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The complete mitochondrial genome of *Pseudoterranova azarasi* and comparative analysis with other anisakid nematodes

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ABSTRACT

Anisakiasis/anisakidosis caused by anisakid nematodes is an emerging infectious disease that can cause a wide range of clinical syndromes and are difficult to diagnose and treat in humans. In spite of their significance as pathogens, the systematics, genetics, epidemiology and biology of these parasites remain poorly understood. In the present study, we sequenced the complete mitochondrial (mt) genome of *Pseudoterranova azarasi*, which is one of the most important zoonotic anisakid parasites. The circular mt genome is 13,954 bp in size and encodes of 36 genes, including 12 protein-coding, 2 ribosomal RNA and 22 transfer RNA genes. The mt gene order of *P. azarasi* is the same as those of *Ascaris* spp. (Ascarididae), *Toxocara* spp. (Toxocaridae) and *Anisakis simplex* (Anisakidae), but distinct from those of *Ascaridia* spp. (Ascaridiidae) and *Cucullanus robustus* (Cucullanidae). Phylogenetic analyses based on concatenated amino acid sequences of 12 protein-coding genes by Bayesian inference (BI) showed that *Pseudoterranova* were more closely related to *Anisakis* than they were to *Contracaecum* with strong a posterior probability support. This mt genome provides a novel genetic markers for exploring cryptic/sibling species and host affiliations, and should have implications for the diagnosis, prevention and control of anisakidosis in humans.

1. Introduction

Anisakid nematodes infect a wide range of invertebrates and vertebrates with a cosmopolitan distribution. Human anisakiasis/anisakidosis caused by the members of genura *Anisakis*, *Contracaecum* and *Pseudoterranova* is an emerging infectious disease that can be associated with mild to severe gastrointestinal disease and allergic responses (Audicana and Kennedy, 2008; Pravettoni et al., 2012). Humans are infected by eating raw or undercooked marine fish with third-stage larva (L3). Given the hot popularity of consuming raw or undercooked fish (e.g., sushi and sashimi), it is possible that anisakidosis is increasing and it is under diagnosed due to limited diagnostic test (Shamsi, 2014). Unfortunately, to date, no effective drug are available, and treatment only relies on surgery.

The accurate identification and differentiation of anisakid nematodes has important implications for studying their systematics, genetics, epidemiology and biology. Recently, although morphological features have been used to identify adults and some L3 of anisakid nematodes to type or species levels (Mattiucci et al., 2005), these criteria are sometimes insufficient for specific identification and differentiation of anisakid nematodes, particularly at the larval stages (Mattiucci and Nascetti, 2008). Various DNA techniques have provided powerful complementary tools to overcome the limitations of morphological approach, which have often been used to distinguish species from one another (Ondrejicka et al., 2014). The internal transcribed spacers (ITS) of nuclear ribosomal DNA (rDNA) region (Garbin et al., 2013; Pekmezci et al., 2014) and mitochondrial (mt) DNA (mtDNA) (Lin et al., 2013; Quiazon et al., 2013) have been used as genetic markers for studying genetic variation in anisakid nematodes, including some cryptic/sibling species. However, some studies have emphasized that it is better to use the complete mt genome datasets for the exploring cryptic/sibling species of anisakid nematodes (Mattiucci and Nascetti, 2008). In spite of the availability of advanced sequencing and bioinformatic methods, there is still relatively limited knowledge about complete mt genomes of many anisakid nematodes of socio-economic importance. In the family Anisakidae, to date, the complete mt genomes have been reported for only four species (Kim et al., 2006; Lin et al., 2012; Mohandas et al., 2014), and in

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the genus *Pseudoterranova* (an important etiological agents for zoonotic human anisakidosis), no information is available about the complete mt genome datasets.

The objectives of the present study were (i) to determine the complete mt genome of *Pseudoterranova azarasi*, which is one of the most important anisakid parasites, (ii) to compare this mt sequence with those of other anisakid nematodes, (iii) and to re-examine phylogenetic relationships among the infraorder Ascaridomorpha using the protein-coding amino acid sequences.

2. Materials and methods

2.1. Parasites and DNA extraction

The adult worm of *P. azarasi* was obtained from gastrointestinal tract of *Eumetopias jubata* (Steller's sea lion) in Iwanai, Japan. This specimen was washed in physiological saline, identified morphologically to species according to existing keys and descriptions (Skrjabin et al., 1991), fixed in 70% (v/v) ethanol and stored at −20 °C until use.

Total genomic DNA was isolated from this specimen using sodium dodecyl sulfate/proteinase K treatment, followed by spin-column purification (Wizard® SV Genomic DNA Purification System, Promega). The molecular identity of this specimen was then verified by PCR using previously reported primers (Zhu et al., 2002) and sequenced directly.

2.2. PCR amplification and sequencing

A fragment of the *cox*1, *nad*1, *nad*4, *cyt*b and *rrn*L was amplified by PCR with primers described in Table 1 (Bowles et al., 1995; Hu et al., 2007; Cao et al., 2005, 2006), respectively, and the amplicons directly sequenced. Based on the partial *cox*1, *nad*1, *nad*4, *cyt*b and *rrn*L sequences, five pairs of primers (Table 1) were designed in the conserved regions to amplify the complete mt genome by PCR as five overlapping amplicons from the genomic DNA. PCR reactions (25 μl) were performed in 2 mM $MgCl_2$, 0.2 mM each of dNTPs, 2.5 μl 10× r*Taq* buffer, 2.5 μM of each primer, 1.25 U r*Taq* polymerase (Takara), and 1 μl of DNA sample in a thermocycler (BioRad) under the following conditions: 94 °C for 5 min (initial denaturation), then 94 °C for 30 s (denaturation), 45–55 °C for 30 s (annealing), and 72 °C for 2–4 min (extension) for 35 cycles, followed by 72 °C for 10 min (final extension). One microliter (5–10 ng) of genomic DNA was added to each PCR reaction. Samples without genomic DNA (no-DNA controls) were included in each amplification run, and in no case were amplicons detected in the no-DNA controls (not shown). Each amplicon (5 μl) was examined by (1%) agarose gel electrophoresis to validate amplification efficiency. PCR products were sent to Sangon company (Shanghai, China) for sequencing using a primer walking strategy (Hu et al., 2007).

