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Transcriptomic analysis of four developmental stages of *Strongyloides venezuelensis*

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ABSTRACT

Strongyloides venezuelensis is one of some 50 species of genus *Strongyloides*, obligate gastrointestinal parasites of vertebrates, responsible for strongyloidiasis in humans and other domestic/companion animals. Although *S. venezuelensis* has been widely used as a model species for studying human/animal strongyloidiasis, the sequence information for this species has been quite limited. To create a more comprehensive catalogue of expressed genes for identification of genes potentially involved in animal parasitism, we conducted a de novo sequencing analysis of the transcriptomes from four developmental stages of *S. venezuelensis*, using a Roche 454 GS FLX Titanium pyrosequencing platform. A total of 14,573 contigs were produced after de novo assemblies of over 2 million sequencing reads and formed a dataset "Vene454". BLAST homology search of Vene454 against proteome and transcriptome data from other animal-parasitic and non-animal-parasitic nematode species revealed several interesting genes, which may be potentially related to animal parasitism, including nicotinamide phosphoribosyltransferase and ferrochelatase. The Vene454 dataset analysis also enabled us to identify transcripts that are specifically enriched in each developmental stage. This work represents the first large-scale transcriptome analysis of *S. venezuelensis* and the first study to examine the transcriptome of the lung L3 developmental stage of any *Strongyloides* species. The results not only will serve as valuable resources for future functional genomics analyses to understand the molecular aspects of animal parasitism, but also will provide essential information for ongoing whole genome sequencing efforts in this species.

1. Introduction

Members of the genus *Strongyloides* are gastrointestinal parasites of vertebrates, some of which are pathogens of medical and veterinary importance. The life cycle of *Strongyloides* is rather complex but fascinating from a biological perspective [1]. Infective 3rd stage larvae (L3i) live in soil and infect the host animal by penetrating the skin. The larvae migrate through the connective tissue, enter the circulation, and then reach the lung. They escape into the alveolar space, ascend the tracheobronchial tree, get swallowed, and finally reach the small intestine, where they molt twice into parasitic adults. Eggs produced by the adult worms hatch into first stage larvae (L1) in the host intestine or in the host feces depending on the species. These larvae either develop directly into L3i through 2 molts (direct development) or into free-living worms of either sex (indirect development). In case of the indirect development, adult worms of male and female mate to produce eggs in the soil. The L1 progeny of this generation develops into L3i in most species. However, more than one free-living cycle are known to occur in some species [2].

More than 52 *Strongyloides* species are recognized so far. Among them, two species are known to cause human diseases (strongyloidiasis), affecting an estimated 50–100 million individuals worldwide [3]. Of the 2 species, *Strongyloides stercoralis* is much more widespread than the other species, *S. fuelleborni* [4]. Several species infect livestock, including *S. ransomi* in swine [5], *S. westeri* in horses [6] and *S. papillosus* in ruminants [7]. Companion animals (dogs and cats) are also affected by some *Strongyloides* species, such as *S. stercoralis* and *S. planiceps* [4,8].

For laboratory studies, *Strongyloides ratti* and *Strongyloides venezuelensis* are the most widely used [9]. Both the species are native to rats but can also infect mice. Large scale EST analysis of *Strongyloides* parasites was first conducted for *S. ratti* using L1, L2, free-living adult/L3i, and parasitic females [10]. A total of 4152 clusters were obtained from this analysis which were estimated to be the 20% of the *S. ratti* genes and later used for developing a microarray assay system [11,12]. Large scale EST analysis was also conducted for *S. stercoralis*, which generated 3311 EST clusters, using L1- and L3i-stage libraries [13]. More recently, a transcriptome analysis of *S. stercoralis* L3i using 454/Roche pyrosequencing technology [14] was also reported [15]. However for *S.*

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venezuelensis, only limited EST data (162 clusters from an L3i library) have been reported so far [16].

In this study, our aim was to create a more comprehensive catalogue of expressed genes for *S. venezuelensis*, which enables us to perform comparative analyses with the rapidly expanding transcriptome data from other free-living and parasitic nematodes.

Using the 454/Roche pyrosequencing technology, we analyzed non-normalized cDNA libraries constructed from four developmental stages, including lung third-stage larvae (LL3) which were not studied in the previous *S. ratti* and *S. stercoralis* EST analyses. The LL3 stage is present in a wide variety of animal-parasitic nematodes belonging to different phylogenetic clades, such as *Ascaris* and *Toxocara* in clade III, *Necator* and *Nippostrongylus* in clade V and *Strongyloides* in clade IV. However, the biological significance of this stage is not well understood [17]. In *S. venezuelensis*, both L3i and LL3 are in the third stage, but they show considerable biological differences. For example, L3i can penetrate the host's skin but they are unable to do so at the LL3 stage [17]. Moreover, L3i cannot settle down in the intestinal mucosa but LL3 can, secreting adhesion molecules [17,18]. Therefore a major developmental change seems to take place during the transition from L3i to LL3.

At present, our understanding on many aspects of biology of *Strongyloides* parasites at the molecular level is quite limited, so the large-scale transcriptome analysis carried out in this study will help in prioritizing and accelerating researches in this understudied genus.

