ITS Sequence Analysis of *Ophidascaris Baylisi* from Burmese Pythons

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Abstract

Members of genus *Ophidascaris* are common parasitic roundworms in snakes that cause gastric granulomas, adenocarcinomas, intestinal obstruction, death, and serious economic losses in snakes and their products. To identify molecular marker of *Ophidascaris baylisi* from the Burmese python (*Python molurus bivittatus*), we amplified internal transcribed spacer (ITS) sequence from this roundworm and analyzed their homology and phylogeny. The amplified ITS sequence length was 1140 bp, comprising the complete ITS1, 5.8S, and ITS2 sequences and a partial 28S sequence. The ITS1+ sequence was homologous (85.8% homology) to the related species *Ophidascaris robertsi*. The phylogenetic tree revealed a close genetic distance between *O. baylisi* and *O. robertsi*, which formed a separate branch within family Ascaridae clade. The results indicated that the identified ITS sequence could be a good molecular marker for further study on the molecular classification and genetic variation of *Ophidascaris* species.

1. Introduction

Ophidascaris sp. is a group of gastrointestinal ascaridoid nematodes parasitic on snakes. Pythons commonly acquire this parasitic infection through ingestion of infected intermediate hosts, such as mice and frogs (Sprent, 1988; Lettoof et al., 2019). Even after the intermediate host had died, *Ophidascaris* sp. larvae survived carcass putrefaction, remained viable in water for a minimum of three weeks, and retained their infectivity to snakes (Sprent, 1970). In pythons, the migration of *Ophidascaris* larvae resulted in tissue bleeding, inflammation, and secondary infection, while adult parasitism caused severe gastric granulomas and adenocarcinoma, intestinal obstruction, anorexia, malnutrition, and even death (Elbihari and Hussein, 1973; Hamir, 1986; Baron et al., 2018; Suwanti et al., 2018). Thus, this parasitic nematode can threaten the health of snakes and the economic value of their products. Until now, snake infection with *Ophidascaris* sp. has been reported in many countries, including China, Iran, Indonesia, Thailand, and Australia (Li et al., 2014; Ganjali et al., 2015; Ngamniyom et al., 2015; Baron et al., 2018; Suwanti et al., 2018; Zhou et al., 2021; Zhao et al., 2021).

Ophidascaris sp. identification has been controversial (Sprent, 1969; Sprent, 1970; Sprent, 1988). Although the classification of *Ophidascaris* sp. has been documented for a long time, it is limited to host characteristics, geographical distribution, and a few morphological features. Morphologically, *Ophidascaris* species infecting pythons belong to the 'filaria' group of genus *Ophidascaris*, including eight species, *O. filaria*, *O. baylisi*, *O. papuanus*, *O. robertsi*, *O. infundibulicola*, *O. amucronata*, *O. niuginiensis*, and *O. moreliae* (Sprent, 1969). However, all these species are morphologically similar and lack epidemiological data. Moreover, individual morphological differences often exist within the same species (Sprent, 1988). With the development of scanning electron microscope, Li et al. (2016) noted that the number of postcloacal papillae of *Ophidascaris* sp. could also be used as a classification standard, but this technique is complicated. Thus, further studies are required to explore an alternative accurate and fast method for *Ophidascaris* species identification.

Molecular markers have proven to be more valuable and efficient than morphological methods for helminth identification. The ribosomal internal transcribed spacer (ITS) has been shown as a helpful population genetic marker to classify and identify helminths (Liu et al., 2015; Shi et al., 2018; Ran et al., 2020). However, *Ophidascaris* sp. ITS data are lacking. Therefore, PCR amplification and sequence analysis of ITS sequence of *Ophidascaris baylisi* from Burmese pythons could lay a scientific basis for rapid identification of these roundworms.

2. Materials And Methods

Ophidascaris baylisi adult worm was collected from an albendazole-treated Burmese python (*Python molurus bivittatus*) at Guangzhou Zoo. The parasite sample was identified according to the method described by Zhao et al. (2021) and stored in 70% alcohol at -20°C. One-third of this worm was separated in a centrifuge tube, washed with sterile double-distilled water five times, and then transferred into a sterile 1.5 mL Eppendorf tube. Before DNA extraction, the worm tissue was macerated into small pieces with clean scissors. The whole genomic DNA of the worm was extracted according to the instructions of the Wizard® SV Genomic DNA Purification System (Promega, Madison, USA).

The ITS sequence of *Ophidascaris baylisi* was amplified using universal primers, NC5 (5'-GTAGGTGAACCTGCGGAAGGATCATT-3') and NC2 (5'-TTAGTTTCTT TTCCTCCG-CT-3') (Zhu et al., 1999). These primers were synthesized by Qingke Biotech Company (Guangzhou, China). The PCR reaction (25 µL) comprised 12.5 µL ExTaq enzyme (Shenggong, Shanghai, China), 0.5 µL of each primer (50 pmol/µL), 2 µL template DNA, and 9.5 µL ddH₂O. A PCR reaction with a negative control sample was included. The used PCR program was as follows: 94°C for 5 min; 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and then 72°C for 7 min. All PCR products were analyzed by 1.5% agarose gel electrophoresis stained with ethidium bromide, visualized under UV light, and photographed.

