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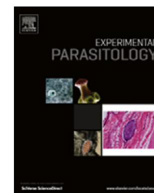

Oesophagostomum dentatum and *Oesophagostomum quadrispinulatum*: Characterization of the complete mitochondrial genome sequences of the two pig nodule worms

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ABSTRACT

In the present study, the complete mitochondrial DNA (mtDNA) sequences of the pig nodule worm *Oesophagostomum quadrispinulatum* were determined for the first time, and the mt genome of *Oesophagostomum dentatum* from China was also sequenced for comparative analysis of their gene contents and genome organizations. The mtDNA sequences of *O. dentatum* China isolate and *O. quadrispinulatum* were 13,752 and 13,681 bp in size, respectively. Each of the two mt genomes comprises 36 genes, including 12 protein-coding genes, two ribosomal RNA and 22 transfer RNA genes, but lacks the ATP synthetase subunit 8 gene. All genes are transcribed in the same direction and have a nucleotide composition high in A and T. The contents of A + T are 75.79% and 77.52% for the mt genomes of *O. dentatum* and *O. quadrispinulatum*, respectively. Phylogenetic analyses using concatenated amino acid sequences of the 12 protein-coding genes, with three different computational algorithms (maximum likelihood, maximum parsimony and Bayesian inference), all revealed that *O. dentatum* and *O. quadrispinulatum* represent distinct but closely-related species. These data provide novel and useful markers for studying the systematics, population genetics and molecular diagnosis of the two pig nodule worms.

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1. Introduction

Parasitic nematodes belonging to the genus *Oesophagostomum* are commonly known as 'nodule' worms of ruminants, pigs and primates. They can cause serious clinical diseases (e.g., oesophagostomiasis) in these hosts (McCarthy and Moore, 2000; Legesse and Erko, 2004; Weng et al., 2005; Krief et al., 2010). Human oesophagostomiasis is a serious public health problem in northern Togo and Ghana (McCarthy and Moore, 2000; Verweij et al., 2001). *Oesophagostomum* infection in pigs is quite common and has a global geographical distribution (Joachim et al., 2001; Weng et al., 2005). A few species of *Oesophagostomum* have been found parasitizing pigs, but only two species, namely *Oesophagostomum dentatum* and *Oesophagostomum quadrispinulatum* were identified to be the main causative nodule worms (Cutillas et al., 1999; Lin et al., 2008). Meanwhile, *O. dentatum* was proposed as a potential model for genomic studies of strongylid nematodes (Gasser et al., 2007). Therefore, relevant studies on the biological, biochemical and molecular biological and genomic information of 'nodule' worms in pig would be of help for the control of oesophagostomiasis and diseases caused by other strongylid nematodes.

The metazoan mitochondrial (mt) genomes, ranging in size from 14 to 18 kb, are typically circular and usually encode 36–37 genes, including 12–13 protein-coding genes, 22 transfer RNA genes, and two ribosomal RNA genes (Wolstenholme, 1992). Due to its maternal inheritance, rapid evolutionary rate, lack of recombination and relatively conserved genome structures, mtDNA sequences have been widely used as genetic markers not only for studying the taxonomy, systematics and population genetics of

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animals, but also for phylogenetic and evolutionary analyses (Li et al., 2008; Catanese et al., 2010; Liu et al., 2011, 2012; Xie et al., 2011; Lin et al., 2011, 2012). In spite of significant advances in mt genomics, there are gaps in our knowledge of mt genomes for many groups of metazoan parasites. In the family Chabertiidae, the mt genomes of only two species, namely *Chabertia ovina* and *O. dentatum*, were sequenced (Jex et al., 2010). This lack of enough knowledge of mt genomics for parasitic nematodes in this family forms a major limitation for population genetic and phylogenetic studies of pathogens in this family including members of the genus *Oesophagostomum*.

The objectives of the present study were to determine and analyze the mt genomes of the two pig nodule worms *O. dentatum* and *O. quadrispinulatum*, and to re-construct the phylogenetic relationships of members of the Chabertiidae using mtDNA sequences.

2. Materials and methods

2.1. Samples and DNA extraction

One nodule worm representing *O. dentatum* used in the present study was from Daqing city, Heilongjiang Province, China, and one specimen representing *O. quadrispinulatum* was collected from Chongqing city, China. These specimens were obtained from the feces of slaughtered fatten pigs, washed in physiological saline, identified primarily based on morphological characters to species, fixed in 70% (v/v) ethanol and stored at −20 °C until use. Total genomic DNA was isolated from individual nematodes using sodium dodecyl-sulfate/proteinase K treatment, followed by spin-column purification (Wizard Clean-Up, Promega). The identity of the specimen was confirmed as *O. dentatum* or *O. quadrispinulatum* by a multiplex PCR assay following protocols reported previously (Lin et al., 2008).

