by nodes are the posterior probabilities recovered from the Bayesian analysis.

the Bayes analysis, the preferred models are GTR and HKY for ITS-1 and ITS-2, respectively. Both topologies of the Bayesian 50% consensus tree based on two genes support that all the *O. asperum* isolates clustered together sistered to *O. venulosum*.

In the present study, the *O. asperum* was characterized at molecular level for the first time. Both the ITS-1 and ITS-2 rDNA sequences provide suitable genetic markers for the identification of *O. asperum* isolates. However, due to limited sample size, the low genetic variability in the ITS rDNA sequences was observed among *O. asperum* isolates from cashmere goats in Shaanxi province, with nearly equal variation in the ITS-1 and ITS-2 rDNA. Therefore, sequence comparison of large numbers of samples is needed in the future to assess the level of genetic divergence between specimens and hosts and the haplotypic variation and within regions in this province.

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