Target DNA fragments were recovered by the E.Z.N.A.® DNA Gel Extraction Kit (Omega Bio-tek, Norcross, USA) following the manufacturer's protocol. The purified PCR products were connected with pMD19-T vector (TaKaRa, Dalian, China) and transformed into *Escherichia coli* DH5α competent cells (100 µL, Shenggong, Shanghai, China). After heat shock, bacteria were shaken for 1 h in 890 µL of LB (Luria-Bertani) broth at 37°C, 150 r/m. Putatively transformed bacteria in LB broth (100 µL) was spread on LB agar plates containing 100 µg/mL Ampicillin and incubated overnight at 37°C. White colonies were picked up and used for PCR validation. Positive clones were sent to Qingke Biotech Company (Guangzhou, China) for sequencing.

Homologous sequences were identified and downloaded by searching the Basic Local Alignment Search Tool (BLAST) on NCBI combined with Bioedit7.1 (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>). The corresponding fragments were intercepted by Bioedit7.1 before sequence homology analysis and alignment by ClustalW (Thompson et al., 1994). For the ITS phylogenetic tree, 23 Ascaridata members were aligned, representing three families (Ascaridae, Toxocaridae, and Anisakidae). The nematode *Onchocerca volvulus* sequence (GenBank AF228575) was used as outgroup to produce rooted tree. The

phylogenetic tree was reconstructed from the aligned sequences by the maximum likelihood (ML) and neighbor-joining (NJ) methods. In the ML method, we used PhyML 3.0 software (Dang et al., 2014) based on AIC (Akaike information criterion). Nodal support and the reliability of the phylogenetic tree were estimated by a bootstrap of 1000 replicates. The NJ method was performed using MEGA5.0 software (Tamura et al., 2011), based on AIC. The phylogenetic tree was drawn after correction of sequence alignment and 1000 bootstrap resampling.

3. Results And Discussion

PCR product of the ITS sequence of *O. baylisi* showed a specific band at 1000 bp (Fig. 1), consistent with the expected fragment size. Figure 2 shows the sequence composition of *O. baylisi* ITS sequence (MW837142). The amplified ITS fragment was 1140 bp, including a part of 18S (26 bp), ITS1 (514 bp), 5.8S (157 bp), ITS2 (373 bp), and a part of 28S sequence (70 bp). Comparing the ITS1+ sequence of *O. baylisi* with that of 23 Ascaridata nematodes in GenBank detected the highest homology percent (85.8%) between *O. baylisi* ITS1+ sequence and *O. robertsi* ITS1+ (AJ007457.1 for ITS1 and AJ001502.1 for 5.8S) from Bettong in Queensland, Australia. In the phylogenetic tree, the Guangzhou isolate of *O. baylisi* clustered into a single branch with *O. robertsi*, which belonged to Ascaridae nematodes clade (Fig. 3).

Molecular marker with high specificity represents an essential genetic tool, which could be used for rapid species identification and as an effective complement to traditional classification methods (Hebert et al., 2003; Zhang et al., 2018). The ITS sequence have been proven as specific genetic markers that can not only be used for the rapid detection of parasites but also have diverse applications in analyzing parasites polymorphism and genetic variation (Zhu et al., 1998; Shamsi et al., 2008). Out of more than 30 available *Ophidascaris* sp, GenBank contains only one ITS1 (AJ007457.1) and 5.8S (AJ001502.1) sequences recovered from *O. robertsi* from Bettong (*Bettongia tropica*), Australia (Zhu et al., 2000). The length of the 5.8S sequence isolated by Zhu et al. (2000) resembled that of *O. baylisi* 5.8S sequence. However, ITS1 isolated from our sample was complete and longer than that identified by Zhu et al. (2000). Homology analysis between the ITS1+ sequence and the corresponding sequences in GenBank showed that *O. baylisi* ITS1+ sequence had high homology (85.8%) with *O. robertsi* (Zhu et al., 2000). The phylogenetic tree showed that the Guangzhou isolate of *O. baylisi* clustered as a sister group in the same branch with *O. robertsi*, separated from other Ascaridae nematodes, suggesting that *O. baylisi* is related to *O. robertsi*. In this study, the ribosomal ITS1 and ITS2 sequences of *O. baylisi* were amplified for the first time, which would enrich the molecular marker data of *Ophidascaris* sp.

Declarations

Acknowledgments

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Conflict of interests

The authors declare that they have no conflicts of interest.