2.2. Long-PCR amplification and sequencing

Based on the partial sequences of mt cytochrome c oxidase subunit (*cox*1) and NADH dehydrogenase subunit 1 (*nad*1) genes available in GenBank (Accession Nos. FM161897–FM161886 and FM163322–FM163311, respectively), primers (Table 1) were designed in the conserved regions to amplify the entire mt genome of each species in two overlapping long fragments. PCR reactions (50 μL) were performed in 2.5 mM MgCl2, 0.4 mM each of dNTPs, 5 μL 10× LA *Taq* buffer, 0.5 μM of each primer, 2.5 U r*Taq* polymerase (Takara) and 2 μL of DNA sample in a thermocycler (Biometra) under the following conditions: 92 °C for 2 min (initial denaturation), then 92 °C for 10 s (denaturation), 50 °C for 30 s (annealing), and 60 °C for 10 min (extension) for five cycles, followed by 92 °C for 10 s, 50 °C for 30 s, and 66 °C for 10 min for 25 cycles, and a final extension at 66 °C for 10 min. Samples without DNA (no-DNA controls) were included in each amplification run, and in no case were amplicons detected in the no-DNA controls (data not shown). Each amplicon (3 μL) was examined by agarose (0.8%) gel electrophoresis, stained with ethidium bromide and photographed using a gel documentation system (UVItec). PCR products were sent to Sangon Company (Shanghai, China) for sequencing using a primer walking strategy.

2.3. Sequence analyses

Sequences were assembled manually and aligned against the complete mt genome sequences of *Ancylostoma duodenale* (GenBank Accession No. NC_003415) and *O. dentatum* (Denmark isolate, GenBank Accession No. GQ888716) available using the computer program Clustal X 1.83 to identify gene boundaries (Thompson et al., 1997). The open-reading frames and codon usage profiles of protein-coding genes were analyzed using the program MacVector 4.1.4 (Kodak, version4.0). Gene annotation, genome organization, translation initiation, translation termination codons and the boundaries between protein-coding genes of mt genomes of the two *Oesophagostomum* species were identified based on comparison with mt genomes of other nematodes reported previously (Hu et al., 2002a; Jex et al., 2010). The amino acid sequences inferred for the mt genes of the two *Oesophagostomum* species were aligned with those of other nematodes by using Clustal X 1.83. Based on pair-wise alignments, amino acid identity (%) was calculated for homologous genes. Codon usage was examined based on the relationships between the nucleotide composition of codon families and amino acid occurrence, and the genetic codons are grouped into AT rich codons, GC-rich codons and unbiased codons. For analyzing ribosomal RNA genes, putative secondary structures of 22 tRNA genes were identified using tRNAscan-SE (Lowe and Eddy, 1997), or by recognizing potential secondary structures and anticodon sequences by eye by aligning mtDNA sequences of *O. dentatum* and *O. quadrispinulatum*.

Table 1
Sequences of primers used to amplify long-PCR fragments from two *Oesophagostomum* species.

Name of primer	Sequence (5′ to 3′)
For O. dentatum	
ODmt*NAD*1F	TAGTTATGATTATTGCTGAG
ODmt*COX*1R	ATAAAACCTAACACCCAC
ODmt*COX*1F	TTCTCGTGCTTATTTTAC
ODmt*NAD*1R	AAACTCCCATATTCACTT
For O. quadrispinulatum	
OQmt*NAD*1F	TTTATAGGTGTATTACAAGCRTT
OQmt*COX*1R	CAGTAAAATAAGCACGAGAATC
OQmt*COX*1F	ACAGTGGGTATGGATTTA
OQmt*NAD*1R	AAAAGAAACACCTGGAAC

2.4. Phylogenetic analyses

Phylogenetic relationship among representative members of the orders Rhabditida and Strongylida (Table 2), plus the two mtDNA sequences obtained in the present study, were performed, using an Enoplean species *Trichinella spiralis* (GenBank Accession No. NC_002681) as the outgroup, based on amino acid sequences of 12 protein-coding genes. Each gene was translated into amino acid sequence using the invertebrate mitochondrial genetic code in MEGA 5.0 (Tamura et al., 2011), and aligned based on its amino acid sequence using default settings, and ambiguously aligned regions were excluded using Gblocks online server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) (Talavera and Castresana, 2007), using the options for a more stringent selection. The final amino acid sequences of the 12 protein-coding genes were then concatenated into single alignments for phylogenetic analyses. Three methods, namely, maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference (Bayes), were employed for phylogenetic re-constructions. ML analyses were performed using PhyML 3.0 (Guindon and Gascuel, 2003), and the mtREV model with its parameter for the concatenated dataset was determined for the ML analysis using ProtTest based on the Akaike information criterion (AIC) (Abascal et al., 2005). Bootstrap support for ML trees was calculated using 100 bootstrap replicates. MP analysis was performed using PAUP 4.0 Beta 10 programme (Swofford, 2002), with indels treated as missing character states. A total of 1000 random addition searches using TBR were performed for each MP analysis. Bootstrap probability (BP) was calculated from 1000 bootstrap replicates with 10 random additions per

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