Table 1
Sequences of primers for amplifying mitochondrial DNA regions from *Pseudoterranova azarasi*.

Primer	Sequence (5′ to 3′)	Amplified sequences	References
JB3	TTTTTTGGGCATCCTGAGGTTTAT	*cox*1	Bowles et al. (1995)
JB4.5	TAAAGAAAGAACATAATGAAAATG	*cox*1	Bowles et al. (1995)
MH21F	TCAAATGTTTTTTAAAGACTTAGG	*rrnL*	Hu et al. (2007)
MH22R	CAAGATAAACAATTCTATCTCAC	*rrnL*	Hu et al. (2007)
CZH1	TATGAGCGTCATTTATTRGG	*nad*1	Cao et al. (2005)
CZH2	TATCATAACGAAAACGAGG	*nad*1	Cao et al. (2005)
CZH3	GCGGCTTTTTGTTCTATGCC	*nad*4	Cao et al. (2006)
CZH4	ATATGAGTAACAGAAGAATAA	*nad*4	Cao et al. (2006)
CF	TATTATACTAATGATGGTGCTTCT	*cytb*	This study
CR	AACATTGACCCAACCAACT	*cytb*	This study
N1-F	AGGGGAATATGGAGCTTTGTT	*nad*1 to *cytb*	This study
Cb1-R	AAAAACTCATCTGGGCTCATACTA	*nad*1 to *cytb*	This study
Cb-2F	AGTCATGTTAAGTTGGTTGG	*cytb* to *nad*4	This study
N4-2R	AAAGTCAAAATAAACCCTC	*cytb* to *nad*4	This study
N4-3F	TTGTTGGCTGGTTTGTTATTGA	*nad*4 to *cox*1	This study
C1-3R	ATTCTTAAAATAGCATACACCATCC	*nad*4 to *cox*1	This study
C1-4F	GGTTTGACGGGAGTTGTT	*cox*1 to *rrnL*	This study
Rl-4R	GCTACCTTAATGTCCTCACGCTA	*cox*1 to *rrnL*	This study
Rl-5F	CGGAGTTAACAGAAAATCATGTC	*rrnL* to *nad*1	This study
N1-5R	AGCACCTACTATTCCGTACTTAG	*rrnL* to *nad*1	This study

2.3. Sequence analyses

Sequences were assembled manually and aligned against the complete mt genome sequences of *Anisakis simplex s.l.* (Kim et al., 2006) available using the computer program MAFFT 7.122 (Katoh and Standley, 2013) to identify gene boundaries. Translation initiation and translation termination codons were identified based on comparison with those of reported previously (Kim et al., 2006; Mohandas et al., 2014). For analyzing tRNA 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, and two rRNA genes were predicted by comparison with those of reported previously (Kim et al., 2006; Mohandas et al., 2014).

2.4. Phylogenetic analyses

The amino acid sequences conceptually translated from individual genes of the mt genome of *P. azarasi* were concatenated. Selected for comparison were concatenated amino acid sequences predicted from published mt genomes of key ascaridoid nematodes representing the Ascaridomorpha, including the superfamily Ascaridoidea [*Ascaris suum* (Liu et al., 2012), *A. simplex* (Kim et al., 2006), *Baylisascaris ailuri*, *Baylisascaris schroederi*, *Baylisascaris transfuga* (Xie et al., 2011a), *Baylisascaris procyonis* (Xie et al., 2011b), *Contracaecum rudolphii* B (Lin et al., 2012), *Toxocara canis*, *Toxocara cati*, *Toxocara malaysiensis* (Li et al., 2008b), *Toxascaris leonina* (Liu et al., 2014a), *Parascaris univalens* (Jabbar et al., 2014), *A. simplex s.s.*, *Contracaecum osculatum s.s.* (Mohandas et al., 2014)] and the superfamily Heterakoidea (*Ascaridia galli*, *Ascaridia columbae* and *Ascaridia* sp. (Liu et al., 2013), and the superfamily Seuratoidea (*Cucullanus robustus* (Park et al., 2011), using *Dirofilaria immitis* as an outgroup (Tamura et al., 2007). All amino acid sequences were aligned using MAFFT 7.122, and ambiguously aligned regions were excluded using Gblocks online server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) with the default parameters (Talavera and Castresana, 2007) using the options for a less stringent selection, and then subjected to phylogenetic analysis using Bayesian inference (BI). BI was conducted with four independent Markov chains run for 1,000,000 metropolis-coupled MCMC generations, sampling a tree every 100 generations in MrBayes3.1.1 (Ronquist and Huelsenbeck, 2003). The first 25% (2500) trees were omitted as burn-in and the remaining trees were used to calculate Bayesian posterior probabilities (BPP). The analysis was performed until the potential scale reduction factor (PSRF) approached 1 and the average standard deviation of split frequencies was less than 0.01. Phylograms were drawn using the program FigTree v.1.4 (Rambaut, 2012).