2. Materials and methods

2.1. Parasites and animals

S. venezuelensis used in this study was originally isolated at Naha, Okinawa, Japan by Hasegawa and others in 1988 [19]. Since then, this isolate has been maintained by serial passages in laboratory rats: feces from infected rats were cultured using the filter paper method [20] for 3–4 days and infective third stage larvae (L3i) which came out of the feces were collected. These larvae were used to infect next rats. Because one infection cycle typically takes 10 days, it was estimated that this *S. venezuelensis* isolate had been maintained for roughly 800 generations before the sample collection (done in 2010) for this study. At the time of the original isolation, it was already noticed that this strain of *S. venezuelensis* produced very few numbers of free-living stages [19]. In our hands, no free-living stage has been observed so far. Therefore, it appears that the ability to undertake free-living development has been lost for this strain of *S. venezuelensis* during the long laboratory maintenance. For this reason, free-living stages were not included in the present study.

ICR mice and Wistar rats were purchased from Kyudo Co. Ltd. (Kumamoto, Japan). All animals were kept and handled under the approval of the Animal Experiment Committee of the University of Miyazaki. The third-stage infective larvae (L3i) were obtained from fecal culture by the filter paper method [21]. For collection of the lung third-stage larvae (LL3), male ICR mice were subcutaneously inoculated with approximately 30,000 L3i, and the lungs were removed 72–75 h post infection (p.i.), homogenized with Polytron PT-MR3000 (Kinematica AG, Littau, Switzerland) at 20,000 rpm for a few seconds. The lung homogenates were wrapped with KimWipe papers and incubated in phosphate-buffered saline (PBS) at 37 °C for 1.5 h and worms emerging out through the paper were collected [22]. Mixtures of eggs and first stage larvae (L1) were collected as follows: eggs were separated from feces by the flotation method with saturated salt solution [23], washed extensively with water, and incubated at 27 °C for 24 h in PBS. About half of the eggs hatched into L1. This egg/L1 mixture was collected by centrifugation. Parasitic adult female worms were collected from infected rats 8–10 days p.i. [22].

2.2. Preparation of transcriptome libraries and 454 sequencing

Frozen worms were crushed manually using a freeze-crushing apparatus (SK Mill, Tokken, Chiba, Japan), followed by total RNA isolation with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) [16].

Four cDNA libraries from each of the 4 different developmental stages (Egg/L1, L3i, LL3, and adults) were prepared for 454 sequencing according to the manufacturer's instruction [24], with each library having different MID (Titanium Multiplex Identifier) adaptors. Two sequencing runs were performed with the Roche 454 GS FLX Titanium platform to generate 543,713 reads, 622,248 reads, 679,257 reads and 638,728 reads for Egg/L1, L3i, LL3 and adult stages, respectively.

2.3. Assembly of 454 reads

Pooled sequencing reads from the four stages were subjected to assembling by the Roche's de novo GS assembler (Newbler, release 2.3). The initial 14,892 contig sequences were first searched against the NCBI nucleotide database (nt) and the NCBI Rat UniGene sequences using the BLASTn program. Contigs which showed BLAST hits to bacterial, viral or *Rattus norvegicus* nucleotide sequences and ribosomal RNA sequences ($<1e^{-40}$) were removed from the dataset. The remaining 14,573 contigs formed a dataset "Vene454" and were subjected for further analyses.

2.4. Functional gene classification based on gene ontology and protein family/domain search

Functional annotation by the gene ontology (GO) terms was carried out using the Blast2GO (B2G) program [25]. InterPro (InterProScan, EBI) search was performed remotely from B2G via the InterPro EBI webserver [26,27].

2.5. BLAST homology search

Sequences in the Vene454 dataset were subjected to BLAST [28] analysis against predicted protein data from the following nematode genome projects: *Caenorhabditis briggsae* (nematode clade V) [29], *Caenorhabditis elegans* (V) [30], *Caenorhabditis japonica* (V) [31], *C. remanei* (V) [31], *Caenorhabditis brenneri* (V) [31], *Meloidogyne incognita* (IV) [32], *Meloidogyne hapla* (IV) [33], *Bursaphelenchus xylophilus* (IV) [34], *Brugia malayi* (III) [35], *Ascaris suum* (III) [36], and *Trichinella spiralis* (I) [37]. BLAST analyses were also performed against nematode ESTs (expressed sequence tags) from NEMBASE4 [38]. In this case, from the original NEMBASE4, which contains 237,154 EST clusters from 62 nematode species, 2 sub-datasets were generated. One was composed of 114,356 clusters from 33 animal-parasitic species and the other contained 101,450 from 25 non-animal-parasitic (i.e. free-living or plant parasitic) species. Species included in each sub-dataset are listed in Table S1.

Contig sequences showed BLAST hits (BLAST score cut-off = 50) to protein or EST sequences of multiple animal-parasitic species that belong to multiple clades in the absence of BLAST hits (BLAST score cut-off = 40) to the following free-living and plant parasitic nematode species: *C. elegans*, *C. briggsae*, *C. brenneri*, *C. japonica*, *M. hapla*, *M. incognita*, and *B. xylophilus* (protein sequences), and species listed as non-animal-parasitic in Table S1 (EST sequences), were considered to be potential animal parasitism related genes. Among such *S. venezuelensis* contigs, only those with sequence descriptions by the Blast2GO program were selected. The original sequence descriptions generated by the Blast2GO program were sometimes uninformative, so we manually curated them when necessary.

2.6. Expression pattern profiling during development

Expression pattern profiling of each gene was conducted with CLC genomics workbench 5.1 (CLC bio, Aarhus, Denmark). In this analysis,