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Figures

Figure 1
PCR amplifications of ITS sequence (A) and PCR identification of recombinant plasmid (B) of *O. baylisi*.
M: DL 2000 DNA Marker; 1: sample; 2: negative control

1	<i>GTAGGTGAAC</i>	<i>CTGCGGAAGG</i>	<i>ATCATTATCG</i>	<i>AGCCAAAAAA</i>	<i>AAAAAGTCTC</i>
51	<i>CGATCGTGCA</i>	<i>TATAAATGGT</i>	<i>←18S][ITS1→</i> <i>ATTTGTGCGT</i>	<i>AAACGTGAGC</i>	<i>CATACAATGG</i>
101	<i>GTAGCAAATA</i>	<i>CACATACACA</i>	<i>CATCCCCATC</i>	<i>CCTACGCAAG</i>	<i>GTAGGTGATG</i>
151	<i>GTTTTTGGTG</i>	<i>GTGGTGGTGG</i>	<i>TGATGATGAT</i>	<i>AGCAGTCAGC</i>	<i>TGAATTTTTG</i>
201	<i>CAGACAATTG</i>	<i>CATGCTACCC</i>	<i>CGATTGTATG</i>	<i>TTGAGGGTAA</i>	<i>ATAAGTGACA</i>
251	<i>CGTTGGGCTA</i>	<i>GTTAGAAAGA</i>	<i>CGTGTCGATA</i>	<i>GCGCTTATTT</i>	<i>GCTCGTTACT</i>
301	<i>TTGTAACAAC</i>	<i>GGTGTCCATG</i>	<i>TTGGCGTCTA</i>	<i>CGCCTCACCG</i>	<i>AGTTATCGCC</i>
351	<i>TGGACCGTCG</i>	<i>ATAGCGATGA</i>	<i>AAGGTGCGGA</i>	<i>GAAAGCTCCT</i>	<i>CGTTTTTTTA</i>
401	<i>TATGATGATG</i>	<i>AGTTGAGTAG</i>	<i>ACTTAATGAG</i>	<i>CCTCAGCTAG</i>	<i>GAGGCCGCCA</i>
451	<i>ACAAAAGCCA</i>	<i>AACACAAACA</i>	<i>CAATATGATT</i>	<i>TCATTTTTTT</i>	<i>TTTTCTTTAA</i>
501	<i>CGATGAAAGT</i>	<i>TGTTATGATG</i>	<i>CTTTCACATT</i>	<i>TAACAATGTA</i>	<i>CAAATCTTAG</i>
551	<i>CGGTGGATCA</i>	<i>CTTGGTTCGT</i>	<i>GGATCGATGA</i>	<i>←ITS1]</i> <i>AGAACGCAGC</i>	<i>[5.8S→</i> <i>TAGCTGCGAT</i>
601	<i>AAATAGTGCG</i>	<i>AATTGCAGAC</i>	<i>ACATTGAGCA</i>	<i>CTAAAAATTC</i>	<i>GAACGCACAT</i>
651	<i>TGCGCCATCG</i>	<i>GGTTCATTCC</i>	<i>CGTTGGCACG</i>	<i>TCTGGCTGAG</i>	<i>GGTCGAAATG</i>
701	<i>TTTGCAAAAA</i>	<i>TAATAAACCG</i>	<i>TTTATTAATG</i>	<i>ATGGATGGAT</i>	<i>←5.8S][ITS2→</i> <i>GGATGAATGA</i>
751	<i>ATGAATGAAT</i>	<i>GCTTGATAAA</i>	<i>TAGGCGGCTA</i>	<i>GCCAATCAAT</i>	<i>GTGTTACAT</i>
801	<i>ACAAACATCA</i>	<i>TCATCATCAT</i>	<i>ATATTAACGG</i>	<i>TGTATGCGGT</i>	<i>TGTATGCGGT</i>
851	<i>GCTTACGTAT</i>	<i>ATATATATAT</i>	<i>ATGCCATAC</i>	<i>CTTACTTAGT</i>	<i>CTTCTAATAT</i>
901	<i>TTTCGTTGTT</i>	<i>GTCGCTCAAA</i>	<i>TGAGGCGCAA</i>	<i>AAATGTGGCT</i>	<i>ATCAATGTTT</i>
951	<i>TTTCTATTGC</i>	<i>TCTACAATGC</i>	<i>AACGCCTTCA</i>	<i>GCATTGTTAG</i>	<i>CTGCCGGTTG</i>
1001	<i>GTTGTTGGA</i>	<i>GGTTGTTGCA</i>	<i>TACGCAACGC</i>	<i>GAGTGAACGA</i>	<i>AACAAACAAA</i>
1051	<i>TAGTCACAAC</i>	<i>ATTTAGTTGT</i>	<i>TTTGACCTCA</i>	<i>GCTCAGTCGT</i>	<i>GACTACCCGC</i>
1101	<i>TGAATTTAAG</i>	<i>←ITS2]</i> <i>CATATAACGA</i>	<i>[28S→</i> <i>AGCGGAGGAA</i>	<i>AAGAAACTAA</i> <i>← NC2]</i>	

Figure 2

Composition of *O. baylisi* ITS sequence. NC5 and NC2 are a pair of primers.

Figure 3

Phylogenetic tree based on ITS1+ of *O. baylisi* and other Ascaridae nematodes by maximal likelihood (ML) and neighbor joining (NJ) method.